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RESEARCH ARTICLE

Genetic diversity and population structure of two endemic *Cupressus* (Cupressaceae) species on the Qinghai-Tibetan plateau

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Abstract. *Cupressus gigantea* and *C. torulosa* are ecologically and economically important endemic species of the conifer family Cupressaceae on the Qinghai-Tibetan plateau. *C. gigantea* was previously classified as a subspecies of *C. torulosa* because of their similar morphological characteristics and close distribution. In this study, 401 individuals were sampled from 16 populations of the two *Cupressus* species. The specimens were genotyped using 10 polymorphic microsatellite loci through fluorescence polymerase chain reaction (PCR). The genetic diversity of *C. gigantea* and *C. torulosa* populations was generally low, with the highest genetic diversity detected in the population LLS of *C. gigantea*. Distance-based phylogenetic and principal co-ordinates analyses indicated a clear genetic structures for the 16 populations of the two *Cupressus* species. Moreover, Mantel test results showed indistinctive correlations between population-pairwise F_{st} values and geographic distances, as well as between genetic distances and geographic distances in *C. gigantea* and *C. torulosa*, respectively. AMOVA suggested that genetic variation mostly resided within populations. Sixteen natural populations were evidently clustered into two major groups in the constructed neighbour-joining tree. The results demonstrated that *C. gigantea* and *C. torulosa* are different *Cupressus* species. The genetic information provided important theoretical references for conservation and management of the two endangered *Cupressus* species.

Keywords. genetic diversity; genetic structure; microsatellite; *Cupressus gigantea*; *Cupressus torulosa*.

Introduction

Quaternary climatic oscillations have played an important role in shaping the distributions and population genetic structure of species at the Qinghai-Tibetan plateau (QTP) (Shi *et al.* 1998; Hewitt 2000). The QTP (area of 2.5 × 106 km^2 and mean elevation > 4000 m above sea level) is the highest and largest plateau in the world (Zhang *et al.* 2002; Geng *et al.* 2009).

Cupressus gigantea and *C. torulosa* are endemic species of the conifer family Cupressaceae on the QTP. The trees of *C. gigantea* could grow up to 40–50 m tall, occurs sparsely in a short-range (a distance of only 350 km from discovery to the end) sandy soil, and are restricted to the dry valleys of Nyang River and Yarlung Tsangpo River (Li *et al.* 2014). *C. torulosa* D. Don (Cupressaceae), also called Himalayan cypress (Sellappan *et al.* 2007), is another *Cupressus* species that is distributed on the

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southeast plateau along the Yigong River. The *C. torulosa* trees could grow up to 30–40 m tall (Zheng and Fu 1978). *C. gigantea* and *C. torulosa* exhibit comparable patterns of variations in tree shape and branch characteristics, although *C. gigantea* contains markedly thicker and shorter branches than *C. torulosa* (figure 1 in electronic supplementary material at http://www.ias.ac.in/jgenet/). In the past decades, several botanists classified *C. gigantea* as a subspecies of *C. torulosa* because of their similar morphological features and relatively close distribution area (Farjon 2007). However, several studies classified some *C. torulosa* plants on the QTP as *C. gigantea* species (Wu 1983).

C. gigantea and *C. torulosa* plants are ecologically and economically valuable because of their common use in afforestation, traditional Tibetan medicine, construction industry and Tibetan incense by locals (Malizia *et al.* 2000). However, they are presently on the verge of extinction primarily because of geographic isolation, extreme climatic condition, anthropogenic disturbance, and slow natural renewal (Hao *et al.* 2006). Additionally, efforts towards conservation of these species are urgently needed because they were also categorized as threatened species in the International Union for Conservation of Nature's Red List (IUCN 2016). The genetic diversity, genetic differentiation, and structure of *C. gigantea* and *C. torulosa* plants should be effectively evaluated to develop efficient conservation strategies (Hedrick 2004; Kurokochi *et al.* 2015). The genetic diversity of *C. gigantea* was previously reported by using AFLP (Tsering 2008), ISSR and RAPD markers (Xia *et al.* 2008). However, only partial populations of *C. gigantea* have been examined, and no genetic information of *C. torulosa* is available. Simple sequence repeat (SSR) markers identify each individual as heterozygote or homozygote through multiple alleles at each locus (Fageria and Rajora 2014) and are widely used in genetic studies because of their codominant inheritance, high reproducibility, and high throughput in genotyping for precise analyses (Martín *et al.* 2012).

In this study, the genetic information, including genetic diversity, genetic differentiation and genetic structure of 16 *Cupressus* populations obtained from all distributions on the QTP were evaluated using 10 polymorphic fluorescent-labelled SSR markers.

Materials and methods

Sample collection

We collected 326 individuals from 13 populations of *C. gigantea* and 75 individuals from three natural populations of *C. torulosa* throughout their entire natural distributions on the QTP. We collected 21–27 samples from each population, and sampling sites were >50 m apart. Details of the 16 populations are described in table 1 and figure 1.

Genomic DNA isolation and detection

DNA from leaves was isolated using the CTAB method. DNA was electrophoresed in 0.8% agarose gel for concentration estimation, and the purity of DNA (containing 260/230 and 260/280) was determined using a micro-UV spectrophotometer (NanoDrop2000C, USA).

Microsatellite genotyping

Ten relatively high polymorphic SSR loci (Cg13, Cg16, Cg23, Cg25, Cg27, Cg35, Cg37, Cg54, Cg59 and Cg61) were selected from previously identified 16 loci screened (Li *et al.* 2014). The fluorescent-labelled SSR technique was performed for population genetic analyses. The primers were optimized to avoid the mutual interference of the fluorescence signal from the 10 loci. New primers for the same loci are listed in table 1 in electronic supplementary material material. Each forward primer was modified using 5′ fluorophore label FAM (standard) (5-FAM) or HEX (standard) (5-HEX) for multiplex product fragment analyses (table 1 in electronic supplementary material). PCRs were conducted in 20 μL volume mixtures including 2× *Taq* buffer, 0.1 μM forward and reverse primer, 1 U HotsrarTaq polymerase (Qiagen), and 1 μL of template DNA. PCR products were analysed using the fragment analyses function of Genetic Analyzer ABI 3730xl (Applied Biosystems, USA), and the alleles were mapped using GeneMapper 4.0 (Applied Biosystems).

Data analyses

GenAlEx v6.5 (Peakall and Smouse 2012) was used to estimate the genetic diversity of all populations by calculating the percentage of polymorphic loci (P), the number of alleles (N_A), the number of effective alleles (N_E), observed (H_O) and expected (H_E) heterozygosities, Shannon's Information Index (I), and fixation index (F). Allelic richness (R_S) (Mousadik and Petit 1996) and inbreeding coefficients (F_{IS}) were detected using FSTAT 2.9.3.2 (Goudet 2002), which was also used to test the significance of F_{IS} deviations from 0 by 1000 random permutations. Analyses of molecular variance (AMOVA) (Excoffier *et al.* 1992) was performed using an allelic distance matrix to calculate variance distribution based on F-statistics (Wright 1978). P values were based on 999 standard permutations. Principal co-ordinates analyses (PCoA) was used with data standardization via a covariance matrix (Peakall and Smouse 2012) to estimate genetic divergence among populations. AMOVA and PCoA were calculated using GenAlEx v6.5.

Population structure was further assessed using STRUCTURE v2.3.4 to investigate the genetic

Table 1. Details of sampled and genetic diversity varied among the populations of *C. gigantea* and of *C. torulosa.*

Species	Location	Code	Sample size	Latitude (N)	Longitude (E)	Altitude (m)	*P*/%	N_A	N_E	*I*	H_o	H_E	R_s	F_{IS}	*F*
C. gigantea	MiLin, MLX	MLN	25	29°20′43.4″	94°23′09.9″	2949	60.0000	2.100	1.307	0.320	0.156	0.180	2.036	0.082	0.120
	LiLong, MLX	LLN	26	29°07′35.2″	93°48′57.8″	3007	60.0000	2.100	1.499	0.393	0.177	0.232	2.048	0.168	0.268
	LiLong, MLX	LLS	26	29°07′35.7″	93°51′12.8″	2980	70.0000	2.600	1.882	0.653	0.281	0.386	2.566	0.202	0.278
	RiCun, MLX	RCS	21	29°04′27.3″	93°23′04.4″	3038	50.0000	2.100	1.465	0.393	0.187	0.229	2.095	0.126	0.234
	ReCun, MLX	RES	26	29°00′44.6″	93°18′58.1″	3055	50.0000	1.900	1.423	0.323	0.192	0.192	1.831	0.103	0.193
	LieShan, LX	LSS	25	28°59′43.5″	93°14′41.9″	3065	70.0000	2.600	1.536	0.450	0.192	0.241	2.498	0.210	0.153
	DongGa, LX	DGN	25	29°01′10.6″	93°10′24.3″	3134	70.0000	2.300	1.467	0.392	0.188	0.226	2.225	0.199	0.270
	XinZhaCun, LX	XZS	26	29°02′19.1″	93°09′09.8″	3089	70.0000	2.400	1.440	0.406	0.215	0.225	2.315	0.077	0.094
	XinZhaCun, LX	XZN	27	29°04′17.0″	93°09′40.9″	3119	60.0000	2.100	1.291	0.316	0.130	0.178	2.033	0.159	0.250
	LangXian, LX	LCS	25	29°04′02.7″	93°04′36.1″	3087	60.0000	2.300	1.377	0.361	0.164	0.200	2.228	0.124	0.191
	LangXian, LX	LCN	23	29°02′59.2″	93°03′43.7″	3126	60.0000	2.100	1.363	0.320	0.157	0.173	2.081	0.142	0.220
	GunDui, LX	GDC	25	29°00′14.0″	93°15′35.6″	3146	80.0000	2.200	1.446	0.395	0.252	0.226	2.154	0.025	0.015
	BaJie, LZX	BJC	26	29°37′20.5″	94°24′03.7″	3014	60.0000	2.100	1.436	0.358	0.200	0.200	2.062	0.092	0.138
							63.0769	2.223	1.456	0.391	0.192	0.222	2.167	0.131	0.186
C. torulosa	TongMai, BMX	YTM	25	30°06′41.1″	95°04′13.7″	2059	80.0000	2.400	1.293	0.347	0.140	0.190	2.305	0.205	0.242
	YiGong, BMX	YCC	25	30°10′47.9″	94°54′30.4″	2282	80.0000	2.200	1.381	0.383	0.168	0.231	2.119	0.219	0.262
	YiGong, BMX	YGC	25	30°08′01.7″	95°01′08.9″	2115	70.0000	2.100	1.229	0.273	0.148	0.150	2.032	0.144	0.193
							76.6667	2.233	1.301	0.334	0.152	0.190	2.152	0.189	0.232

P, percentage of polymorphic loci; N_A, average number of alleles; N_E, number of effective alleles; *I*, Shannon's information index; H_o, observed heterozygosity; H_E, expected heterozygosity; R_s, allelic richness; F_{IS}, inbreeding coefficients; *F*, fixation index.

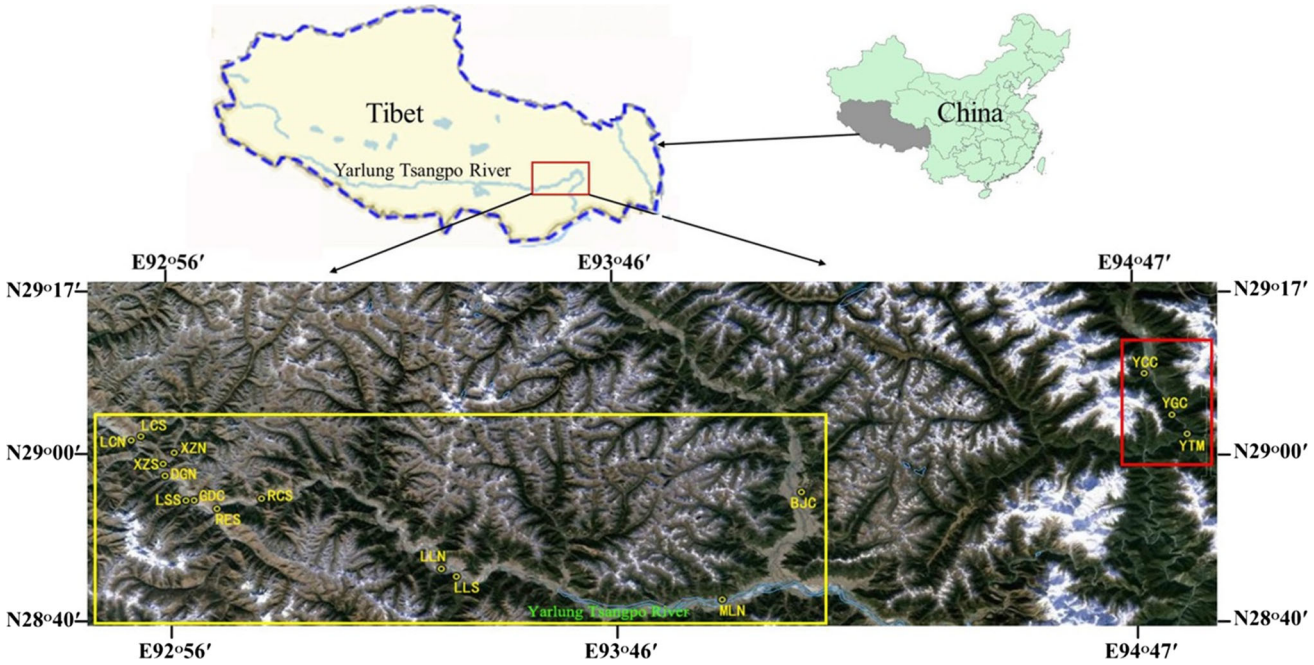

Figure 1. Geographic distribution of 13 populations of *C. gigantea* and three populations of *C. torulosa* that were adopted in this study. The populations of *C. gigantea* and *C. torulosa* were circled in thick yellow and red full line, respectively.

differentiation and assignment of individuals (Pritchard *et al.* 2000). The optimal K value was calculated using the delta K method (Evanno *et al.* 2005). Simulation was run 20 times for each number of clusters (K) from one to 17 for the whole microsatellite dataset (10 loci over 401 individuals). The initial burn-in period was 50,000, and the Markov chain Monte Carlo (MCMC) was 100,000. Genetic structure analyses was based on the LOCPRIOR model described by Hubisz *et al.* (2009).

Population differentiation was evaluated using the profiles of isolation-by-distance. Mantel (1967) test was implemented using GenAlEx v6.5 in *C. gigantea* and *C. torulosa* populations to detect relationships between population-pairwise F_{st} values and geographic distances and between genetic distances and geographic distances (natural logarithms), respectively (Peakall and Smouse 2012). Genetic relationships among populations were depicted by an unrooted neighbour-joining (NJ) tree (Saitou and Nei 1987), which was estimated using the PHYLIP 3.695 package (Felsenstein 2004). Allele frequencies were created using the SEQBOOT subroutine in PHYLIP 3.695 with 1000 bootstraps. Then, the correlated genetic distance was calculated using the GENDIST subroutine. Distance matrix trees were created using the NEIGHBOUR subroutine in PHYLIP 3.695. The input order was randomized to ensure that the final tree was not dependent on the sample order entry. Consequently, a consensus tree was constructed using the CONSENSE subroutine within PHYLIP 3.695, and the TREEVIEW 1.6.6 program was used to display the unrooted tree (Page 1996).

Results

Genetic diversity, genetic distance and differentiation

Genetic diversity varied among the populations of *C. gigantea* and *C. torulosa* (table 1). The percentage of polymorphic loci ranged from 50% (populations RCS and RES) to 80% (GDC) across 13 populations of *C. gigantea*, with a mean of 63.077%. Similarly, the ratio of polymorphic loci ranged from 70% (YGC) to 80% (YCC and YTM) across three populations of *C. torulosa*, with an average of 76.6667%. N_A ranged from 1.9 (RES) to 2.6 (LLS and LSS) for *C. gigantea* and from 2.1 (YGC) to 2.4 (YTM) for *C. torulosa*. N_E ranged from 1.291 in XZN to 1.882 in LLS for *C. gigantea* and from 1.229 in YGC to 1.381 in YCC for *C. torulosa*. The mean values of I per population were 0.391 for *C. gigantea* and 0.334 for *C. torulosa*. The H_O and H_E values varied from 0.130 (XZN) to 0.281 (LLS) and from 0.173 (LCN) to 0.386 (LLS) for *C. gigantea*, respectively. The highest H_O (0.168) and H_E (0.231) were observed in YCC for *C. torulosa*. The mean values of H_O and H_E were higher in *C. gigantea* populations than in *C. torulosa* populations. The allelic richness ranged from 1.831 (RES) to 2.566 (LLS) across 13 populations of *C. gigantea*, with a mean of 2.167. Corresponding value for the three populations of *C. torulosa* ranged from 2.032 (YGC) to 2.305 (YTM), with a mean of 2.152. The mean values of F_{IS} were 0.131 and 0.189 for each of the 13 populations of *C. gigantea* and three populations of *C. torulosa*, respectively. The F values varied from

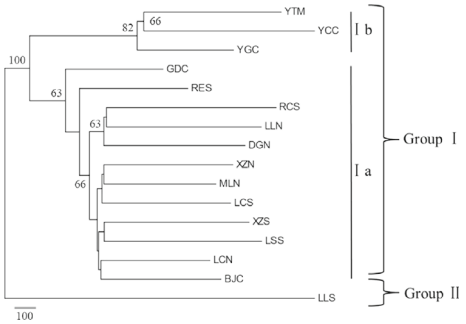

Figure 2. Neighbor-joining (NJ) tree based on DCE distance for 16 natural populations of two *Cupressus* species in China. Only bootstrap values >60 were indicated at each node. Most of the populations were clustered into group I, and only one population (LLS) was clustered into group II.

0.015 (GDC) to 0.278 (LLS), with an average of 0.186 for *C. gigantea*, and from 0.193 (YGC) to 0.262 (YCC), with an average of 0.232 for *C. torulosa*. The *F* values for all populations were evidently positive. The genetic diverdity parameters (N_A, N_E, H_O, H_E and R_S) for each locus over populations of the two species are listed in table 2 in electronic supplementary material.

Genetic differentiation among populations was expressed through pairwise F_{st} values (table 3 in electronic supplementary material). Among all populations, pairwise F_{st} values were almost significant for all pairs ($P < 0.05$, table 3 in electronic supplementary material). Nevertheless, pairwise F_{st} values were all significantly higher than zero ($P < 0.001$) among populations between the two *Cupressus* species (detailed values are marked in red in table 3 in electronic supplementary material). At two randomly picked Cg16 and Cg54 loci (figure 2 in electronic supplementary material), our genotyping results using the ABI 3730xl method were confirmed by those obtained using the PAGE method.

A phylogenetic tree (figure 2) was constructed to examine intergroup relationships. The populations were divided into groups I and II. All populations of *C. torulosa* and *C. gigantea* were categorized into group I with maximum bootstrap support (100%), except LLS, which belongs to *C. gigantea.* Only the population of LLS constituted group II. Group I branched into two subgroups, Ia and Ib (figure 2). The Ia subgroup consisted of *C. gigantea* populations, namely, BJC, DGN, GDC, LCN, LCS, LLN, LSS, MLN, RCS, RES, XZN and XZS. Meanwhile, all populations of *C. torulosa* were grouped in the Ib subgroup in the tree with a relatively high bootstrap support (82%). The populations showed genetic similarities within the Ib subgroup. The higher the similarity in the genetic structure among populations, the easier would be the clustering would be into one group.

PCoA was performed to analyse each individual among the populations. The PCoA result revealed that the first three principal co-ordinated contribute 54.54% of the total variations (22.76%, 20.85% and 10.93% for PCo1, PCo2 and PCo3, respectively). The 16 populations, which were based on the biplot created using the first two PCs (43.61% of the total variability), fell into three sides of the plot (figure 3). Twelve populations of *C. gigantea* were placed into the right side of the plot, three populations of *C. torulosa* were positioned into the left side of the plot, and the population LLS clustered into the middle of the plot (figure 3: groups A, C and B, respectively). The clustering result was also consistent with the intergroup relationship analyses (figure 2).

Genetic structure based on Bayesian analysis

Bayesian clustering was implemented using the STRUCTURE v2.3.4 software. When default settings and the LOCPRIOR model were used, the high peak of delta *K* was found at $K = 2$ based on the delta *K* method (Evanno *et al.* 2005). Thus, the most likely value of *K* would be 2. Population stratification for $K = 2$ showed clear differences between group I (red bars) and group II (green bars) (figure 4). All 13 populations of *C. gigantea* were classified into group I (figure 4, red bars), and all three populations of *C. torulosa* were fell into group II (figure 4, green bars). A relatively high level of admixture was detected in the *C. gigantea* population of LLS, which was consistent with the results of intergroup relationship analyses and PCoA (figures 2 and 3).

Genetic variation at different levels

AMOVA of the 16 wild populations of the two *Cupressus* species indicated that most existing genetic variation was distributed within populations. For *C. gigantea* populations, 93% genetic variation was presented within populations (table 2), with the remaining 7% variation observed among populations ($F_{st} = 0.074$, $P < 0.001$; table 2). Similarly, 95% of the genetic variation for *C. torulosa* populations was observed within populations, with the remaining 5% variation detected among populations ($F_{st} = 0.046$, $P < 0.002$; table 2). Additionally, only 23% variation was observed between the two species ($F_{st} = 0.287$, $P < 0.001$; table 2).

Geographic and genetic correlations

No significant correlation was detected between pairwise F_{st} values and pairwise geographic distances among the 13 populations of *C. gigantea*, and three populations of *C. torulosa* ($r^2 = 0.0008$ ($P = 0.258$) and 0.2024 ($P = 0.495$), respectively) (figure 3 in electronic supplementary material). Similar result was found between pairwise genetic distance and pairwise geographic distances among the populations of *C. gigantea* and *C. torulosa* ($r^2 = 0.0012$

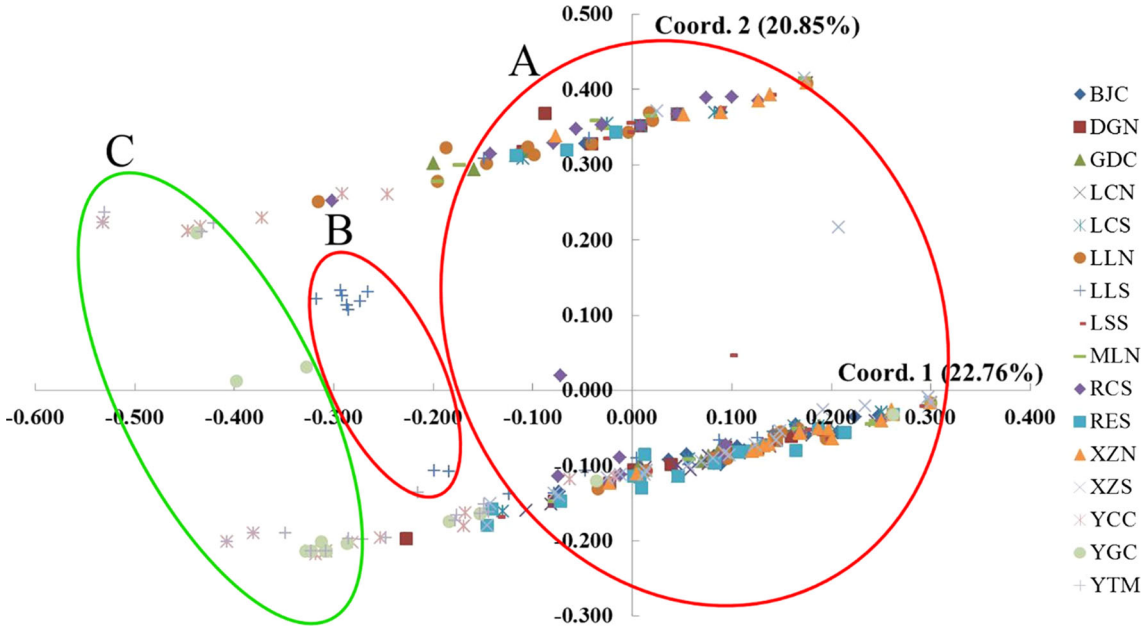

Figure 3. Principal co-ordinates analyses (PCoA) of 16 natural populations for *C. gigantea* and *C. torulosa* resulted as intercomparison of individual groups. Twelve populations in group A were from *C. gigantea* (BJC, DGN, GDC, LCN, LCS, LLN, LSS, MLN, RCS, RES, XZN and XZS), population LLS in group B were also from *C. gigantea*, and all *C. torulosa* populations (YCC, YGC and YTM) were in group C.

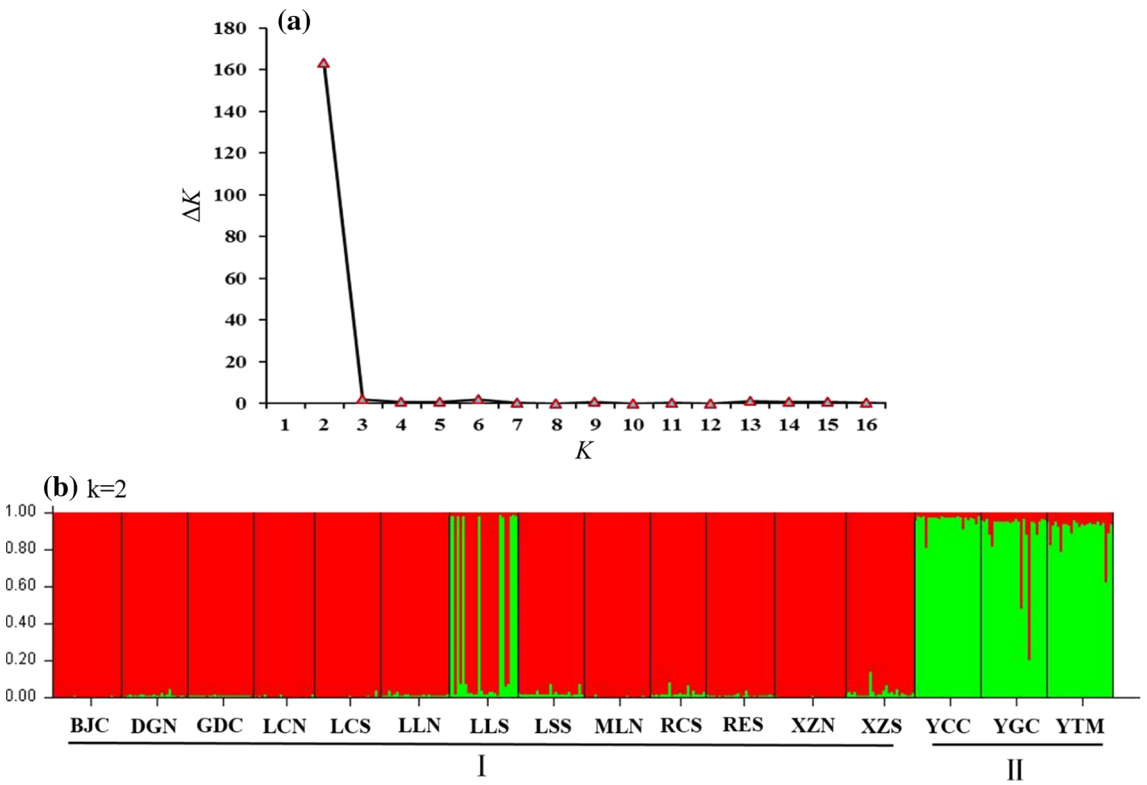

Figure 4. Genetic structure of the two *Cupressus* species obtained in the Bayesian analyses with the LOCPRIOR option using the program STRUCTURE v2.3.4. (a) The delta *K* shows a clear peak at the true value of *K*, suggesting two is the most likely number of cluster.

Table 2. AMOVA for the wild populations of two *Cupressus* species.

Cupressus species grouping	Source of variation	df	SS	*VC*	%V	*P* value
C. gigantea	Among populations	12	68.474	0.091	7	< 0.001
	Within populations	639	726.916	1.138	93	
C. torulosa	Among populations	2	6.613	0.047	5	< 0.002
	Within populations	147	142.700	0.971	95	
Total	Among species	1	93.073	0.360	23	< 0.001
	Among populations	14	75.087	0.085	6	
	Within populations	786	869.616	1.106	71	

Df, degrees of freedom; SS, sum of squares; VC, variance component; %V, percentage of variance; *P* value estimated by a permutation procedure based on 1000 replicates.

($P = 0.256$) and 0.0052 ($P = 0.505$), respectively) (figure 3 in electronic supplementary material).

Discussion

Genetic diversity

Heterozygosity is widely used an as indicators of genetic diversity in wild populations (Freeland *et al.* 2011). These indicators ($I = 0.391$, $H_O = 0.192$, and $H_E = 0.222$) were higher in the 13 populations of *C. gigantea* were higher than in *C. torulosa* ($I = 0.334$, $H_O = 0.152$, and $H_E = 0.190$) (table 1). However, genetic diversity was lower in the natural populations of *C. gigantea* or *C. torulosa* compared with other *Cupressus* species reported, such as *C. funebris* ($H_O = 0.506$, and $H_E = 0.637$), *C. duclouxiana* ($H_O = 0.539$, and $H_E = 0.622$), *C. chengiana* ($H_O = 0.528$, and $H_E = 0.669$), and *C. gigantea* ($H_O = 0.778$, and $H_E = 0.587$) (Lu *et al.* 2013). However, the genetic diversity among these species was not directly compared in this study because of differences in sampling and SSR loci. The difference observed was caused by the narrow and isolated distributions of the threatened species with lower genetic diversity than widespread taxa (Nybom 2004; Poudel 2012). The QTP began a severe uplift in the Pliocene, and the uplift continued through the quaternary glaciations. Many research showed that the entire plateau was covered by a huge ice sheet during the glacial ages, forcing most species to retreat to refugia on the edge of the plateau during glacial maxima (Trinkler 1930; Kuhle 1988; Gupta *et al.* 1992). The harshness of the climatic condition possibly affected plant survival, and the machinery involved in DNA replication and other processes was very robust. Hence, despite sampling over a large geographical area, the genetic diversity was very low among the populations. Moreover, population XZN for *C. gigantea* showed the lowest genetic diversity because of its remoteness from the Yarlung Tsangpo River. The distribution of the plant was relatively isolated because of the reduced population density resulting from highly fragmented distribution of individuals and populations.

Previous studies established a very high genetic diversity of the six populations of *C. gigantea* through the SSR technique with different SSR primers identified from related species, namely, *C. funebris* (Li *et al.* 2013a), *C. chengiana* (Xu *et al.* 2008), and *C. sempervirens* (Sebastiani *et al.* 2005). By contrast, in our previous study, we first screened all SSR primers reported from six related species, including the three aforementioned species and three other related species, namely, *Juniperus cedrus* (Rumeu *et al.* 2013), *Juniperus przewalskii* (Zhang *et al.* 2008), and *Thujopsis dolabrata* (Mishima *et al.* 2012). None of the amplified SSR primers were polymorphic for *C. gigantea*, Thus, in the current study, we used the partial genomic sequences of *C. gigantea* to screen polymorphic SSR primers (Li *et al.* 2014). The high genetic diversity of *C. gigantea* from the literature was primarily attributed to the low number of samples analysed or other unknown reasons. Although outcrossing, long-lived species showed a moderate or high level of genetic diversity (Hamrick and Godt 1996a; Nybom 2004). Our results showed that the populations of the two *Cupressus* species in the QTP still contained relatively low levels of genetic diversity.

Genetic differentiation and population structure

Genetic differentiation, an important indicator of the genetic structure, among plant populations is caused by long-term evolution, which is related to the distribution, habitat fragmentation and isolated populations of species (Schaal *et al.* 1998). F_{st} values > 0.25 reflect strong genetic differentiation (Wright 1949), whereas F_{st} values between 0.05 and 0.15 among populations indicate moderate genetic differentiation (Wright 1978). In this study, most of the pairwise F_{st} values among the populations of *C. gigantea* and *C. torulosa* were lower than 0.05 (table 3 in electronic supplementary material); hence, weak genetic differentiation existed among the populations of the two *Cupressus* species. Moreover, the overall population differentiation observed in the two species was lower than the average F_{st} observed in gymnosperms (Nybom 2004). Nevertheless, higher F_{st} values were observed between the

two species rather than within each species (table 3 in electronic supplementary material), which resulted from the limited wind-mediated or insect-mediated pollen flow caused by complex mountainous areas and long-distance separation in the QTP.

AMOVA results showed that the average genetic variation was higher within populations rather than among populations for both *C. gigantea* and *C. torulosa*. This finding is also commonly observed among other outcrossing and perennial species that generally maintain high levels of genetic variations within populations (Poudel 2012; Zhang *et al.* 2015). The fixation index (F) among all populations for *C. gigantea*, and *C. torulosa* was positive (table 1). This characteristic showed heterozygote deficiency in these two *Cupressus* species, indicating a restricted gene flow among the populations with a strong genetic drift in the small isolated populations (Shah *et al.* 2008; Dubreuil *et al.* 2010). Thus, a relatively high and significant proportion of the total variation was found (23% of the total variation, $P < 0.001$; table 2) among the species in this study, which was lower than that for *Tilia cordata* and *Tilia platyphyllos* (Logan *et al.* 2015). The lower species differentiation might have been caused by later diversion of the two *Cupressus* species compared with the two *Tilia* species. Additionally, the variance within all populations of *C. gigantea* and *C. torulosa* that contributed to total genetic variation was 71%. The obtained results are consistent with the observation that woody species with a predominantly outcrossing breeding system maintain a high genetic diversity within populations (Yoichi and Tomaru 2014).

The Bayesian clustering analysis, the distance-based phylogeny analysis, and the PCoA indicated clear genetic structures for the 16 populations of the two *Cupressus* species, which showed that *C. gigantea* and *C. torulosa* were clearly categorized into two genetic clusters (figures 2–4). This result suggested that high genetic differentiation developed between the two species. High F_{st} values observed between the populations of the two species also explained the remarkable genetic differentiation between *C. gigantea* and *C. torulosa*. In addition, this phenomenon was characterized by decreased gene flow between the two species with increasing geographical distances without the river (Crispo and Hendry 2005). The genetic discrepancy between the two species may have resulted from the adaptive selection to varied environmental factors and geographic isolation (Hamrick and Godt 1996b). However, a low genetic differentiation was observed among the populations of *C. gigantea* and *C. torulosa*, as shown by the low F_{st} value, many of which were not significant, except for population LLS (table 3 in electronic supplementary material). No significant correlation was found between population pairwise F_{st} values and geographic distances (figure 3 in electronic supplementary material) and between genetic distances and geographic distances (figure 3 in electronic supplementary material) for the two species, respectively. Hence, gene flow may not be limited by geographical distances within each species. Natural individuals of *C. gigantea* are mainly distributed in the Yarlung Tsangpo River basin, which can facilitate seed dispersal by river flow and wind. The conditions mentioned were the most probable explanation for gene exchange between all populations. This result is similar to that of *C. torulosa*, with natural individuals of *C. torulosa* distributed on the southeast QTP along the Yigong River.

Liepelt *et al.* (2009) reported that the genetic background of population adapted to the environment of the migrated plants. Genetic recombination may occur along the path, resulting in the current population. The QTP uplift and climatic fluctuation influenced the biodiversity formation in the area, causing isolated population, the initiation of allopatric speciation and divergence, and then gene flow reduction were then induced (Li *et al.* 2013b). Numerous high mountains, freezing temperature, and retreat of glaciers from the quaternary in the QTP created potential refugia for cold-tolerant species (Wen *et al.* 2014). Quaternary climatic changes further accelerate the biodiversity formation of the QTP through isolation of species distributions during glacial periods (Liu and Tian 2007; Qiu *et al.* 2011). In this study, the LLS population was clustered into a single group (figures 2 and 3). This result may be due to the fact that the LLS population is located in the middle reaches of Yarlung Tsangpo River, and it is very close to river bank. The LLS population may be a front population in the present expansion by river flow, forming the current situation. Although many genetic resources from ancestries spread to the migration front by subsequent dispersal, most front populations did not contain sufficient genetic diversity. Another possibility was the divergence of plant lineages following the early uplifts of the QTP or even prior to the formation of the plateau. Therefore, the rapid uplifts of the QTP led to the geologic changes that altered the original distribution pattern and collected several populations of *Cupressus* species. After a long period of natural hybridization, these recently expanded populations had relatively high genetic diversity and genetic differentiation, similar to LLS.

In conclusion, this study first revealed the genetic diversity and genetic structure of two *Cupressus* species, *C. gigantea* and *C. torulosa*, from the QTP by using microsatellite markers. The results still support that *C. gigantea* and *C. torulosa* are different *Cupressus* species. Basic genetic information provided basis for conservation of these two valuable germplasms. Low genetic differentiation among populations (table 3 in electronic supplementary material) and clear genetic structures were revealed among populations within the two species (figures 2–4). Thus, *in situ* conservation is a priority to safeguard genetic variation in the two *Cupressus* species. Decreasing local disturbance is also necessary to allow

the regeneration of wild populations. The high level of genetic diversity for populations should be utilized when selecting genetic materials for tree improvement or conservation programmes. Populations with high microsatellite diversity, such as populations LLS and GDC of *C. gigantea* and population YCC of *C. torulosa* should be prioritized for conservation.

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