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Plant recolonization in the Himalaya from the southeastern Qinghai-Tibetan Plateau: Geographical isolation contributed to high population differentiation

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ABSTRACT

The Himalaya–Hengduan Mountains region (HHM) in the southern and southeastern Qinghai-Tibetan Plateau (QTP) is considered an important reservoir and a differentiation center for temperate and alpine plants in the Cenozoic. To reveal how plants responded to the Quaternary climatic oscillations in the QTP, the phylogeographical histories of a few subalpine and alpine plants have been investigated, but nearly all studies used only uniparentally inherited cytoplasmic DNA markers, and only a couple of them included sampling from the Himalaya. In this study, range-wide genetic variation of the Himalayan hemlock (*Tsuga dumosa*), an important forest species in the HHM, was surveyed using DNA markers from three genomes. All markers revealed genetic depauperation in the Himalaya and richness in the Hengduan Mountains populations. Surprisingly, population differentiation of this wind-pollinated conifer is very high in all three genomes, with few common and many private nuclear gene alleles. These results, together with fossil evidence, clearly indicate that *T. dumosa* recolonized the Himalaya from the Hengduan Mountains before the Last Glacial Maximum (LGM), accompanied with strong founder effects, and the influence of the earlier glaciations on demographic histories of the QTP plants could be much stronger than that of the LGM. The strong population differentiation in *T. dumosa* could be attributed to restricted gene flow caused by the complicated topography in the HHM that formed during the uplift of the QTP, and thus sheds lights on the importance of geographical isolation in the development of high plant species diversity in this biodiversity hotspot.

1. Introduction

The uplift history of the Qinghai-Tibetan Plateau (QTP) and effects of the uplift on local climate and biological distribution have drawn tremendous interest from the scientific community (e.g. Hsü, 1981; Wu, 1987; Molnar et al., 1993; An et al., 2001; Spicer et al., 2003; Song et al., 2003; Liu et al., 2006; Owen et al., 2008; Royden et al., 2008; Wang et al., 2008; Hampe and Petit, 2010; Opgenoorth et al., 2010; Shimono et al., 2010). It is reported that the QTP has played an important role in development of the Asian climate system and the East Asian biodiversity (An et al., 2001; Guo et al., 2002; Sun and Wang, 2005). The Himalaya–Hengduan Mountains region (HHM), which extends along the southern frontier to the southeastern rim of the QTP, contains over 20,000 species of vascular plants, and harbors the richest alpine flora on the earth with the prosperity of endemic species (Wu, 1988; Li and Li, 1993). Of great interest is to unravel the mechanisms for the species differentiation in the HHM, one of the world's biodiversity hotspots (Mittermeier et al., 2005).

On the other hand, as the most glaciated mountain region outside of the polar realms (Lehmkuhl and Owen, 2005), the QTP experienced several glacial and interglacial cycles since the middle Pleistocene (ca. 1.17 mya) (Zheng et al., 2002). How did plants in the QTP respond to climatic changes in the Quaternary? The past population processes, such as range fragmentation, refugial isolation and range expansion, could have left footprints on the tempo-spatial distribution of genetic variation in extant populations that could be revealed by the phylogeographical study (Abbott et al., 2000; Avise, 2000; Hewitt, 2000).

In recent years, a few subalpine and alpine plants in the QTP have been phylogeographically surveyed (e.g. Song et al., 2003; Zhang et al., 2005; Meng et al., 2007; Chen et al., 2008; Yang et al., 2008; Wang et al., 2009a,b; Opgenoorth et al., 2010; Shimono et al., 2010), suggesting different demographic histories of these species. All these studies used only uniparentally inherited cytoplasmic DNA markers except direct sequencing of the nrDNA ITS in *Aconitum gymnandrum*, and only a couple of them included a wide sampling from the central QTP and the Himalaya (Yang et al., 2008; Opgenoorth et al., 2010; Shimono et al., 2010). In

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addition, although most of the previous studies suggest that the influence of the LGM on flora is weaker in the QTP than in the high latitudes of Europe and North America, the influence of the earlier glaciations is controversial. Furthermore, in the Hengduan Mountains that served as a refugium for some plants such as *Pedicularis longiflora* (Yang et al., 2008) and *Aconitum gymnandrum* (Wang et al., 2009a) during the Quaternary Ice Ages, the mechanisms for the origin and development of high genetic diversity remain unresolved. Obviously, in order to better understand the Quaternary evolutionary history of the QTP flora, further phylogeographic studies are needed, and DNA markers from different genomes could be used (Avise, 2009).

Tsuga dumosa (Himalayan hemlock) is a long-lived, monoecious species of the family Pinaceae, with wind-dispersed pollen and seeds. It is also an important forest species in the Hengduan Mountains and the eastern and central Himalaya, with an altitude range of ca. 2000–3600 m a.s.l. (Hara, 1971; Stainton, 1972; Fu et al., 1999; Farjon, 2001). This species originated around the Oligocene/Miocene boundary based on the molecular clock estimation (Havill et al., 2008). In the pine family, the predominantly paternal inheritance of chloroplast (cp) and the strictly or predominantly maternal inheritance of mitochondria (mt) present a unique opportunity for evolutionary and population genetic studies (Neale and Sederoff, 1989; White, 1990; Mogensen, 1996; Guo et al., 2005). The maternal mtDNA markers bear imprints of the colonization history of a species by seed that are unaffected by subsequent pollen movements (Petit et al., 1993). In combined with the paternal cpDNA markers, it would be very efficient to study the effects of differential levels of gene flow through seed and pollen and the impact of historical factors on population genetic diversity and structure (e.g. Richardson et al., 2002; Burban and Petit, 2003; Song et al., 2003; Jaramillo-Correa et al., 2006). Compared with the cytoplasmic genomes, the nuclear genome has a larger effective population size, and thus is less sensitive to founder effects (Petit et al., 1993). Single-copy nuclear genes could provide valuable information about population histories (Avise, 2009), and the nuclear gene phylogeography offers an opportunity for a more complete understanding of the mosaic of genealogical patterns evolving in genomes as a response to the history and environment that populations have experienced (Hare, 2001). Additionally, there are rich Cenozoic fossil records of *Tsuga* in the QTP. Therefore, *T. dumosa* provides an excellent material for the phylogeographical study that aims to investigate the biotic response to historical environmental changes in the QTP.

In this study, we used DNA markers from three genomes to investigate genetic variation and its spatial–temporal distribution in *T. dumosa* based on a range-wide population sampling. Our main objectives are: (i) to explore effects of the extremely complex topography in the HHM on plant population genetic structure and species differentiation and (ii) to retrieve the Quaternary history of subalpine tree species in the QTP, especially the change of its distribution in response to the Quaternary glacial and interglacial cycles in the Himalaya, based on both molecular data and fossil records.

2. Materials and methods

2.1. Population sampling

Foliage samples of 503 trees were collected from 18 populations, covering the entire range of *Tsuga dumosa* (D. Don) Eichler. Although *T. dumosa* also occurs in the northernmost areas of some neighboring countries of China along the eastern and central Himalaya that are difficult to access, the five populations CN, YD, DJ, NLM and JL nearly cover its distribution in the Himalaya (Fig. 1, Supplementary Table S1). Each population was represented by 16–33 individuals that were at least 100 m apart, and needles were dried in silica gel. To better understand the phylogeographical history of *T. dumosa*, 35 trees from eight allopatric or sympatric populations of its two congeners, *T. forrestii* and *T. chinensis*, were additionally included in the study as outgroups. Although the systematic position of *T. dumosa* has not been completely resolved (Farjon, 2001; Havill et al., 2008), this species can be easily distinguished from the other two species based on the leaf and cone morphology according to our extensive field investigation. In *T. dumosa*, seed scales are thin and slightly recurved distally, and the leaf apex is obtuse and entire. In contrast, in *T. forrestii* and *T. chinensis*, seed scales are thick and not recurved distally, and the leaf apex is emarginate. The designation, location, elevation and sample size of each population are shown in Supplementary Table S1.

2.2. DNA extraction, PCR amplification, cloning and sequencing

Total DNA extraction and PCR amplification followed the protocols of Yang et al. (2008), except that an annealing temperature of 55 °C was used and the elongation time was adjusted according to the length of different markers. For cytoplasmic genomes, two mtDNA fragments, *nad*5 intron 1 (Wang et al., 2000) and *cox*1 (Duminil et al., 2002), and two cpDNA intergenic spacers, *trn*S/fM (Shaw et al., 2005) and *atp*H/I (Grivet et al., 2001), were amplified and directly sequenced for all 503 individuals of *T. dumosa* and 35 of *T. forrestii* and *T. chinensis*. To amplify the first intron of the *LEAFY* gene, one of the key regulatory genes that is involved in the formation of flower meristem and exists as a single copy in the nuclear genome in most seed plants (Frohlich and Parker, 2000; Peng and Wang, 2008), the primers *LEY*E1F (forward, 5′TGTTGATGGAAAACGCAAGATTG3′) and *LEY*E2R2 (reverse, 5′CCTTTGCAATATGTTGCACATC3′) were designed at two regions conserved among all Pinaceae genera. This primer pair was used to amplify all 503 samples of *T. dumosa*, but amplification failed in 68 individuals, including all individuals from the population GGS, due to DNA degradation. Two distinct bands (approximately 1400 bp and 700 bp, respectively) were consistently obtained from each of the rest 435 individuals, and the BLASTN showed that the sequences of both bands have homology to the *LEAFY* gene. To save cost and to minimize PCR artifacts, only the short copy (≈700 bp) was used in the population genetic analysis (Supplementary Table S2). When this copy was directly sequenced, 188 out of the 435 individuals showed one or more double-peaks in the chromatograms. All these potential heterozygotes and an additional of 34 individuals without double-peaks in the chromatograms were cloned with the pGEM®-T Easy Vector System II (Progmega), and 6–12 clones per individual were sequenced using the primer T7. The results showed that each double-peak represents a polymorphic site, and only one or two different alleles occur in each individual. In addition, 11 trees of *T. forrestii* and *T. chinensis* were included in the nuclear gene analysis. Details of the five DNA markers we used are shown in Supplementary Table S2.

Sequencing was performed using the DYEnamic ET Terminator Kit (Amersham Pharmacia Biotech). The sequencing products were separated on a MegaBACE 1000 automatic sequencer (Amersham Biosciences, Buckinghamshire, UK). The sequences reported in this study are deposited in GenBank under Accession Nos. HM162940–HM163073.

2.3. Data analyses

Molecular diversity indices, including the number of segregating sites (S), number of haplotypes (nh), haplotype diversity (Hd), expected heterozygosity (H_E) and nucleotide diversity (π), were estimated for the species, groups of populations and each population using ARLEQUIN 3.11 (Excoffier et al., 2005). The haplo-

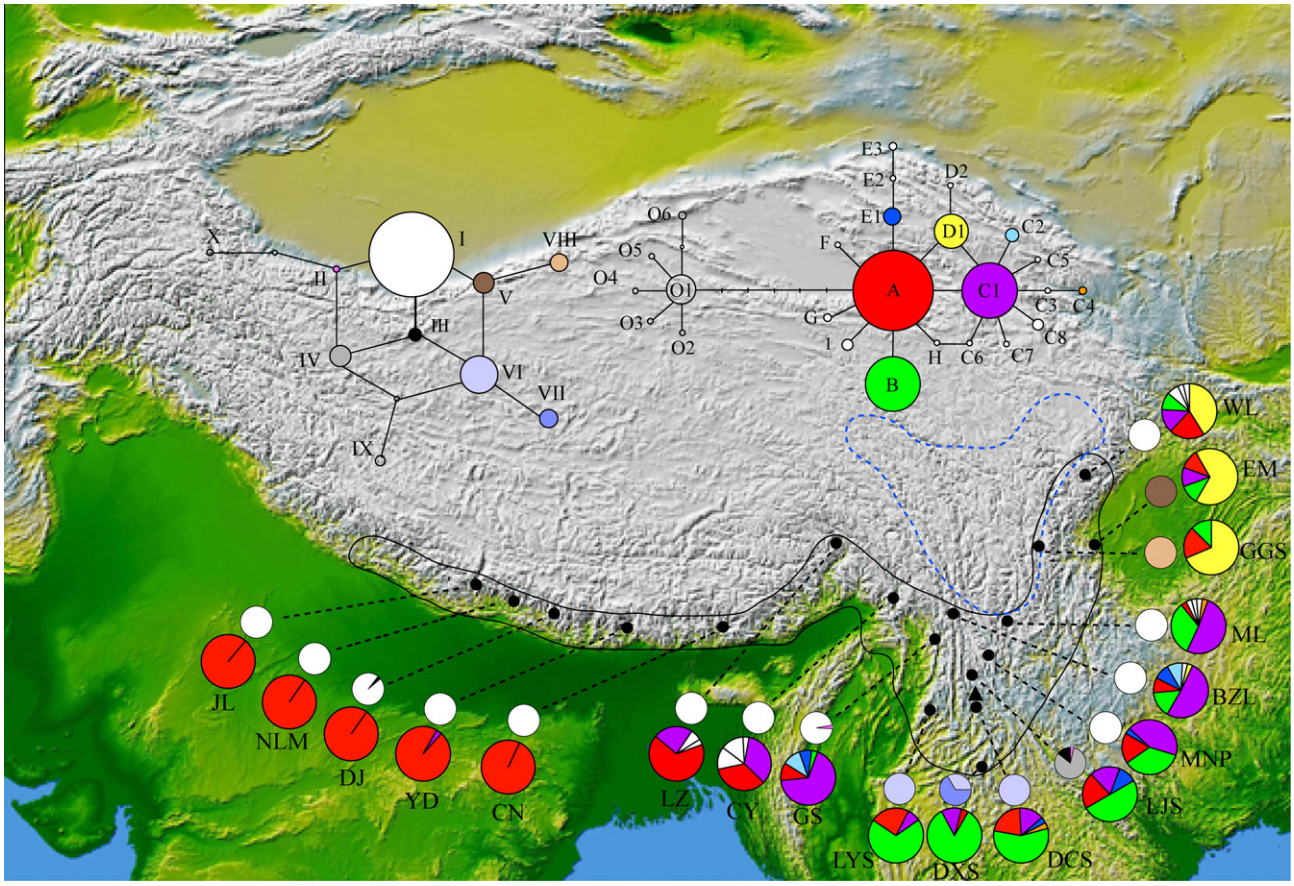

Fig. 1. Sampling locations and distribution frequencies of chlorotypes (in large circles) and mitotypes (in small circles) in *Tsuga dumosa* populations, with networks of the mitotypes (upper left) and chlorotypes (upper right) constructed by using TCS 1.21. Color haplotypes are shared by two or more populations, and blank ones are private haplotypes. Mitotypes IX and X, and chlorotypes O1–O6 occur exclusively in the outgroups. The sizes of the circles in the network are proportional to the observed frequencies of the haplotypes. The blue dashed line indicates the location of the Western Sichuan Plateau, and the black triangle indicates the location of the Diancang Mountain. Population names correspond to those in Supplementary Table S1.

type richness (A) was calculated by dividing the number of haplotypes by the sample size, and the observed frequency of heterozygotes (H_O) was estimated by dividing the number of heterozygotes by the number of sampled individuals. Haplotype networks were constructed using the software TCS 1.21 (Clement et al., 2000).

Based on the physio-geographical boundaries (Compilation group of the Yunnan Vegetation, 1987; Li and Li, 1993), we divided the distribution range of *T. dumosa* into four regions. That is, the Himalaya (Hi) consists of five populations (JL, NLM, DJ, YD, CN); the eastern Hengduan Mountains (EHe) includes populations WL, EM and GGS; the western and central Hengduan Mountains (W&CHe) harbors populations LZ, CY, BZL, GS, ML, MNP and LJS; and the southern Hengduan Mountains (SHe) comprises populations DXS, LYS and DXS. The latter three regions are separated by the Western Sichuan Plateau and the Diancang Mountain in Yunnan province (Fig. 1). In the southwest of the Diancang Mountain, there are few mountains over 3000 m a.s.l., while in the north many mountains reach 4000–4500 m a.s.l. To test whether the predefined regions are consistent with genetically differentiated population groups, the program SAMOVA version 1.0 (Dupanloup et al., 2002) was used to identify groups of populations that are geographically homogenous and maximally differentiated from each other based on a simulated annealing procedure. Moreover, the cluster analysis was conducted to partition genetic groups based on genetic distance (D_A) matrices of cpDNA and nuclear DNA (nDNA), respectively, using unweighted pair-group method with arithmetic averages (UPGMA) (Sneath and Sokal, 1973) with the PHYLIP 3.67 package (Felsenstein, 1993).

An analysis of molecular variance (AMOVA) (Excoffier et al., 1992) was performed to partition variation within and among the defined regions (population groups) and populations using ARLEQUIN. Isolation by distance was tested by regressing D_A, i.e. net nucleotide divergence between populations (Nei and Li, 1979), against geographic distance between populations, using a Mantel test (Mantel, 1967) implemented in the program IBD 1.52 (Bohonak, 2002). We used partial Mantel tests (Smouse et al., 1986) to determine whether a putative physical-barrier could explain a significant component of the variance in D_A.

Mismatch distributions of pairwise differences among alleles of *LEAFY* were calculated using ARLEQUIN for species and groups of populations. If the null hypothesis is not rejected, the formula $\tau = 2ut$ was used to estimate the age of expansion (t), where $u = m_T\mu$ (m_T is the number of nucleotides and μ is the mutation rate of the sequence). To test whether population expansions have occurred with an east–west axial trend, molecular diversity indices were plotted against longitude using EXCEL 2003 (Microsoft). Spearman's rank correlation coefficients were calculated in SPSS 15.0 for Windows (SPSS Inc.) with molecular diversity as the dependent variable and longitude as the independent variable.

3. Results

3.1. Mitochondrial genealogy

The two mtDNA fragments *nad*5 intron 1 and *cox*1 are conserved in length in *Tsuga dumosa*, being 1304 bp and 1293 bp,

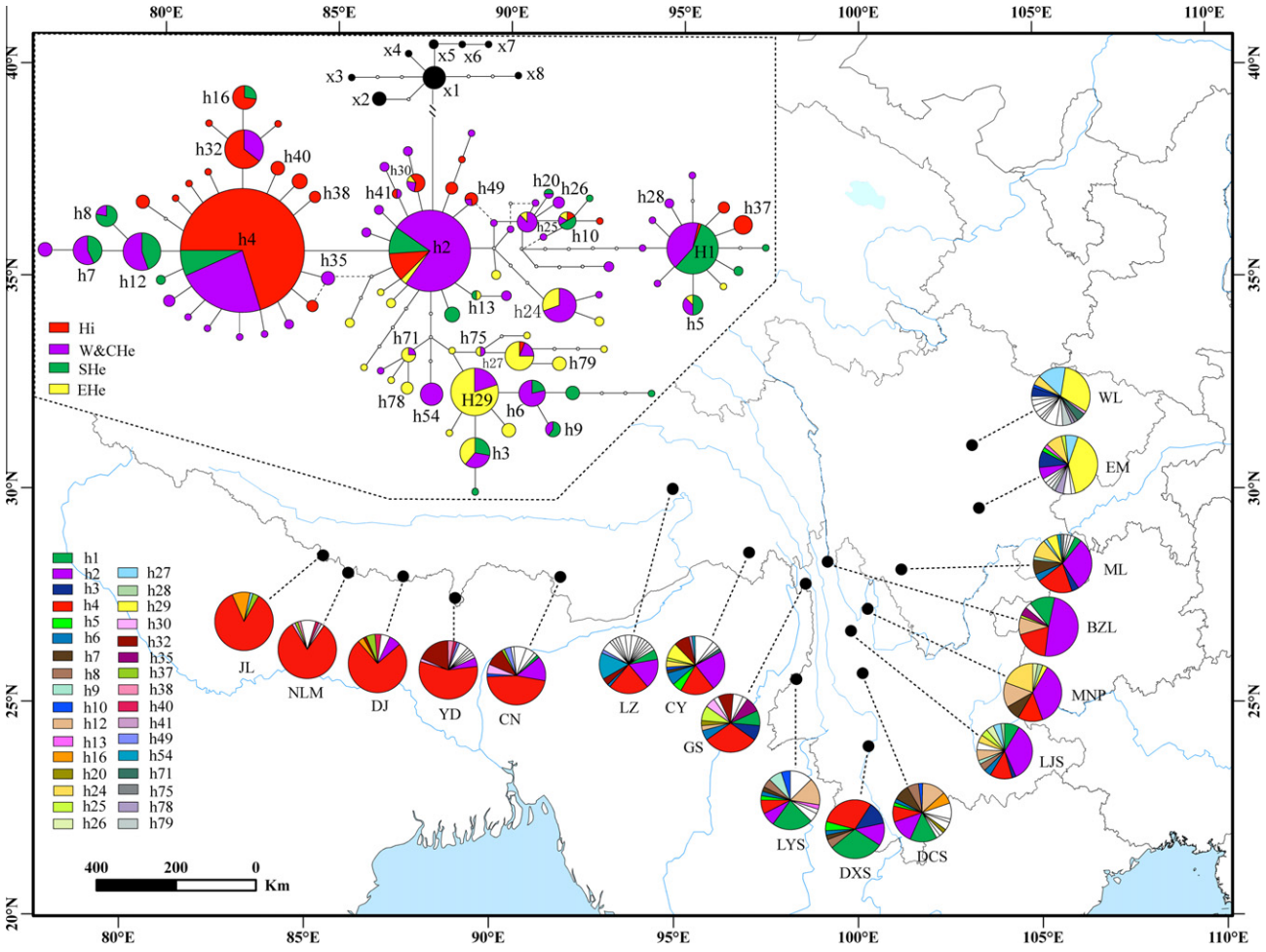

Fig. 2. Geographical distributions and frequencies of the *LEAFY* gene alleles detected in *Tsuga dumosa* populations. Color alleles are shared by two or more populations, and blank ones are private alleles. In the allele network (upper left), the sizes of the circles are proportional to the observed frequencies of the alleles; the numbered alleles occur in two or more populations and the unnumbered ones are private alleles; red, purple, green and yellow colors indicate the allele distribution in the Hi, W&CHe, SHe and EHe regions, respectively. Alleles x1–8 occur in the outgroups. Population names correspond to those in Supplementary Table S1.

respectively. Five single nucleotide polymorphisms were detected, allowing the designation of eight distinct mitochondrial haplotypes (mitotypes). Only two mitotypes have frequencies >5%, and the four mitotypes IV, V, VII and VIII are population-specific (Supplementary Table S3). The mitotypes I–VI occupy central positions in the network (Fig. 1). All eight mitotypes occurred in the Hengduan Mountains, although most populations in this region harbored pure mitotypes. In contrast, all five populations in the Himalaya, i.e. CN, YD, DJ, NLM and JL, were fixed for the mitotype I except for one tree from population DJ (Fig. 1).

3.2. Chloroplast genealogy

Two indels and 12 substitutions were detected from the two cpDNA regions *trn*S/fM (977–979 bp) and *atp*H/I (758–783 bp) of *T. dumosa* samples, and were used to designate 19 chloroplast haplotypes (chlorotypes), none of which occurred in the outgroups. Only four chlorotypes were common (frequency >5%), including A (43.5%), B (20.3%), C1 (20.7%) and D1 (8.0%), and the frequencies of the remaining 15 rare chlorotypes ranged from 0.2% to 2.0% (Supplementary Table S3). The network of chlorotypes indicates that A is ancestor to the others and away from B, C1 and D1 by a single mutation (Fig. 1). When we replaced *T. forrestii* and *T. chinensis* with *T. canadensis* as outgroup, the position of chlorotype A in the network did not change. Similar to the distribution of mitotypes, all the five Himalayan populations were fixed for chlorotype A except one individual from population YD. Although all 19 chlorotypes had distribution in the Hengduan Mountains region, only A, C1 and B were widespread. In particular, 12 of the 19 chlorotypes were population-specific and occurred in five Hengduan Mountains populations (LZ, CY, BZL, ML, WL) (Fig. 1).

3.3. Nuclear genealogy

Among the 435 individuals of *T. dumosa* that were finally used in the *LEAFY* gene analysis, 247 and 188 were homozygotes and heterozygotes, respectively. Only one or two distinct sequences were detected in each individual, and a total of 90 alleles (haplotypes), ranging from 675 bp to 683 bp in length, were designated based on 92 substitutions and three indels in the sequence alignment (685 bp, excluding outgroups). Thirty-three alleles were shared among populations, but only four of them had frequencies >5%, including h1 (5.7%), h2 (14.8%), h4 (32.4%) and h29 (5.2%). The rest 57 private alleles were distributed in 14 populations, respectively, with a total frequency of 11.6% (Fig. 2, Supplementary Table S4). In the network rooted with the eight sequences from *T. chinensis* and *T. forrestii*, the allele h2 is ancestral to the others, and the majority of the private haplotypes (51/57) are tip alleles. When *T. chinensis* and *T. forrestii* were replaced by *T. mertensiana* as outgroup, the position of the allele h2 in the network did not

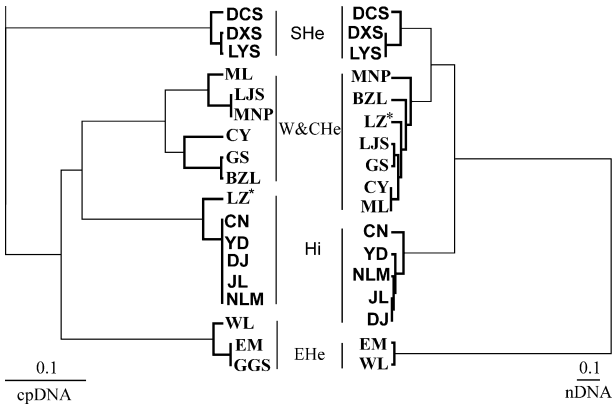

Fig. 3. UPGMA clustering of *Tsuga dumosa* populations based on net nucleotide divergence (D_A; Nei and Li, 1979) of cpDNA (left) and nDNA (right) markers. Hi, Himalaya; W&CHe, western and central Hengduan Mountains; SHe, southern Hengduan Mountains; EHe, the eastern Hengduan Mountains.

change. In the Hengduan Mountains 75 alleles were detected, while in the Himalaya only 25 alleles were found. The allele h4 occurred in all populations, except WL and EM in the eastern Hengduan Mountains, and was dominant in the Himalaya region. In contrast, h1, h2 and h29 were dominant in the Hengduan Mountains except that h2 also had a high frequency in the two populations from the east of the Himalaya. Overall, the *LEAFY* gene shows great allele diversity, richness of population-specific allele, and clear geographic structure.

3.4. Genetic differentiation and population structure

The clustering of populations in the UPGMA dendrogram based on genetic distance (D_A) of nuclear DNA is completely in accordance with the four regions (Hi, W&CHe, EHe, SHe) predefined according to the physic–geographic boundaries. The UPGMA clustering of populations based on cpDNA also coincides with that based on nuclear DNA except that population LZ was classified into the Hi group (Fig. 3). The SAMOVA analysis indicated that the F_{CT} value, the proportion of total genetic variation among groups, reached a plateau ($F_{CT} = 0.35332$, $P < 0.001$) when the number of groups (K) was set to 4 for the cpDNA data (Fig. 4a), while it reached the maximum ($F_{CT} = 0.32237$, $P < 0.01$) at $K = 2$ for the nDNA data (Fig. 4b). Thus, most evidence suggests that the populations of *T. dumosa* be divided into four groups, one in the Himalaya (Hi) and the other three in the Hengduan Mountains (W&CHe, EHe, SHe). It is of particular interest that the distributions of the four

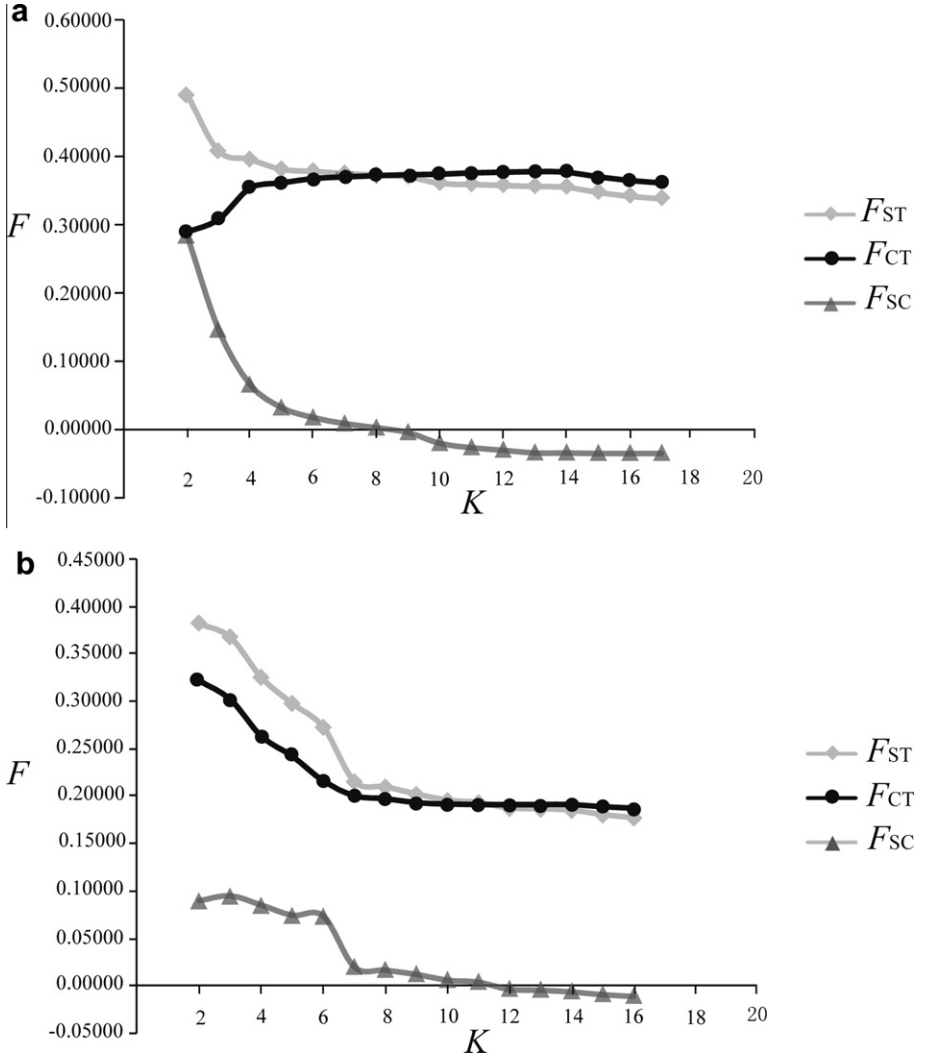

Fig. 4. Plots of the values of fixation indices (F) obtained from SAMOVA as a function of the user-defined number of groups (K). (a) Based on chlorotypes; (b) based on the nuclear *LEAFY* gene alleles. F_{ST}, differentiation among populations; F_{CT}, differentiation among groups of populations; F_{SC}, differentiation among populations within groups.

Table 1
Results of analyses of molecular variance (AMOVAs), and Mantel tests for different DNA markers and groups of populations in *Tsuga dumosa*.

Region		mtDNA	cpDNA		nDNA	
		$F_{ST}(F_{CT})$	$F_{ST}(F_{CT})$	r	$F_{ST}(F_{CT})$	r
Himalaya	Hi	−0.00000^{NS}	−0.00000^{NS}	–	0.03147**	0.8076*
Hengduan Mountains	SHe	0.67324	0.02568^{NS}	–	−0.01031^{NS}	–
	EHe	1.00000	0.00825^{NS}	–	−0.00378^{NS}	–
	W&CHe	0.89372	0.10956	0.5398*	0.01624^{NS}	−0.0558^{NS}
	Divided into 3 groups	0.96189 (0.62684)	0.33295 (0.27157)	0.5369	0.18926 (0.17529)	0.6339
Entire range	Divided into 4 groups	0.95781 (0.62032)	0.39550 (0.34030)	0.4074	0.22050 (0.20464)	0.5116**
	Treated as 1 group	0.94974	0.33712		0.17604	

^{NS} $P \geq 0.05$.
* $0.05 > P \geq 0.01$.
** $0.01 > P \geq 0.001$; and for the remaining estimates, $P < 0.001$.

Table 2
Genetic parameters of 18 *Tsuga dumosa* populations.

Region	Pop.	Organelle DNA								nDNA						
		mtDNA			cpDNA											
		n	S	h	S	h	A	π	H_d	n	S	h	A	π	H_E	H_O
Hengduan Mountains	DXS	25	1	2	2	3	0.120	0.000297	0.2900	20	19	8	0.400	0.008760	0.8026	0.1500
	LYS	26	0	1	2	3	0.115	0.000350	0.4800	20	25	14	0.700	0.009431	0.9064	0.4000
	DCS	28	0	1	4	5	0.179	0.000609	0.6270	23	27	16	0.696	0.009062	0.9266	0.3043
	SHe	79	1	2	4	5	0.063	0.000433	0.4823	63	33	22	0.587	0.009106	0.8999	0.2857
	LJS	28	2	3	3	4	0.143	0.000668	0.7381	23	28	14	0.609	0.006646	0.8541	0.2609
	MNP	28	0	1	3	4	0.143	0.000626	0.6799	18	21	8	0.444	0.004689	0.8063	0.3333
	GS	28	1	2	4	5	0.179	0.000449	0.5291	18	28	13	0.722	0.007497	0.8841	0.5000
	BZL	33	0	1	5	7	0.212	0.000691	0.7064	32	13	7	0.219	0.004191	0.7093	0.3438
	ML	31	0	1	6	7	0.226	0.000738	0.6452	25	29	15	0.600	0.006357	0.8596	0.2800
	CY	29	0	1	4	5	0.172	0.000639	0.7488	29	30	16	0.552	0.006406	0.8917	0.7931
	LZ	30	0	1	3	4	0.133	0.000335	0.5216	27	34	20	0.741	0.006069	0.9022	0.7778
	W&CHe	207	2	4	12	16	0.077	0.000657	0.7267	127	61	50	0.541	0.006027	0.8712	0.4826
	EM	22	0	1	2	4	0.182	0.000370	0.4675	22	32	15	0.682	0.006421	0.8171	0.5909
	GGS	16	0	1	2	3	0.188	0.000410	0.5083	–	–	–	–	–	–	–
	WL	29	0	1	6	7	0.241	0.000764	0.7759	29	34	18	0.621	0.006485	0.8724	0.5517
	EHe	67	2	3	6	7	0.104	0.000555	0.6232	51	44	27	0.647	0.006465	0.8517	0.5686
	HM	353	5	8	14	19	0.054	0.000722	0.7796	286	86	75	0.262	0.007592	0.9167	0.4545
Himalaya	JL	30	0	1	0	1	0.033	0.000000	0.0000	30	17	4	0.133	0.001804	0.2706	0.1000
	NLM	30	0	1	0	1	0.033	0.000000	0.0000	30	17	8	0.267	0.001112	0.3299	0.3000
	DJ	30	1	2	0	1	0.033	0.000000	0.0000	30	14	7	0.233	0.002076	0.4328	0.3667
	YD	30	0	1	1	2	0.067	0.000040	0.0667	30	13	12	0.400	0.001529	0.6480	0.5333
	CN	30	0	1	0	1	0.033	0.000000	0.0000	29	19	12	0.414	0.004057	0.7538	0.6552
	Hi	150	1	2	1	2	0.013	0.000008	0.0133	149	34	25	0.168	0.002163	0.5149	0.3893
Entire range	Total	503	5	8	14	19	0.038	0.000573	0.7207	435	95	90	0.207	0.006131	0.8551	0.4322

The parameters include sample size (n), number of segregating sites (S), number of haplotypes (h), haplotype richness (A), nucleotide diversity (π), haplotype diversity (H_d), expected heterozygosity (H_E) and the observed heterozygosity (H_O). HM, Hengduan Mountains.

common nuclear *LEAFY* alleles (h1, h2, h4, h29) are very similar to those of the four common chlorotypes (A, B, C1, D1), i.e. A and h4 dominating in the Hi region, C1 and h2 in W&CHe, B and h1 in SHe, and D1 and h29 in EHe (Figs. 1 and 2).

T. dumosa populations were significantly differentiated throughout the HHM region. The AMOVA analysis showed that the differences among the four groups (Hi, W&CHe, EHe, SHe) explained 62.0% of the total mtDNA variation, 34.0% of cpDNA and 20.5% of nDNA (Table 1). For mtDNA, which varied in only four of the 18 surveyed populations, 33.7% of the total variation occurred among populations within regions and only 4.2% within populations. In contrast, for cpDNA and nDNA that are mainly distributed by pollen-flow, a large portion of the total variation existed within populations (cpDNA: 60.5%; nDNA: 78.0%). Nevertheless, in the Hengduan Mountains, the mtDNA, cpDNA and nDNA variations among populations still account for 96.2%, 33.3% and 18.9%, respectively (Table 1). Interestingly, all the five Himalayan populations except one tree were fixed for one mitotype (I) and one chlorotype (A) (Fig. 1), and only a small proportion (3.1%) of the nDNA variation occurred among populations (Table 1), besides that the nDNA diversity ($\pi = 0.002163$; $A = 0.168$; $H_E = 0.515$) was much lower in the Himalaya than in the Hengduan Mountains populations ($\pi = 0.007592$; $A = 0.262$; $H_E = 0.917$) (Table 2). A significant association between genetic and geographical distances was detected with the Mantel test for the entire range (cpDNA: $r = 0.407$, $P < 0.001$; nDNA: $r = 0.512$, $P < 0.01$) and for the Hengduan Mountains populations (cpDNA: $r = 0.537$, $P < 0.001$; nDNA: $r = 0.634$, $P < 0.001$). In particular, from population CN in the east to population JL in the west, the nDNA diversity gradually decreased while the frequency of allele h4 gradually increased ($r = 1.000$; $P < 0.001$) along the Himalaya, and significantly positive correlations were detected between the genetic diversity indices (A, H_E, H_O) and longitude ($r = 0.900$, 1.000, and 0.956, respectively; all $P < 0.05$) (Fig. 5).

Partial Mantel tests based both on cpDNA and nDNA showed that the Western Sichuan Plateau had served as a barrier to gene flow between the two regions EHe and W&CHe (cpDNA: $r = 0.715$, $P < 0.01$; nDNA: $r = 0.898$, $P < 0.05$) (Table 3). In addition,

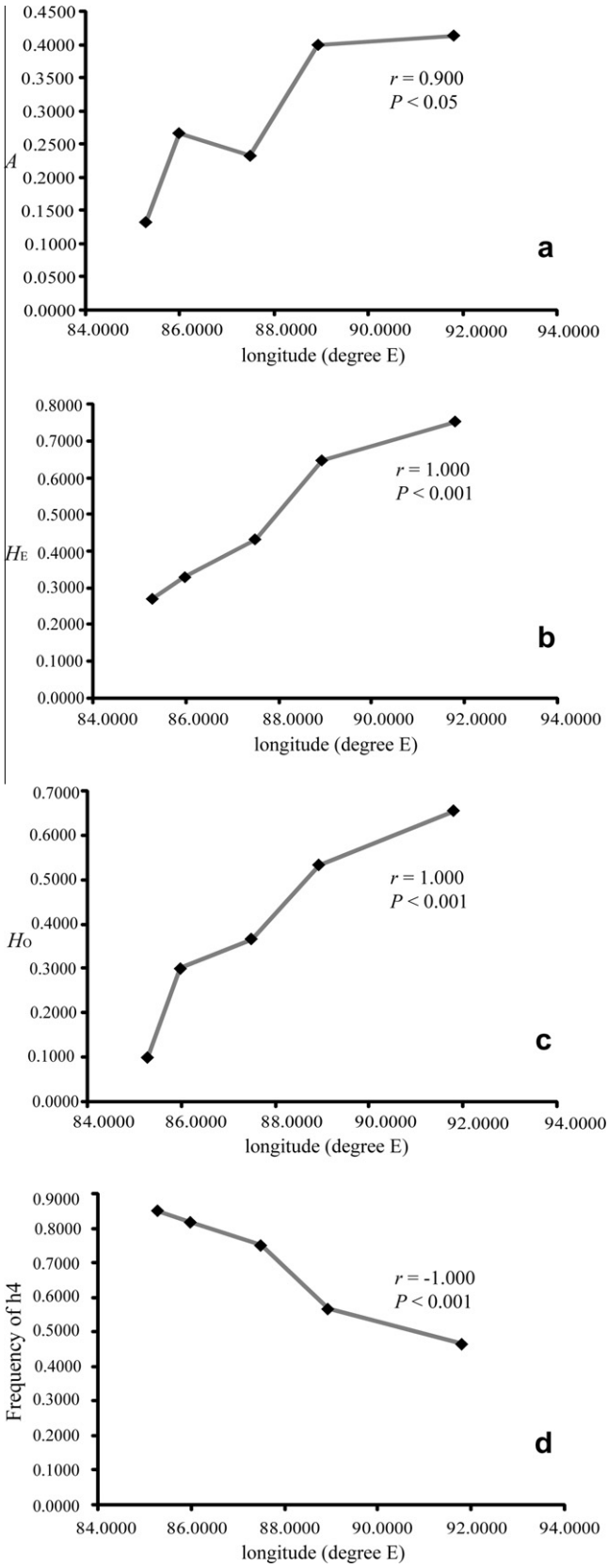

Fig. 5. The Spearman's correlation coefficient between longitude and genetic diversity indices estimated from the *LEAFY* sequences for the Himalaya populations of *Tsuga dumosa*. (a) Haplotype richness within population (*A*). (b) Expected heterozygosity within population (H_E). (c) Observed heterozygosity within population (H_O). (d) The frequency of the allele h4.

Table 3
Results of partial Mantel tests for the effects of hypothesized biogeographical barriers in the range of *Tsuga dumosa*.

Pair of regions	r_1[a]		r_2[b]	
	cpDNA	nDNA	cpDNA	nDNA
Hi vs. W&CHe	0.3522*	0.6735***	0.1775NS	0.3077*
W&CHe vs. SHe	0.4456*	0.2223NS	0.6143**	0.6377**
W&CHe vs. EHe	0.0647NS	0.1157NS	0.7152**	0.8981*

[a] Partial correlation between genetic and geographical distances when controlling for indicator matrix.
[b] Partial correlation between genetic distance and indicator matrices when controlling for geographical distance.
NS $P \geq 0.05$.
* $0.05 > P \geq 0.01$.
** $0.01 > P \geq 0.001$.
*** $P < 0.001$.

the scatterplot showing the relationship between genetic and geographical distances suggests that the divergence between the EHe region and the remaining range of *T. dumosa* is significant (Fig. 6a). When the presence of hypothesized physical-barrier between SHe and W&CHe was controlled, the correlation between geographical and genetic (cpDNA) distances remained significant ($r = 0.446$, $P < 0.05$), indicating that the barrier alone is not sufficient to account for the observed genetic structure (Table 3).

3.5. Population expansion and population's demographic history

In the Himalayan populations, mismatch distributions of nuclear alleles fitted the demographic expansion model ($P_{SSD} = 0.748$), showing two peaks in the distribution curve, i.e. the steep main peak at zero-difference and the other minor peak with a value of 10-differnece (Fig. 6b). The expansion time was estimated to be 0.730 Ma (0–2.796 Ma in 95% CI), when a divergence time of 19.76 Ma between *T. dumosa* and its two close relatives *T. chinensis* and *T. forrestii* was used as the calibration point (Havill et al., 2008). Although a sudden expansion model was also supported for the whole sample of *T. dumosa* ($P_{SSD} = 0.818$) (Supplementary Fig. S1a), the Hengduan Mountains ($P_{SSD} = 0.835$) (Fig. 6c) and its three parts, i.e., W&CHe ($P_{SSD} = 0.841$), EHe ($P_{SSD} = 0.407$) and SHe ($P_{SSD} = 0.533$) (Supplementary Fig. S2b, c, d), the curves of the mismatch distributions included three or four modes.

4. Discussion

4.1. Recolonization of Tsuga dumosa into the Himalaya region from its glacial refugia in the Hengduan Mountains

Previous phylogeographical studies of a few subalpine and alpine plants found that these plants postglacially recolonized the central platform of the QTP from their LGM refugia in the lower altitudes of the eastern or southeastern edge of the plateau (Zhang et al., 2005; Meng et al., 2007; Chen et al., 2008), or probably survived the LGM, even the earlier glaciations, on the platform (Yang et al., 2008; Wang et al., 2009a,b; Hampe and Petit, 2010; Opgenoorth et al., 2010; Shimono et al., 2010). However, the recolonization route and the estimated time of population expansion differ in different groups. For instance, Yang et al. (2008) suggested that the population expansion of *Pedicularis longiflora* on the platform from its refugia in the Hengduan Mountains occurred before the LGM, probably during the last interglaciation, while Shimono et al. (2010) found that *Potentilla fruticosa* contracted to the interior of the QTP during interglaciations and expanded its range during periods of climatic cooling. All these studies focused on the Quater-

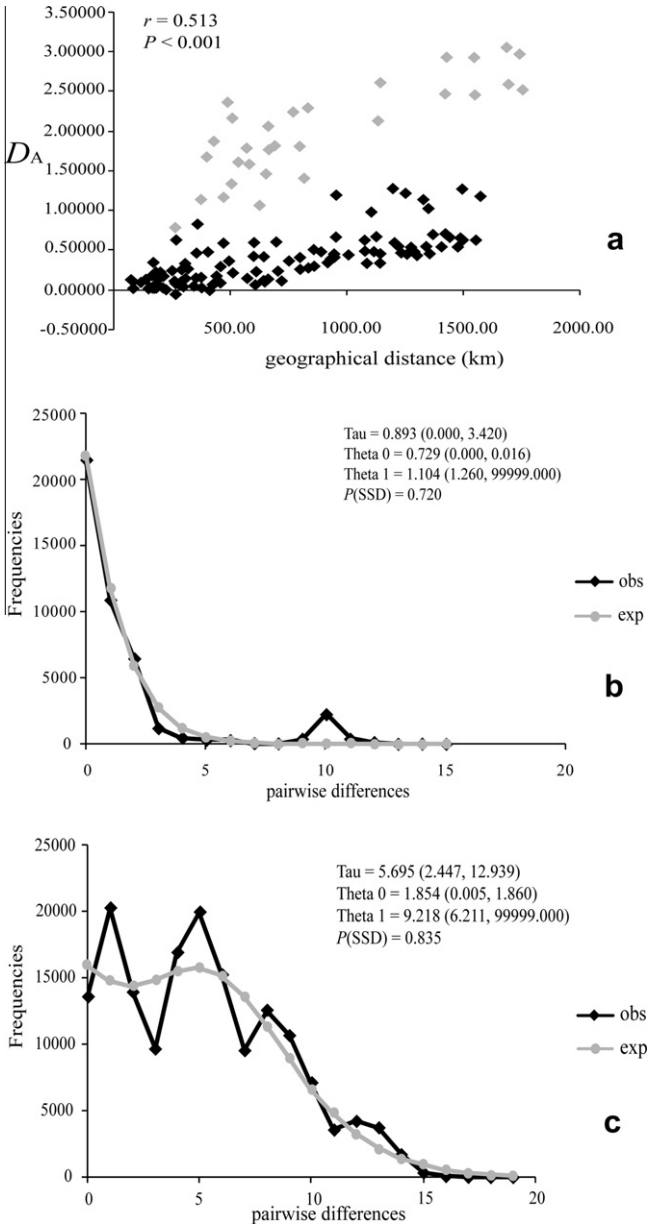

Fig. 6. A scatterplot showing the relationship between genetic distance (D_A, based on nDNA) and geographical distance for all *Tsuga dumosa* populations investigated (a), and the mismatch distributions for the Himalayan (Hi) populations (b) and the Hengduan Mountains populations (c). Gray squares in (a) represent D_A values between populations EM, WL and the remaining populations. The gray and black lines in (b) and (c) indicate the expected and observed mismatch distributions, respectively, based on segregating sites of the aligned *LEAFY* sequences.

nary history of the populations on the QTP platform, with less attention paid to the subalpine vegetation zone where species diversity is much higher, and only a couple of them included sampling from the Himalaya (Yang et al., 2008; Opgenoorth et al., 2010; Shimono et al., 2010).

In the present study, range-wide genetic variation of *Tsuga dumosa* was surveyed using markers from paternal chloroplast, maternal mitochondrial and biparental nuclear genomes. We found that all five Himalayan populations are fixed for the mitotype I (except one tree from DJ) and the chlorotype A (except one individual from YD), whereas all eight mitotypes and 19 chlorotypes we detected occur in the Hengduan Mountains populations. In addition, both mitotype I and chlorotype A are ancestral haplotypes (Fig. 1). The distributions of cytoplasmic DNA haplotypes, together with fossil evidence (discussed later), strongly suggest that the Hengduan Mountains provided refugia for *T. dumosa* during the Quaternary glaciations, and that strong founder effects occurred during the recolonization of this species into the Himalaya region. This inference is corroborated by the findings in the nuclear gene analysis, such as the much lower allele diversity in the Himalaya (25 alleles) than in the Hengduan Mountains (75 alleles), and the gradual increase in frequency of the allele h4 along the Himalaya from east to west (Fig. 2). Furthermore, according to the mismatch distribution for the *LEAFY* gene, the Himalaya populations could have undergone recent demographic expansions (Fig. 6b). The steep peak at zero-difference in the mismatch distribution curve suggests sudden population expansion following bottleneck (Rogers and Harpending, 1992).

It is very likely that the recolonization of *T. dumosa* along the Himalaya from the Hengduan Mountains, covering a distance of ca. 1000 km, experienced the leading-edge leptokurtic dispersal, and sequential bottlenecks occurred along the migration route (Hewitt, 1996; Ibrahim et al., 1996). When a species underwent sequential founding events, allelic diversity and expected heterozygosity would significantly decrease as the number of founder events separating populations increased (Clegg et al., 2002; Pruett and Winker, 2005). As expected, the three genetic diversity indices A, H_E and H_O of *T. dumosa* populations show a gradual east-to-west decrease along the Himalaya (Table 2), and a significant positive correlation exists between these indices and the longitudes of sampling sites (Fig. 5a–c). In addition, the frequency distribution of allele h4 provides corroborative evidence for the effects of cumulative founding events during the westward recolonization of *T. dumosa* (Fig. 5d). This allele was gradually fixed along the Himalaya from population CN to population JL.

The recolonization history of *T. dumosa* in the Himalaya is also supported by the fossil records (Supplementary Fig. S2, Table S5). Hemlock pollen was found in almost all formations of the mid-Miocene to the early Quaternary in the southern QTP, including both southern and northern slopes of the Himalaya (Lukose, 1968; Hsü et al., 1973, 1976; Nandi, 1975; Li and Guo, 1976; Song and Liu, 1982; Huang and Liang, 1983; Li and Liang, 1983; Mathur, 1984; Sarkar et al., 1994; Qian, 1999; Spicer, 2003). In particular, palynological analyses in two independent lacustrine deposition sequences (ca.7.0–1.67 Ma) in the Gyirong Basin of central Himalaya continuously found hemlock pollen with high frequencies, even up to 10% in the interval of 4.1–4.5 Ma in one sequence (Wang et al., 1998; Sun et al., 2007). According to these palynological data and the macrofossil floras from Wulong (ca. 15 mya), Yebokangjiale and Jiabula Formations (late Pliocene) (Hsü et al., 1973, 1976; Li and Guo, 1976; Qian, 1999; Spicer et al., 2003), coniferous and broad-leaved mixed forests, containing hemlock, were predominant in the southern QTP from the mid-Miocene to the early Quaternary (Hsü, 1981; Tang and Shen, 1996). Then the vegetation succession in the QTP was primarily influenced by climatic oscillations during the cycles of glaciation and interglaciation, and the coniferous and broad-leaved mixed forests retreated from the Himalaya since the middle Quaternary (Tang and Shen, 1996). However, *T. dumosa* very likely recolonized the Himalaya from the Hengduan Mountains before the LGM and survived the LGM in the southern slope of the Himalaya, considering the wide occurrence of its late Pleistocene pollen in the Thimi Formation and the older Gokarna Formation in the Kathmandu valley of central Nepal (Paudayal and Ferguson, 2004; Paudayal, 2005, 2006) and other *Tsuga* fossils in the late Quaternary sediments of Nepal (Bhandari et al., 2009; Schlütz and Zech, 2004). Interestingly, the population expansion time (0. 730 Ma) of *T. dumosa* that we estimated largely corresponds to the middle Pleistocene transition (Maasch, 1988), and it does not violate the palynological data of hemlock in the Himalaya mentioned above. Although the esti-

mated time may be not very accurate due to the long coalescence time and potential reticulate evolution of nuclear gene alleles (Bermingham and Moritz, 1998), it is much earlier than the LGM.

One may argue that *T. dumosa* had also survived the earlier glaciations in situ in the Himalaya. However, it is unlikely that there was a refugium for the species in the northwest Himalaya considering its fossil records and present distribution (Supplementary Fig. S2, Table S5), and it is also nearly impossible that there was a large refugium in the other region of the Himalaya due to the complicated topography. If *T. dumosa* survived the earlier glaciations in isolated small populations in the Himalaya by shifting altitude in response to the Quaternary climatic oscillations, we would expect random fixation of the DNA haplotypes rather than single cytoplasmic haplotype and a gradual decrease in nuclear gene diversity in the Himalayan populations from east to west (Figs. 1 and 2).

The QTP experienced four major glaciations during the Quaternary (Zheng et al., 2002), and, as suggested by increasing studies, the glaciations had become less extensive after the most extensive Naynayxungla Glaciation (ca. 0.72–0.5 Ma) (Zheng et al., 2002; Owen and Benn, 2005; Ehlers and Gibbard, 2007), particularly after 0.17 Ma (Schäfer et al., 2002). Recent studies also suggest that there were only limited glacier advances during the LGM in the QTP, including the Himalaya (summarized in Lehmkuhl and Owen, 2005). Currently, it is generally accepted that there was not a unified ice sheet covering the QTP, at least not during the last few glacial cycles of the Quaternary (Shi, 2002; Owen et al., 2008), and thus potential habitats for cold-tolerant species could be found in the plateau in that period. Recent phylogeographical studies suggest that *Pedicularis longiflora* (Yang et al., 2008), *Juniperus tibetica* complex (Opgenoorth et al., 2010) and *Potentilla glabra* (Wang et al., 2009b) survived the LGM on the QTP platform, while *Potentilla fruticosa* (Shimono et al., 2010) and *Aconitum gymnandrum* (Wang et al., 2009a) could have survived the recent few or all Quaternary glaciations on the platform. Based on the chlorotype distribution and molecular clock estimation, Yang et al. (2008) suggested that *Pedicularis longiflora* recolonized the QTP platform probably during the last interglaciation. Therefore, the phylogeographical history of the alpine herb *P. longiflora* and our present study of the subalpine conifer *T. dumosa* indicate that the influence of the earlier glaciations on the demographic histories of the QTP plants could be much stronger than that of the LGM. This reference is also supported by the phylogeographic patterns recovered from *Pyrgilauda ruficollis* (Qu et al., 2005) and *Pseudopodoces humilis* (Yang et al., 2006), two bird species recolonizing the QTP platform before the LGM.

As mentioned earlier, the Hengduan Mountains region would have served as a refugium for *T. dumosa* during the Quaternary Ice Ages, given the distribution of all cytoplasmic haplotypes and 75 of the 90 *LEAFY* alleles in this area (Figs. 1 and 2; Supplementary Table S4). Although *T. dumosa* occurs sympatrically with *T. forrestii* or *T. chinensis* in some localities at the eastern rim of the Hengduan Mountains, it inhabits higher altitudes. In addition, all the six chlorotypes detected in *T. forrestii* and *T. chinensis* do not occur in *T. dumosa*, and all 12 polymorphic sites found in the two cpDNA markers of *T. dumosa* are species-specific (Supplementary Table S3). In particular, all the *LEAFY* alleles of *T. dumosa* form a monophyly (Fig. 2). Therefore, the high genetic diversity in the Hengduan Mountains populations of *T. dumosa* could not have originated from interspecific gene flow among the three species.

Furthermore, the fossil evidence also strongly suggests that *T. dumosa* was widely distributed in the Hengduan Mountains in the Pliocene (Tao and Kong, 1973; Tao and Du, 1982; Liu and Li, 2002; Xu et al., 2003, 2004; Yi et al., 2005; Supplementary Fig. S2, Table S5). The fossil woods of *T.* cf. *dumosa* were found in the Yangyi Formation in western Yunnan (Yi et al., 2005). Of great interest is the temporal change of species composition in the Mula Formation (Pliocene to early Pleistocene, ca. >1.24 mya), Litang County, Sichuan Province (Chen et al., 1986). *Tsuga* sp., including its cones, twigs and leaves, was the major element in the early stage, but was not found in the middle to late stages, during which alpine shrub taxa and cold temperate conifers, very similar to the present flora, were dominant (Supplementary Fig. S2). Therefore, the geographical distribution of *T. dumosa* in the Hengduan Mountains before the Quaternary Ice Ages is likely similar to that in the present, although a history of altitude shift accompanied with population size fluctuation was reflected by the palynological assemblages in the Heqing deep drilling core (2.78 mya to present), northwest Yunnan (Xiao et al., 2007).

4.2. Geographical isolation contributed to high plant population differentiation and speciation in the Hengduan Mountains

Tropical montane regions are thought to be sources and reservoirs of genetic and species diversity (Fjeldså and Lovett, 1997; Hewitt, 2000), since these regions harbored topographic complexity and relatively ecoclimatic stability throughout the Quaternary climatic oscillations, which are important for speciation to occur and survival of relict taxa (Fjeldså and Lovett, 1997; Hewitt 2000; Tzedakis et al., 2002). In the present study, we detected a high genetic diversity of *T. dumosa* from the Hengduan Mountains, which is consistent with previous findings in other plants (Chen et al., 2008; Yang et al., 2008; Wang et al., 2009a). Surprisingly, population differentiation of this wind-pollinated conifer is also very high in all of the three genomes, with 95.0%, 33.7% and 17.6% of the mt-, cp- and nDNA variation occurring among populations, respectively (Table 1). These values are significantly higher than the averages (mtDNA: 0.764; cpDNA: 0.165; nDNA: 0.116) summarized by Petit et al. (2005) based on 20, 37 and 33 conifers, respectively. Moreover, it is notable that only four of the 90 *LEAFY* alleles have frequencies higher than 5% and 57 (63.3%) alleles are private to 14 populations, respectively (Fig. 2, Supplementary Table S4). The high levels of genetic differentiation and the unique phylogeographic structure of *T. dumosa* could be attributed to restricted gene flow caused by the complex topography in the Hengduan Mountains. Such an inference is also supported by the high population differentiation documented by cytoplasmic DNA markers in some other conifers distributed in the QTP (Song et al., 2003; Gao et al., 2007). The strong geographical isolation might also have contributed to rapid species diversification in the Hengduan Mountains, since the factors underlying population differentiation are representative of those leading to species divergence to some extent (Avise et al., 1987). In fact, fast radiation has been reported in some plant groups in the QTP (Wei and Wang, 2004; Liu et al., 2006; Ran et al., 2006). For example, in the genus *Larix*, the rapid species and variety differentiation in the HHM region was accompanied with strong founder effects in the evolution of nrDNA paralogues (Wei et al., 2003; Wei and Wang, 2004).

A range of geological studies suggest that the quick uplift of the eastern QTP occurred ca. 13 to 5 Ma, although uplift of the central QTP probably started much earlier (Harris, 2006; Royden et al., 2008). *T. dumosa* likely originated in or first migrated into the W&CHe region following the uplift of the eastern QTP in the mid-Miocene, considering that the most primitive allele h2 is dominant in this region (Fig. 2). The upthrust of the eastern QTP and the ensuing intense denudation created extremely intricate topography, containing numerous lofty ridges and deep gorges. These geographic barriers, together with the Western Sichuan Plateau, would have greatly restricted gene flow and led to relatively independent evolution of gene pools in isolated montane blocks. In *T. dumosa*, 10 of the 13 Hengduan Mountains populations are fixed for a single mitotype, respectively, suggesting that seed flow between these populations is negligible (Fig. 1). Also, the distributional patterns

of the four common *LEAFY* alleles (h1, h2, h4, h29) indicate that long-distance pollen-flow is extremely low. All of the four alleles occur in the W&CHe region, the distribution center of the species, but h1 and h4 are not present in the EHe region, and h29 not in the Himalaya and the SHe region (Fig. 2). Moreover, the effects of biogeographical barriers for gene flow might have been further strengthened by cycles of contraction/expansion of plant distribution that responded to the repeated glacial/interglacial cycles in the HHM (Yang et al., 2008), providing additional driving forces for the high rate of speciation in this region.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ympev.2010.05.007.

References

Abbott, R.J., Smith, L.C., Milne, R.I., Crawford, R.M.M., Wolff, K., Balfour, J., 2000. Molecular analysis of plant migration and refugia in the Arctic. Science 289, 1343–1346.

An, Z.S., Kutzbach, J.E., Prell, W.L., Porter, S.C., 2001. Evolution of Asian monsoons and phased uplift of the Himalaya–Tibetan plateau since Late Miocene times. Nature 411, 62–66.

Avise, J.C., 2000. Phylogeography: The History and Formation of Species. Harvard Univ. Press, Cambridge, MA.

Avise, J.C., 2009. Phylogeography: retrospect and prospect. J. Biogeogr. 36, 3–15.

Avise, J.C., Arnold, J., Ball, R.M., Bermingham, E., Lamb, T., Neigel, J.E., Reeb, C.A., Saunders, N.C., 1987. Intraspecific phylogeography: the mitochondrial DNA bridge between population genetics and systematics. Ann. Rev. Ecol. Syst. 18, 489–522.

Bermingham, E., Moritz, C., 1998. Comparative phylogeography: concepts and applications. Mol. Ecol. 7, 367–369.

Bhandari, S., Momohara, A., Paudayal, K.N., 2009. Late Pleistocene plant macrofossils from the Gokarna formation of the Kathmandu Valley, Central Nepal. Bull. Dept. Geol. (Tribhuvan Univ., Kathmandu, Nepal) 12, 75–88.

Bohonak, A.J., 2002. IBD (isolation by distance): a program for analyses of isolation by distance. J. Hered. 93, 153–154.

Burban, C., Petit, R.J., 2003. Phylogeography of maritime pine inferred with organelle markers having contrasted inheritance. Mol. Ecol. 12, 1487–1495.

Chen, M.H., Kong, Z.C., Chen, Y., 1986. Plant fossils from Mula Formation in Litang County and their significance in palaeogeography and palaeoclimatic. In: The Comprehensive Scientific Expedition to the Qinghai-Xizang Plateau, the Chinese Academy of Sciences (Ed.), Studies in Qinghai-Xizang (Tibet) Plateau Special Issue of Hengduan Mountains Scientific Expedition, vol. 2. Science and Technology Press, Beijing, pp. 71–79.

Chen, S.Y., Wu, G.L., Zhang, D.J., Gao, Q.B., Duan, Y.Z., Zhang, F.Q., Chen, S.L., 2008. Potential refugium on the Qinghai-Tibet Plateau revealed by the chloroplast DNA phylogeography of the alpine species Metagentiana striata (Gentianaceae). Bot. J. Linn. Soc. 157, 125–140.

Clegg, S.M., Degnan, S.M., Kikkawa, J., Moritz, C., Estoup, A., Owens, I.P.F., 2002. Genetic consequences of sequential founder events by an island-colonization bird. Proc. Natl. Acad. Sci. USA 99, 8127–8132.

Clement, M.D., Posada, D., Crandall, K., 2000. TCS: a computer program to estimate gene genealogies. Mol. Ecol. 9, 1657–1660.

Compilation Group of the Yunnan Vegetation, 1987. The Vegetation of Yunnan. Science Press, Beijing.

Duminil, J., Pemonge, M.H., Petit, R.J., 2002. A set of 35 consensus primer pairs amplifying genes and introns of plant mitochondrial DNA. Mol. Ecol. Notes 2, 428–430.

Dupanloup, I., Schneider, S., Excoffier, L., 2002. A simulated annealing approach to define the genetic structure of populations. Mol. Ecol. 11, 2571–2581.

Ehlers, J., Gibbard, P.L., 2007. The extent and chronology of Cenozoic Global Glaciation. Quatern. Int. 164–165, 6–20.

Excoffier, L., Laval, G., Schneider, S., 2005. Arlequin version 3.0: an integrated software package for population genetics data analysis. Evol. Bioinf. Online 1, 47–50.

Excoffier, L., Smouse, P.E., Quattro, J.M., 1992. Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. Genetics 191, 479–491.

Farjon, A., 2001. World Checklist and Bibliography of Conifers, Second ed. Royal Bot. Gard, Kew, England.

Felsenstein, J., 1993. PHYLIP (Phylogeny Inference Package) version 3.5c. Distributed by the Author, Dept. Genetics, Univ. Washington, Seattle, WA.

Fjeldså, J., Lovett, J.C., 1997. Biodiversity and environmental stability. Biodiver. Conserv. 6, 315–323.

Frohlich, M.W., Parker, D.S., 2000. The mostly male theory of flower evolutionary origins: from genes to fossils. Syst. Bot. 25, 155–170.

Fu, L.G., Li, N., Mill, R.R., 1999. Tsuga. In: Wu, Z.Y., Raven, P.H. (Eds.), Flora of China (4). Science Press, Beijing and Missouri Botanical Garden Press, St. Louis, pp. 39–41.

Gao, L.M., Möller, M., Zhang, X.M., Hollingsworth, M.L., Liu, J., Mill, R.R., Gibby, M., Li, D.Z., 2007. High variation and strong phylogeographic pattern among cpDNA haplotypes in *Taxus wallichiana* (Taxaceae) in China and North Vietnam. Mol. Ecol. 16, 4684–4689.

Grivet, D., Heinze, B., Vendramin, G.G., Petit, R.J., 2001. Genome walking with consensus primers: application to the large single copy region of chloroplast DNA. Mol. Ecol. Notes 1, 345–349.

Guo, F., Hu, S.Y., Yuan, Z., Zee, S.Y., Han, Y., 2005. Paternal cytoplasmic transmission in Chinese pine (*Pinus tabulaeformis*). Protoplasma 225, 5–14.

Guo, Z.T., Ruddiman, W.F., Hao, Q.Z., Wu, H.B., Qiao, Y.S., Zhu, R.X., Peng, S.Z., Wei, J.J., Yuan, B.Y., Liu, T.S., 2002. Onset of Asian desertification by 22 Myr ago inferred from loess deposits in China. Nature 416, 159–163.

Hampe, A., Petit, R.J., 2010. Cryptic forest refugia on the 'Roof of the World'. New Phytol. 185, 5–7.

Hara, H., 1971. The Flora of Eastern Himalaya, Second Report. Univ. of Tokyo Press, Tokyo. pp. 12.

Hare, M.P., 2001. Prospects for nuclear gene phylogeography. Trends Ecol. Evol. 16, 700–706.

Harris, N., 2006. The elevation history of the Tibetan Plateau and its implications for the Asian monsoon. Palaeogeogr. Palaeocl. 241, 4–15.

Havill, N.P., Campbell, C.S., Vining, T.F., LePage, B., Bayer, R., Donoghue, M.J., 2008. Phylogeny and biogeography of *Tsuga* (Pinaceae) inferred from nuclear ribosomal ITS and chloroplast DNA sequence Data. Syst. Bot. 33, 478–489.

Hewitt, G.M., 1996. Some genetic consequences of ice ages, and their role in divergence and speciation. Biol. J. Linn. Soc. 58, 247–276.

Hewitt, G.M., 2000. The genetic legacy of the Quaternary ice ages. Nature 405, 907–913.

Hsü, J., 1981. Vegetational changes in the past and uplift of Qinghai-Xizang Plateau. In: Liu, D.L. (Ed.), Geological and Ecological Studies of Qinghai-Xizang Plateau, vol. 1. Science Press, Beijing and Gordon and Breach Science Publishes, Inc., New York, pp. 139–144.

Hsü, J., Kong, Z.C., Sun, X.J., 1976. A research of the Quaternary palaeobotany in the Everest region. In: Tibetan Scientific Expedition Team from Chinese Academy of Sciences (Ed.), A Report on Scientific Expedition to Mount Everest Region (1966–1968): Quaternary Geology. Science Press, Beijing, pp. 93–103.

Hsü, J., Tao, J.R., Sun, X.J., 1973. On the discovery of a *Quercus semicarpifolia* Bed in Mount Shisha Pangma and its significance in botany and geology. Acta Bot. Sin. 15, 103–119.

Huang, C.X., Liang, Y.L., 1983. Sporo-pollen assemblages from the deposits of the Qungdogyang Basin, Qusum in the early Pleistocene, and their palaeogeographical significance. In: Li, B.Y., Wang, F.B., Zhang, Q.S. (Eds.), Quaternary Geology in Xizang. Science Press, Beijing, pp. 167–171.

Ibrahim, K.M., Nichols, R.A., Hewitt, G.M., 1996. Spatial patterns of genetic variation generated by different forms of dispersal during range expansion. Heredity 77, 282–291.

Jaramillo-Correa, J.P., Beaulieu, J., Ledig, F.T., Bousquet, J., 2006. Decoupled mitochondrial and chloroplast DNA population structure reveals Holocene collapse and population isolation in a threatened Mexican-endemic conifer. Mol. Ecol. 15, 2787–2800.

Lehmkuhl, F., Owen, L.A., 2005. Late Quaternary glaciation of Tibet and the bordering mountains: a review. Boreas 34, 87–100.

Li, H.M., Guo, S.X., 1976. The Miocene flora from Namling of Xizang. Acta Palaeontol. Sin. 15, 7–18.

Li, W.Y., Liang, Y.L., 1983. Sporo-pollen analysis on the lacustrine deposits in Zanda Basin during the Pliocene. In: Li, B.Y., Wang, F.B., Zhang, Q.S. (Eds.), Quaternary Geology in Xizang. Science Press, Beijing, pp. 132–144.

Li, X.W., Li, J., 1993. A preliminary floristic study on the seed plants from the region of Hengduan Mountains. Acta Bot. Yunnan. 15, 217–231.

Liu, G.W., Li, W.T., 2002. Upper cretaceous and tertiary of western Sichuan Plateau. J. Stratigr. 26, 161–169.

Liu, J.Q., Wang, Y.J., Wang, A.L., Hideaki, O., Abbott, R.J., 2006. Radiation and diversification within the *Ligularia–Cremanthodium–Parasenecio* complex (Asteraceae) triggered by uplift of the Qinghai-Tibetan Plateau. Mol. Phylogenet. Evol. 38, 31–49.

Lukose, N.G., 1968. Microfossils from the Middle Siwalik of Bihar. India. J. Palynol. 4, 107–112.

Maasch, K.A., 1988. Statistical detection of the mid-Pleistocene transition. Clim. Dynam. 2, 133–143.

Mantel, N., 1967. The detection of disease clustering and a generalization regression approach. Cancer Res. 27, 209–220.

Mathur, Y.K., 1984. Cenozoic palynofossils, vegetation, ecology and climate of the north and northwestern Sub-Himalayan region. In: Whyte, R.O. (Ed.), The

Evolution of the East Asian Environment, vol. 2. Center of Asian Studies, Univ. of Hong Kong, pp. 504–551.
Meng, L.H., Yang, R., Abbott, R.J., Miehe, G., Hu, T.H., Liu, J.Q., 2007. Mitochondrial and chloroplast phylogeography of *Picea crassifolia* Kom (Pinaceae) in the Qinghai-Tibetan Plateau and adjacent highlands. Mol. Ecol. 16, 4128–4137.
Mittermeier, R.Z., Gil, P.R., Hoffman, M., Pilgrim, J., Brooks, T., Mittermeier, C.G., Lamoreux, J., da Fonseca, G.A., 2005. Hotspots Revisited: Earth's Biologically Richest and Most Endangered Terrestrial Ecoregions. Cemex, Conservation International and Agrupación Sierra Madre, Monterrey, Mexico.
Mogensen, H.L., 1996. The hows and ways of cytoplasmic inheritance in seed plants. Am. J. Bot. 83, 383–404.
Molnar, P., England, P., Martinod, J., 1993. Mantle dynamics, uplift of the Tibetan Plateau and the Indian Monsoon. Rev. Geophy. 31, 357–396.
Nandi, B., 1975. Palynostratigraphy of the Siwalik group of Punjab. Himalayan Geol. 5, 411–424.
Neale, D.B., Sederoff, R.R., 1989. Paternal inheritance of chloroplast DNA and maternal inheritance of mitochondrial DNA in loblolly pine. Theor. Appl. Genet. 77, 212–216.
Nei, M., Li, W.H., 1979. Mathematical model for studying genetic variation in terms of restriction endonucleases. Proc. Natl. Acad. Sci. USA 76, 5269–5273.
Opgenoorth, L., Vendramin, G.G., Mao, K., Miehe, G., Miehe, S., Liepelt, S., Liu, J., Ziegenhagen, B., 2010. Tree endurance on the Tibetan Plateau marks the world's highest known tree line of the Last Glacial Maximum. New Phytol. 185, 332–342.
Owen, L.A., Benn, D.I., 2005. Equilibrium-line altitudes of the Last Glacial Maximum for the Himalaya and Tibet: an assessment and evaluation of results. Quatern. Int. 138–139, 55–78.
Owen, L.A., Caffee, M.W., Finkel, B.C., Seong, Y.B., 2008. Quaternary glaciation of the Himalayan–Tibetan orogen. J. Quatern. Sci. 23, 513–531.
Paudayal, K.N., 2005. Late Pleistocene pollen assemblages from the Thimi formation Kathmandu Valley, Nepal. Island Arc 14, 328–337.
Paudayal, K.N., 2006. Late Pleistocene pollen assemblages from Gokarna Formation (Dhapasi section) in Kathmandu Valley, Nepal. J. Nepal Geol. Soc. 33, 33–38.
Paudayal, K.N., Ferguson, D.K., 2004. Pleistocene palynology of Nepal. Quatern. Int. 117, 69–79.
Peng, D., Wang, X.Q., 2008. Reticulate evolution in *Thuja* inferred from multiple gene sequences: implications for the study of biogeographical disjunction between eastern Asia and North America. Mol. Phylogenet. Evol. 47, 1190–1202.
Petit, R.J., Duminil, J., Fineschi, S., Hampe, A., Salvini, D., Vendramin, G.G., 2005. Comparative organization of chloroplast, mitochondrial and nuclear diversity in plant populations. Mol. Ecol. 14, 689–701.
Petit, R.J., Kremer, A., Wanger, D.B., 1993. Finite island model for organelle and nuclear genes in plants. Heredity 71, 630–641.
Pruett, C.L., Winker, K., 2005. Northwestern song sparrow populations show genetic effects of sequential colonization. Mol. Ecol. 14, 1421–1434.
Qian, F., 1999. Study on magnetostratigraphy in Qinghai-Tibetan Plateau in late Cenozoic. J. Geomechan. 5, 22–34.
Qu, Y.H., Ericson, P.G.P., Lei, F.M., Li, S.H., 2005. Postglacial colonization of the Tibetan plateau inferred from the matrilineal genetic structure of the endemic red-necked snow finch, *Pyrgilauda ruficollis*. Mol. Ecol. 14, 1767–1781.
Ran, J.H., Wei, X.X., Wang, X.Q., 2006. Molecular phylogeny and biogeography of *Picea* (Pinaceae): implications for phylogeographical studies using cytoplasmic haplotypes. Mol. Phylogenet. Evol. 41, 405–419.
Richardson, B.A., Brunsfeld, S.J., Klopfenstein, N.B., 2002. DNA from bird-dispersed seed and wind-disseminated pollen provides insights into postglacial colonization and population genetic structure of whitebark pine (*Pinus albicaulis*). Mol. Ecol. 11, 215–227.
Rogers, A.R., Harpending, H., 1992. Population growth makes waves in the distribution of pairwise genetic differences. Mol. Biol. Evol. 9, 552–569.
Royden, L.H., Burchfiel, B.C., van der Hilst, R.D., 2008. The geology evolution of the Tibetan Plateau. Science 321, 1054–1058.
Sarkar, S., Bhattacharya, A.P., Sing, H.P., 1994. Palynology of Middle Siwalik sediments (late Miocene) from Bagh Rao, Uttar Pradesh. Palaeobotanist 42, 199–209.
Schäfer, J.M., Tschudi, S., Zhao, Z.Z., Wu, X.H., Ivy-Ochs, S., Wieler, R., Baur, H., Kubik, P.W., Schlüchter, C., 2002. The limited influence of glaciation in Tibet on global climate over the past 170,000 yr. Earth Planet. Sci. Lett. 194, 287–297.
Schlütz, F., Zech, W., 2004. Palynological investigations on vegetation and climate change in the Late Quaternary of Lake Rukche area, Gorkha Himal, Central Nepal. Veg. Hist. Archaeobot. 13, 81–90.
Shaw, J., Lickey, E.B., Beck, J.T., Farmer, S.B., Liu, W.S., Miller, J., Siripun, K.C., 2005. The tortoise and the hare II: relative utility of 21 noncoding chloroplast DNA sequences for phylogenetic analysis. Am. J. Bot. 92, 142–166.
Shi, Y.F., 2002. Characteristics of late Quaternary monsoonal glaciation on the Tibetan Plateau and in East Asia. Quatern. Int. 97–98, 79–91.
Shimono, A., Ueno, S., Gu, S., Zhao, X., Tsumura, Y., Tang, Y., 2010. Range shifts of Potentilla fruticosa on the Qinghai-Tibetan Plateau during glacial and interglacial periods revealed by chloroplast DNA sequence variation. Heredity 104, 534–542.
Smouse, P.E., Long, J.C., Sokal, R.R., 1986. Multiple regression and correlation extensions of the Mantel test of matrix correspondence. Syst. Zool. 35, 627–632.
Sneath, P.H.A., Sokal, R.R., 1973. Numerical Taxonomy: the Principles and Practice of Numerical Classification. W.H. Freeman, San Francisco, CA, USA.
Song, B.H., Wang, X.Q., Wang, X.R., Ding, K.Y., Hong, D.Y., 2003. Cytoplasmic composition in *Pinus densata* and population establishment of the diploid hybrid pine. Mol. Ecol. 12, 2995–3001.
Song, Z.C., Liu, J.L., 1982. The tertiary sporo-pollen assemblages from Namling of Xizang. The Comprehensive Scientific Expedition to the Qinghai-Xizang Plateau, Academia Sinica (Ed.), Palaeontology of Xizang, vol. 5. Science Press, Beijing, pp. 153–164.
Spicer, R.A., Harris, N.B.W., Widdowson, M., Herman, A.B., Guo, S.X., Valdes, P.J., Wolfe, J.A., Kelley, S.P., 2003. Constant elevation of southern Tibet over the past 15 million years. Nature 421, 622–624.
Stainton, J.D.A., 1972. Forests of Nepal. Hafner Publishing Company, New York. pp. 109–111.
Sun, L.M., Yan, T.S., Tang, G.Y., Ding, X.Y., Wang, R.J., Tian, L.F., 2007. Neogene sporopollen assemblages and paleogeography in the Gyirong basin, Tibet. Geology (China) 34, 49–54.
Sun, X.J., Wang, P.X., 2005. How old is the Asian monsoon system? – Palaeobotanical records from China. Palaeogeogr. Palaeocl. 222, 181–222.
Tao, J.R., Du, N.Q., 1982. Neogene flora of Tengchong basin in western Yunnan, China. Acta Bot. Sin. 24, 273–281.
Tao, J.R., Kong, Z.C., 1973. The fossil florule and sporo-pollen assemblage of the Shang-In Coal Series of Erhyuan, Yunnan. Acta Bot. Sin. 15, 120–126.
Tang, L.Y., Shen, C.M., 1996. Late Cenozoic vegetation history and climatic characteristic of Qinghai-Xizang Plateau. Acta Micropalaeontol. Sin. 13, 321–337.
Tzedakis, P.C., Lawson, I.T., Frogley, M.R., Hewitt, G.M., Preece, R.C., 2002. Buffered tree population change in a Quaternary refugium: evolutionary implications. Science 297, 2044–2047.
Wang, C.S., Zhao, X.X., Liu, Z.F., Lippert, P.C., Graham, S.A., Coe, R.S., Yi, H.S., Zhu, L.D., Liu, S., Li, Y.L., 2008. Constraints on the early uplift history of the Tibetan Plateau. Proc. Natl. Acad. Sci. USA 105, 4987–4992.
Wang, F.B., Li, S.F., Shen, X.H., 1998. Records of natural exposures on the northern side of the Mid Himalayas (Jilong Basin). In: Shi, Y.F., Li, J.J., Li, B.Y. (Eds.), Uplift and Environmental Changes of Qinghai-Xizang (Tibetan) Plateau in the Late Cenozoic. Guangdong Science and Technology Press, Guangzhou, China, pp. 115–139.
Wang, L.Y., Abbott, R.J., Zheng, W., Chen, P., Wang, Y.J., Liu, J.Q., 2009a. History and evolution of alpine plants endemic to the Qinghai-Tibetan Plateau: *Aconitum gymnandrum* (Ranunculaceae). Mol. Ecol. 18, 709–721.
Wang, L.Y., Ikeda, H., Liu, T.L., Wang, Y.J., Liu, J.Q., 2009b. Repeated range expansion and glacial endurance of *Potentilla glabra* (Rosaceae) in the Qinghai-Tibetan Plateau. J. Integr. Plant Biol. 51, 698–706.
Wang, X.Q., Tank, D.C., Sang, T., 2000. Phylogeny and divergence times in Pinaceae: evidence from three genomes. Mol. Biol. Evol. 17, 773–781.
Wei, X.X., Wang, X.Q., 2004. Recolonization and radiation in *Larix* (Pinaceae): evidence from nuclear ribosomal DNA paralogues. Mol. Ecol. 13, 3115–3123.
Wei, X.X., Wang, X.Q., Hong, D.Y., 2003. Marked intragenomic heterogeneity and geographical differentiation of nrDNA ITS in *Larix potaninii* (Pinaceae). J. Mol. Evol. 57, 623–635.
Wu, Z.Y., 1987. Origin and evolution of the flora of Tibet. The Comprehensive Scientific Expedition to the Qinghai-Xizang Plateau, Academia Sinica (Ed.), Flora Xizangica, vol. 5. Science Press, Beijing, pp. 874–902.
Wu, Z.Y., 1988. Hengduan Mountain flora and her significance. J. Jpn. Bot. 63, 297–311.
White, E.E., 1990. Chloroplast DNA in *Pinus monticola*. 2. Survey of within-species variability and detection of heteroplasmic individuals. Theor. Appl. Genet. 79, 251–255.
Xiao, X.Y., Shen, J., Wang, S.M., Xiao, H.F., Tong, G.B., 2007. Palynological evidence for vegetational and climatic changes from the HQ deep drilling core in Yunnan Province, China. Sci. China D: Earth Sci. 50, 1189–1201.
Xu, J.X., Ferguson, D.K., Li, C.S., Wang, Y.F., Du, N.Q., 2004. Climatic and ecological implications of Late Pliocene palynofloral from Longling, Yunnan, China. Quatern. Int. 117, 91–103.
Xu, J.X., Wang, Y.F., Du, N.Q., 2003. Late Pliocene vegetation and palaeoclimate of Yangyi and Longling of western Yunnan province. J. Palaeogeogr. 5, 217–223.
Yang, F.S., Li, Y.F., Ding, X., Wang, X.Q., 2008. Extensive population expansion of *Pedicularis longiflora* (Orobanchaceae) on the Qinghai-Tibetan Plateau and its correlation with the Quaternary climate change. Mol. Ecol. 17, 5135–5145.
Yang, S.J., Yin, Z.H., Ma, X.M., Lei, F.M., 2006. Phylogeography of ground tit (*Pseudopodoces humilis*) based on mtDNA: evidence of past fragmentation on the Tibetan Plateau. Mol. Phylogenet. Evol. 41, 257–265.
Yi, T.M., Li, C.S., Jiang, X.M., 2005. Conifer woods of the Pliocene age from Yunnan, China. J. Integr. Plant Biol. 47, 264–270.
Zhang, Q., Chiang, Y., George, M., Liu, J.Q., Abbott, R.J., 2005. Phylogeography of the Qinghai-Tibetan Plateau endemic *Juniperus przewalskii* (Cupressaceae) inferred from chloroplast DNA sequence variation. Mol. Ecol. 14, 3513–3524.
Zheng, B.X., Xu, Q.Q., Shen, Y.P., 2002. The relationship between climate change and Quaternary glacial cycles on the Qinghai-Tibetan Plateau: review and speculation. Quatern. Int. 97–98, 93–101.