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Mitochondrial and chloroplast phylogeography of *Picea crassifolia* Kom. (Pinaceae) in the Qinghai-Tibetan Plateau and adjacent highlands

LIHUA MENG,*,**,†† RUI YANG,*†,†† RICHARD J. ABBOTT,‡ GEORG MIEHE,§ TIANHUA HU¶ and JIANQUAN LIU†

*Key Laboratory of the Qinghai-Tibetan Plateau Ecological Adaptation, Northwest Plateau Institute of Biology, Chinese Academy of Sciences, Xining, Qinghai 810001, China, †Key Laboratory of Arid and Grassland Ecology, Lanzhou University, Lanzhou 730000, China, ‡School of Biology, Mitchell Building, University of St Andrews, St Andrews, Fife KY16 9TH, UK, §Faculty of Geography, University of Marburg, Deutschhaustr.10 35032 Marburg, Germany, ¶Ningxia Administration of Helen Mountain State Natural Reserve Area, Yinchuan, China, **Graduate School of the Chinese Academy of Sciences, Beijing 100039, China*

Abstract

The disjunct distribution of forests in the Qinghai-Tibetan Plateau (QTP) and adjacent Helan Shan and Daqing Shan highlands provides an excellent model to examine vegetation shifts, glacial refugia and gene flow of key species in this complex landscape region in response to past climatic oscillations and human disturbance. In this study, we examined maternally inherited mitochondrial DNA (*nad*1 intron b/c and *nad*5 intron 1) and paternally inherited chloroplast DNA (*trn*C-*trn*D) sequence variation within a dominant forest species, *Picea crassifolia* Kom. We recovered nine mitotypes and two chlorotypes in a survey of 442 individuals from 32 populations sampled throughout the species' range. Significant mitochondrial DNA population subdivision was detected (G_{ST} = 0.512; N_{ST} = 0.679), suggesting low levels of recurrent gene flow through seeds among populations and significant phylogeographical structure ($N_{ST} > G_{ST}$, $P < 0.05$). Plateau haplotypes differed in sequence from those in the adjacent highlands, suggesting a long period of allopatric fragmentation between the species in the two regions and the presence of independent refugia in each region during Quaternary glaciations. On the QTP platform, all but one of the disjunct populations surveyed were fixed for the same mitotype, while most populations at the plateau edge contained more than one haplotype with the mitotype that was fixed in plateau platform populations always present at high frequency. This distribution pattern suggests that present-day disjunct populations on the QTP platform experienced a common recolonization history. The same phylogeographical pattern, however, was not detected for paternally inherited chloroplast DNA haplotypes. Two chlorotypes were distributed throughout the range of the species with little geographical population differentiation ($G_{ST} = N_{ST}$ = 0.093). This provides evidence for highly efficient pollen-mediated gene flow among isolated forest patches, both within and between the QTP and adjacent highland populations. A lack of isolation to pollen-mediated gene flow between forests on the QTP and adjacent highlands is surprising given that the Tengger Desert has been a geographical barrier between these two regions for approximately the last 1.8 million years.

Keywords: cpDNA, gene flow, mtDNA, *Picea crassifolia*, postglacial recolonization, Tibetan Plateau

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Correspondence: Dr Jian-Quan Liu, Fax: 931-8914288; E-mail: liujq@nwipb.ac.cn; ljqdxy@public.xn.qh.cn

L. Meng and R. Yang contributed equally to this work

Introduction

Climatic and environmental changes that have occurred during the last 2 million years have played an important role in shaping the present-day distribution and diversity

of many plants and animals (Hewitt 1996). Population genetic analyses and fossil evidence have provided critical information on the demographic history of these organisms (Hewitt 1996, 2000), showing that many European and North American taxa were restricted to ice-free refugia during glacial periods, and then recolonized deglaciated regions during interglacials (e.g. Webb & Bartlein 1992; Abbott *et al*. 2000; Mardulyn 2001; Despres *et al*. 2002; Petit & Grivet 2002; Burban & Petit 2003; Hamper *et al*. 2003; Jaramillo-Correa & Bousquet 2003; Kropf *et al*. 2003; Petit *et al*. 2003, 2005; Abbott & Comes 2004; Hewitt 2004; Jaramillo-Correa *et al*. 2005; Anderson *et al*. 2006). Genetic divergence in geographically isolated glacial refugia is believed to have promoted intraspecific differentiation in many instances and led to speciation on occasion (Hewitt 2000), although this might have been reduced in wind-pollinated plants because of high levels of gene flow through long-distance pollen dispersal (Liepelt *et al*. 2002).

The Qinghai-Tibetan Plateau (QTP) is the highest and largest plateau in the world with an area of 2.5 million km^2, and is known also to have been subject to large-scale climatic changes in the past (Zhang *et al*. 1996; Shi *et al*. 1998; Ni 2000; Thompson *et al*. 2000). During the Quaternary, such changes might have caused recurrent retreats and advances of some species' ranges, in parallel with shifts in the dominant vegetation type from permafrost-steppe to forest during glacial and interglacial cycles (Tang & Shen 1996). Moreover, the repeated fragmentation of the species' ranges, possibly triggered by Quaternary climatic oscillations and also earlier uplifts of the plateau, might have increased considerably rates of speciation and species diversification in the region (e.g. Liu *et al*. 2006). Very few population genetic analyses have been conducted on QTP organisms, and consequently patterns of past migration, recolonization and intraspecific divergence in key species of the region have not been adequately studied. However, the few studies that have been completed support the retreat and recolonization hypothesis of both plants and animals in this region during past Quaternary climatic oscillations (Qu *et al*. 2005; Zhang *et al*. 2005).

The vegetation of the northeast QTP platform is currently dominated by alpine meadow and desert-steppe, but with disjunct forests scattered within it. The forests are dominated by *Juniperus przewalskii* on southern slopes and by *Picea crassifolia* Kom. on northern slopes. The evolutionary history of disjunct forests and the alpine meadow ecosystem on the QTP platform remains elusive. It has been suggested that the alpine meadow ecosystem developed and replaced original forest vegetation gradually from the late Pliocene onwards, that is following the most recent large-scale uplift of the plateau to an average altitude of 4500 m (Wu 1980; Zhang 1983; Shi *et al*. 1998; Wu *et al*. 2001). However, an alternative hypothesis is that the northeast area was invaded by forests most recently during postglacial stages, and that the widespread forest cover that became established, subsequently became fragmented during the late Holocene as a dominant alpine meadow and desert-steppe vegetation developed in the region (Tang & Shen 1996). If the platform forest patches are relicts of a formerly widespread Pliocene forest, it might be expected that genetic diversity within patches will be low, whereas variation between populations will be high because of the effects of genetic drift fixing different alleles and haplotypes in different populations of reduced size during the Quaternary climatic oscillations. However, if the forests were formed following recent postglacial recolonization from plateau-edge refugia, it is possible that all disjunct populations will share a common haplotype with low levels of genetic diversity occurring both within and between populations. A recent survey of chloroplast DNA (cpDNA) variation in *J. przewalskii* revealed such a pattern of haplotypic diversity in this species (Zhang *et al*. 2005).

Picea crassifolia (Pinaceae), which forms pure stands on north-facing slopes in the northeast QTP platform region, is also distributed at lower altitudes along the northeast edge of the plateau and in the adjacent highlands of Helan Shan and Daqing Shan in north China (Fig. 1). A geographical barrier exists between the populations found in the QTP and Helan/Daqing regions because of the presence of the Tengger Desert, which formed approximately at the start of the Quaternary, 1.8 million years ago (Yang *et al*. 2006). It remains unknown whether forests in these two disjunct regions are derived from a common refugium or from independent refugia that occurred within each region during Quaternary glaciations. If the latter were the case, it would be of interest to know whether gene flow mediated by long-distance pollen dispersal has occurred between forests in the two regions.

The organellar DNA in species of the Pinaceae exhibit contrasting modes of inheritance: mitochondrial DNA (mtDNA) is maternally transmitted via seed and cpDNA is paternally transmitted via pollen (Wagner 1992). This unusual inheritance of organelles provides an excellent model to trace range shifts in the species through seedling establishment and also gene flow via long-distance pollen dispersal. Various studies have suggested that genetic differentiation of these two markers is asymmetrical in that most species show greater differentiation for mtDNA than cpDNA (Latta & Mitton 1997, 1999; Mitton *et al*. 2000; Liepelt *et al*. 2002; Richardson *et al*. 2002). In the study reported here, we examined mtDNA and cpDNA haplotype variation to obtain a better understanding of the phylogeography and gene flow via seed and pollen of *P. crassifolia*. Our survey involved the analysis of 442 trees from 32 populations sampled throughout the entire geographical distribution of *P. crassifolia*. Our objectives were: (i) to discriminate between the two alternative hypotheses

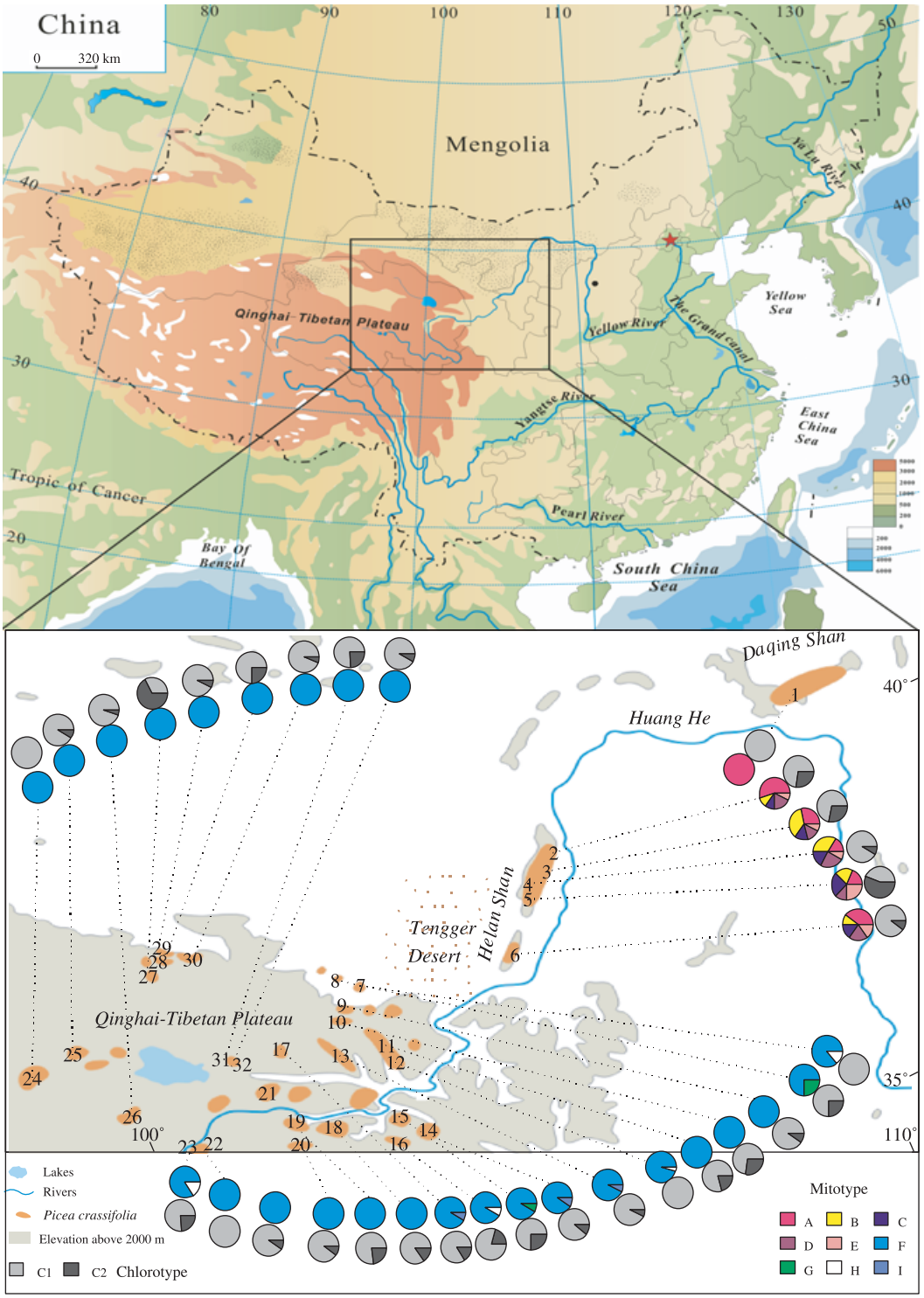

Fig. 1 Sampling sites of *Picea crassifolia* with frequencies of nine mitotypes and two chlorotypes indicated. A key to locations is given in Table 1.

regarding the formation of disjunct forest patches in the QTP platform; (ii) to test whether there may have existed different Quaternary refugia for this species in the QTP and adjacent highlands (Helen/Daqing Shan Mountains) that are isolated from each other by the Tengger Desert; and (iii) to check whether long-distance, pollen-mediated gene flow may have occurred between forests of these two isolated regions.

Table 1 Locations of populations of *Picea crassifolia* sampled, alleles at two mtDNA regions (*nad*1 and *Nad*5), number of each mitotype and chlorotype per population, and estimates of gene diversity for mitotypes (H_{Em}) and chlorotypes (H_{Ec})

						Haplotypes																		
						*nad*1					*nad*5			Mitotype									Chlortotype (*trn*C-D)		
P.	Population/ Location	Latitude	Longitude	Alt. (m)	*N*	a	b	c	d	e	1	2	H_{Em}	A	B	C	D	E	F	G	H	I	H_{Ec}	C1	C2
1	Inner Mongolia	40°54′	111°09′	2120	14	14	0	0	0	0	14	0	0.000	14	0	0	0	0	0	0	0	0	0.000	14	0
2	Helan Mt, NX	39°00′	106°05′	2420	11	6	1	1	2	1	11	0	0.709	6	1	1	2	1	0	0	0	0	0.436	8	3
3	Beisigou,NX	38°58′	105°55′	2185	14	4	5	2	2	1	14	0	0.802	4	5	2	2	1	0	0	0	0	0.396	10	4
4	Helan Mt, NX	38°45′	105°53′	2230	12	2	4	2	3	1	12	0	0.833	2	4	2	3	1	0	0	0	0	0.167	11	1
5	Helan Mt, NX	38°44′	105°55′	2310	16	3	3	4	2	4	16	0	0.842	3	3	4	2	4	0	0	0	0	0.525	7	9
6	Daluoshan, NX	37°17′	106°17′	2390	20	8	2	3	4	3	20	0	0.784	8	2	3	4	3	0	0	0	0	0.189	18	2
7	Maolingshan,GS	37°27′	103°41′	2360	15	13	0	2	0	0	0	15	0.248	0	0	0	0	0	13	0	2	0	0.000	15	0
8	Shoulushan,GS	37°07′	103°44′	2820	12	5	3	4	0	0	0	12	0.409	0	0	0	0	0	9	3	0	0	0.409	9	3
9	Tianzhu, GS	37°24′	102°33′	2650	22	22	0	0	0	0	0	22	0.000	0	0	0	0	0	22	0	0	0	0.173	20	2
10	Mengyuan, QH	37°10′	102°09′	2600	11	11	0	0	0	0	0	11	0.000	0	0	0	0	0	11	0	0	0	0.436	8	3
11	Tianzhu, GS	36°59′	102°55′	2260	10	10	0	0	0	0	0	10	0.000	0	0	0	0	0	10	0	0	0	0.356	8	2
12	Yongdeng, GS	36°42′	102°40′	2530	21	20	0	1	0	0	0	21	0.095	0	0	0	0	0	20	0	1	0	0.000	21	0
13	Huzhu, QH	36°47′	102°41′	2800	14	13	0	0	1	0	0	14	0.143	0	0	0	0	0	13	0	0	1	0.143	13	1
14	Kangle,GS	34°56′	103°44′	2480	10	9	0	0	1	0	0	10	0.200	0	0	0	0	0	9	0	0	1	0.200	9	1
15	Xiahe, GS	35°08′	102°50′	2840	12	11	1	0	0	0	0	12	0.167	0	0	0	0	0	11	1	0	0	0.409	9	3
16	Zhuoni, GS	34°35′	103°30′	2900	12	11	0	1	0	0	0	12	0.167	0	0	0	0	0	11	0	1	0	0.303	10	2
17	Datong, QH	37°02′	101°50′	2900	13	12	0	0	1	0	0	13	0.154	0	0	0	0	0	12	0	0	1	0.282	11	2
18	Tongren, QH	35°32′	102°15′	3100	13	13	0	0	0	0	0	13	0.000	0	0	0	0	0	13	0	0	0	0.282	11	2
19	Tongren, QH	35°18′	101°56′	2910	9	9	0	0	0	0	0	9	0.000	0	0	0	0	0	9	0	0	0	0.389	7	2
20	Tongren, QH	35°16′	101°54′	3120	19	19	0	0	0	0	0	19	0.000	0	0	0	0	0	19	0	0	0	0.199	17	2
21	Ping'an, QH	36°20′	101°54′	2750	11	11	0	0	0	0	0	11	0.000	0	0	0	0	0	11	0	0	0	0.182	10	1
22	Tongde, QH	34°47′	100°49′	3420	12	12	0	0	0	0	0	12	0.000	0	0	0	0	0	12	0	0	0	0.000	12	0
23	Maqin, QH	34°48′	100°14′	3520	13	11	0	2	0	0	0	13	0.282	0	0	0	0	0	11	0	2	0	0.385	10	3
24	Dulan,QH	36°20′	98°15′	3700	16	16	0	0	0	0	0	16	0.000	0	0	0	0	0	16	0	0	0	0.000	16	0
25	Wulan, QH	37°01′	98°46′	3310	12	12	0	0	0	0	0	12	0.000	0	0	0	0	0	12	0	0	0	0.167	11	1
26	Xinghai, QH	35°32′	99°51′	3510	19	19	0	0	0	0	0	19	0.000	0	0	0	0	0	19	0	0	0	0.105	18	1
27	Huangyuan, QH	36°45′	101°08′	2780	9	9	0	0	0	0	0	9	0.000	0	0	0	0	0	9	0	0	0	0.500	3	6
28	Sunan, GS	38°28′	100°24′	2740	13	13	0	0	0	0	0	13	0.000	0	0	0	0	0	13	0	0	0	0.154	12	1
29	Sunan, GS	38°41′	99°31′	3230	12	12	0	0	0	0	0	12	0.000	0	0	0	0	0	12	0	0	0	0.409	9	3
30	Shandan, GS	38°27′	101°20′	3170	16	16	0	0	0	0	0	16	0.000	0	0	0	0	0	16	0	0	0	0.125	15	1
31	Huangyuan, QH	36°38′	101°02′	3520	17	17	0	0	0	0	0	17	0.000	0	0	0	0	0	17	0	0	0	0.382	13	4
32	Qilian, QH	38°09′	100°17′	2750	13	13	0	0	0	0	0	13	0.000	0	0	0	0	0	13	0	0	0	0.154	12	1

Abbreviations: P., the population code; Alt., altitude; QH, Qinghai; GS, Gansu; NX, Ningxia; *N*, number of trees analysed; H_{Em}, total mtDNA diversity; H_{Ec}, total cpDNA diversity.

Materials and methods

Population sampling

We sampled leaf-needles from 9 to 22 trees collected from each of six populations from the Daqing Shan (1) and Helan Shan (2–6) Mountains in north and northwest China, 14 populations (7–20) occurring along the QTP edge and twelve populations (21–32) from the QTP platform (Table 1, Fig. 1). These populations covered the entire geographical distribution of *Picea crassifolia*. Samples were taken from trees at least 100 m apart in each population. The latitude, longitude and altitude at each collection centre were measured using an Etrex GIS monitor (Garmin). The forest distribution (Fig. 1) was drawn using CORELDRAW based on the distribution pattern described in Wu (1980). In total, 442 trees were sampled and following collection, needles were dried and stored in silica gel.

DNA extraction, amplification and sequencing

Genomic DNA was isolated from approximately 50 mg of silica gel-dried, leaf-needle material. DNA was extracted using the cetyltrimethyl ammonium bromide (CTAB) method (Doyle & Doyle 1987). For mtDNA markers, a preliminary universal primer scan of the mitochondrial

genome using four different pairs of primers was conducted on 50 individuals sampled from 10 populations across the distribution region (Demesure *et al*. 1995). Polymerase chain reaction (PCR) was performed in a 25-μL volume, containing 10–40 ng plant DNA, 50 mM Tris-HCl, 1.5 mM $MgCl_2$, 250 μg/mL BSA, 0.5 mM dNTPs, 2 μM of each primer, and 0.75 U of *Taq* polymerase following Zhang *et al*. (2005). The annealing temperatures provided by Demesure *et al*. (1995) were optimized for each pair of primers. The intron b/c of subunit 1 of the nicotinamide adenine dinucleotide (NADH) dehydrogenase gene (*nad*1 intron b/c), the intron 1 of subunit 5 (*nad*5 intron 1) and the intron 1 of subunit 7 (*nad*7 intron 1) of the same gene, were amplified and sequenced along with the V1 region of the small ribosomal subunit of the RNA gene (SSU *rRNA* V1 region), using primers described in Jaramillo-Correa *et al*. (2003). Two pairs of primers, that is those used to amplify *nad*1 intron b/c and *nad*5 intron 1, respectively, revealed sequence polymorphism within the 50 individuals examined, and were used thereafter for the large-scale survey of mitotype variation within *P. crassifolia*.

Similarly, four pairs of cpDNA primers, that amplified the *trn*L-*trn*F, *rps*5-*trn*S, *trn*C-*trn*D and *ndh*K-C pseudogene regions, respectively (Demesure *et al*. 1995; Anderson *et al*. 2006), were used in the initial variation scan with the optimized annealing temperatures. PCRs were performed following Zhang *et al*. (2005). Variation was found only within the *trn*C-*trn*D region among the 50 individuals examined in 10 populations across the total distribution region. Only this region therefore was examined in the large-scale survey of chlorotype distribution within *P. crassifolia*.

PCR products were purified using a CASpure PCR Purification Kit following the recommended protocol (Casarray). Sequencing reactions were carried out in a Biometra thermocycler using DYEnamic Dye Terminator Cycle Sequencing Kit (Amersham Pharmacia Biotech Inc.) also following the recommended protocol. Sequencing products were separated and analysed on a MegaBACE 500 Automated Sequencer (Amersham Pharmacia Biotech Inc.).

Multiple alignments of sequences were obtained using CLUSTAL W (Thompson *et al*. 1994) and subsequent manual adjustments. Two separate matrices, one for mtDNA and the other for cpDNA sequences, were constructed for 442 trees examined and nine different mtDNA sequences (mitotypes) and two different cpDNA sequences (chlorotypes) were identified. All sequences have been deposited in the European Molecular Biology Laboratory (EMBL) GenBank databases under accession nos. AY23406–AY23422.

Data analysis

Relationships between mtDNA haplotypes were examined via a haplotype network constructed using the computer program NETWORK (Weir 1996). In the analysis, both site mutations and indels were hypothesized to evolve with equal possibility and each indel was assumed to have originated independently of other indels.

Estimates of unbiased genetic diversity (H_E) (equivalent to expected heterozygosity for diploid data, Weir 1996) were calculated as H: $H = n/(n-1)(1 - \Sigma_{i=1}^{k} p_i^2)$, in which n is the number of samples in the population, k is the number of haplotypes, and p is the population frequency of the ith haplotype (Nei 1987). Average gene diversity within populations (H_S), total gene diversity (H_T) and two measures of population differentiation, G_{ST} (coefficient of genetic variation over all population, Nei 1973) and N_{ST} (equivalent coefficient taking into account sequence similarities between haplotypes), were calculated using the program PERMUT (available at www.pierroton.inra.fr/genetics/labo/Software/) (Pons & Petit 1996). The occurrence of significant phylogeographical structure was inferred by testing if G_{ST} and N_{ST} were significantly different using PERMUT with 1000 permutations. Population differentiation (G_{ST}) was tested for significance using an exact test (Raymond & Rousset 1995). Genetic structure was further examined across both regions and within each of the QTP and Helan/Daqing regions by analysis of molecular variance (AMOVA, Excoffier *et al*. 1992) as implemented in ARLEQUIN version 3.0 (Excoffier *et al*. 2005).

Because neither mtDNA nor cpDNA variation can be assumed to be in drift–migration equilibrium in our study system, we did not estimate gene flow separately for each of these two marker systems using the infinite-island model. Instead, we compared the different geographical patterns of cpDNA vs. mtDNA haplotype variation by examining correlations between Nei's unbiased genetic distance (Nei 1978) and geographical distances (in kilometres) over all pairs of populations for each organelle marker in turn. For this, Mantel tests (Mantel 1967) employing 999 permutations were performed using the software Tools for Population Genetic Analysis (TFPGA, Miller 1997).

Results

Mitotype distribution

Polymorphism was observed within both mtDNA regions: *nad*1 intron b/c and *nad*5 intron. The five variants of *nad*1 intron b/c differed from each other in the presence of between zero to four repeats of a 34-bp insertion at site 465 in the sequence (Table 2). The two variants for the *nad*5 intron 1 were distinguished by a single nucleotide substitution at site 290 (G ↔ T). The combined data of both intron sequences identified nine different mitotypes, A, B, C, D, E, F, G, H and I (Table 2), over all trees examined. Mitotype A was fixed in the single population examined from the Daqing Shan mountains, and occurred together with mitotypes B-E in all five Helan Shan populations surveyed

Table 2 Variable sites of the aligned sequences of two mitochondrial DNA fragments in nine haplotypes of *Picea crassifolia* (*indicates presence of insertion sequence at site). Sequences are numbered from the 5′- to the 3′ end in each region

	Nucleotide position				
	*nad*1				*nad*5
	4	4	5	5	2
	6	9	3	6	9
Haplotype	5	8	1	4	0
Type A	—	—	—	—	G
Type B	*	—	—	—	G
Type C	*	*	—	—	G
Type D	*	*	*	—	G
Type E	*	*	*	*	G
Type F	—	—	—	—	T
Type G	*	—	—	—	T
Type H	*	*	—	—	T
Type I	*	*	*	*	T

*CCCCCTCCGTTGTCAGGGGAGCGACTTCGTACCT.

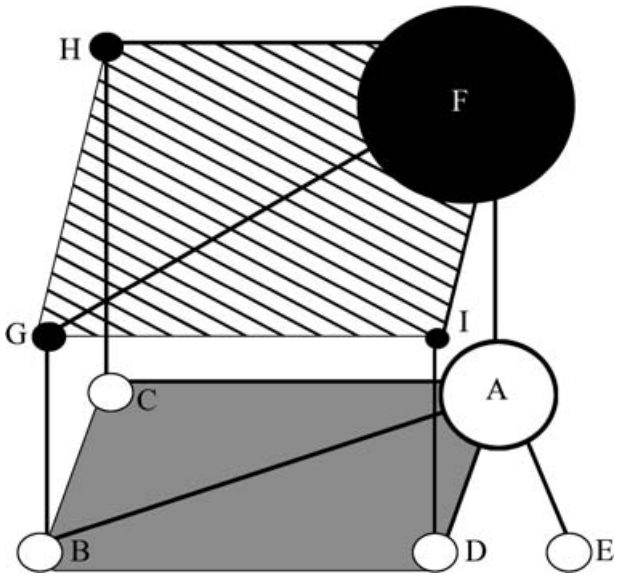

Fig. 2 A network of mitotypes constructed for *Picea crassifolia*. Mitotypes in the lower layer of the diagram occur only in the Helan/Daqing region, while those in the upper layer occur only in the QTP. Circle sizes are proportional to mitotype frequencies over all populations, with the largest circle representing the most abundant mitotype.

(Fig. 1), which exhibited highest gene diversity (H_E) (Table 1). These mitotypes were not found in any of the QTP populations surveyed. Almost all populations on the QTP platform (21, 22, 24–32) were fixed for mitotype F, which was also present in all QTP edge populations (7–20) along with mitotype G in populations 8 and 15, mitotype H in populations 7, 11 and 16 (also present in 23), and mitotype I in populations 12, 13 and 17 (Table 1, Fig. 1). The mitotype network (Fig. 2) reflected a strong geographical pattern with mitotypes found in the Helan/Daqing region appearing to form one related group while those present only in the QTP region formed another group. However, because no outgroup was included and also because loops were present in the network, it was not possible to establish clearly the direction of mitotype evolution in the species.

Chlorotype distribution

A single nucleotide substitution at site 134 (C ↔ A) in the *trn*C-*trn*D cpDNA sequence yielded two chlorotypes (C1 and C2). Both of these chlorotypes were present in most populations. Chlorotype C1 occurred at high frequency in all populations except populations 5 and 32, while chlorotype C2 generally occurred at lower frequency and was absent from five populations (1, 7, 12, 22 and 24) (Table 1 and Fig. 1).

Population genetic parameters and phylogeographical structure

When mitotype variation was examined over all populations surveyed, total gene diversity H_T equalled 0.452, average within-population diversity H_S was 0.220, and G_{ST} and N_{ST} were 0.512 and 0.679, respectively. A permutation test showed that G_{ST} and N_{ST} were significantly different from each other ($N_{ST} > G_{ST}$, $U = 2.11$, $P < 0.05$). In contrast, when chlorotype variation was examined over all populations, values of H_T and H_S were found to be similar (0.272 and 0.247, respectively), while G_{ST} and N_{ST} both equaled 0.093. Within each of the two main regions (Helan/Daqing and QTP), there was no significant difference between H_T and H_S, nor between N_{ST} and G_{ST}, for either mitotype or chlorotype variation. All estimates of diversity for both markers were significantly higher in the Helan/Daqing region than in the QTP region (Table 3). In each case, this was due to the much higher diversity in the Helan Shan populations, as the single Daqing Shan population surveyed contained only one mitotype and one chlorotype.

Analysis of molecular variance (AMOVA) showed that most mitotype diversity occurred between the Helan/Daqing and QTP regions (74.47% of the total variance), while about 2.14% of the total variance was assigned among populations within these regions. In contrast, for chlorotype variation, most diversity was present within populations (89.32%), and only a small amount was partitioned between the two regions (1.25%, Table 4). AMOVAs conducted separately on data within each region showed that mitotype and chlorotype variation was significant among populations in both regions, but was greater in the Helan/Daqing region than in the QTP region (Table 4).

Regions	H_S	H_T	G_{ST}	N_{ST}
Mitotype				
Helan	0.789(0.020)	0.794(0.024)	0.006(0.019)	0.006(0.019)^{NS}
Helan/Daqing	0.662(0.134)	0.754(0.072)	0.122(0.148)	0.122(0.148)^{NS}
QTP	0.072(0.022)	0.075(0.023)	0.048(nc)	0.048(nc)^{nc}
Total distribution	0.220(0.053)	0.452(0.099)	0.512(0.051)	0.679 (0.060)*
Chlorotype				
Helan	0.351(0.073)	0.401(0.083)	0.123(0.039)	0.123 (0.039)^{NS}
Helan/Daqing	0.293(0.083)	0.354(0.094)	0.173(0.059)	0.173 (0.059)^{NS}
QTP	0.236(0.030)	0.254(0.038)	0.071(0.066)	0.071(0.066)^{NS}
Total distribution	0.247(0.028)	0.272 (0.036)	0.093(0.054)	0.093 (0.054)^{NS}

Table 3 Estimates of average gene diversity within populations (H_S), total gene diversity (H_T), interpopulation differentiation (G_{ST}), and the number of substitution types (N_{ST}) (mean ± SE in parentheses) for mitotypes and chlorotypes

*Indicates that N_{ST} is significantly different from G_{ST} ($0.01 < P < 0.05$), ^{NS}, not significantly different; ^{nc} = not computed because of small sample size.

Table 4 Analyses of molecular variance (AMOVAS) for mitotypes and chlorotypes in *Picea crassifolia*

Regions	Source of variation	d.f.	SS	VC	Variation (percentage)	Fixation index
Mitotype						
(i) Both regions (Helan/Daqing and QTP)	Among regions	1	41.737	0.297	74.47	$F_{RT} = 0.745$*
	Among populations	30	6.324	0.009	2.14	$F_{SR} = 0.084$*
	Within populations	411	38.319	0.093	23.39	$F_{ST} = 0.766$*
	Total	442	86.380	0.399		
(ii) Helan Shan	Among populations	4	1.401	0.003	0.85	$F_{ST} = 0.008$
	Within populations	68	27.106	0.399	100.85	
(iii) Helan/Daqing	Among populations	5	4.860	0.044	11.69	$F_{ST} = 0.117$*
	Within populations	81	27.106	0.335	88.31	
(iv) QTP	Among populations	25	1.464	0.002	5.03	$F_{ST} = 0.050$*
	Within populations	330	11.213	0.034	94.97	
Chlorotype						
(i) Both regions (Helan/Daqing and QTP)	Among regions	1	0.522	0.002	1.25	$F_{RT} = 0.013$
	Among populations	30	8.454	0.012	9.43	$F_{SR} = 0.095$*
	Within populations	411	47.191	0.115	89.32	$F_{ST} = 0.107$*
	Total	442	56.167	0.129		
(ii) Helan Shan	Among populations	4	2.362	0.029	14.44	$F_{ST} = 0.144$*
	Within populations	68	11.693	0.172	85.56	
(iii) Helan/Daqing	Among populations	5	3.157	0.034	19	$F_{ST} = 0.190$*
	Within populations	81	11.693	0.144	81	
(iv) QTP	Among populations	25	5.297	0.008	6.63	$F_{ST} = 0.066$*
	Within populations	330	35.498	0.108	93.37	

d.f., degrees of freedom; SS, sum of squares; VC, variance components; *$P < 0.001$. F_{RT}, correlation of haplotypes within regions relative to total; F_{SR}, correlation within populations relative to regions; F_{ST}, correlation within populations relative to total.

However, the greater level of mitotype variation among Helan/Daqing populations was entirely due to the inclusion of the single Daqing population in this set. When this monomorphic population was excluded, and the AMOVA was rerun on only Helan Shan populations, it was evident that there was no significant variation among these five populations, each of which was polymorphic for mitotypes A–E.

The much greater differentiation between regions recorded for mitotype variation relative to chlorotype variation suggests that the dispersal ability of *Picea crassifolia* seeds is low relative to that of pollen. This was supported by Mantel tests, which revealed a highly significant positive correlation for genetic and geographical distances of mtDNA haplotypes ($r = 0.801$, $P < 0.01$) over all populations, but a lack of such a correlation for the cpDNA marker ($r = 0.004$, $P = 0.93$). However, within each region, there appear to be no difference between seed and pollen dispersal based on the values recorded for G_{ST} (Table 3) and the results of within region AMOVAS for mitotype and

chlorotype variation. Although, interestingly, G_{ST} is lower for mitotype than chlorotype variation when only Helan Shan populations are considered (Table 3).

Discussion

Mitotype phylogeographical structure

Significant phylogeographical structure was present for mitotype variation among populations of *Picea crassifolia* sampled throughout the geographical range of the species that extends from the northeast part of the Qinghai-Tibetan Plateau into the adjacent highland areas of Helan and Daqing Shan. An AMOVA showed that 74.47% of mitotype variation occurred among populations, while a comparison of differentiation indices showed that N_{ST} was significantly higher than G_{ST} ($P < 0.05$). Thus, the current pattern of mitochondrial diversity within *P. crassifolia* reflects a strong genetic signature of past population change. One important characteristic of the mitotype distribution is that there is no common mitotype found in both QTP and Helan/Daqing regions. Such a disjunction suggests that populations of the species from these two regions have remained isolated from each other throughout the Quaternary irrespective of climatic oscillations. Thus, the Tengger Desert, which began to form around 1.8 million years ago (Yang *et al*. 2006), is likely to have imposed a significant geographical barrier to seed-mediated gene flow between stands of *P. crassifolia* occurring in the QTP region and adjacent Helan and Daqing highlands.

Only one mitotype (F) was present in most QTP platform populations (21, 22, 24–32), while QTP edge populations possessed additional haplotypes and therefore exhibited an overall higher diversity. Highest diversity was present among QTP populations at the southeast edge of the QTP, that is Gansu, from where four mitotypes (F, G, H and I) were recovered (Table 1, Fig. 1). Other parts of the plateau edge, that is the northeast (Maolingshan and Shoulushan in Gansu province) and southern edges (Guoluo in Qinghai province) often contained at least one additional mitotype to the common F mitotype. In contrast, in the Helan Shan Mountains, each population was polymorphic for five mitotypes and despite the fact that the single population from Daqing Shan was fixed for mitoype A, a relatively high within-region diversity was recorded for the Helan/Daqing region.

These results indicate that within the QTP region, the species might have experienced a more serious bottleneck effect in the past than it did in the Helan/Daqing region. Furthermore, the observed decline in mitotype variation of *P. crassifolia* from the plateau edge to the platform in the QTP region suggests a marked founder effect in the relatively recent past (e.g. Gugerli *et al*. 2001). This, together with the fact that plateau edge populations contain, at high frequency, mitotype F, which is fixed in all but one plateau platform populations, supports the 'expansion' hypothesis that all QTP platform populations are derived from a common colonization event from either a single refugium (e.g. located at the southeast edge) or from a more widely distributed refugial population that existed along the edge of the QTP platform. Thus, our results support this hypothesis rather than the hypothesis that present-day forests are relicts of a once widespread Pliocene forest on the plateau platform, which subsequently became fragmented during the Quaternary.

Such a recolonization is likely to have occurred in the early Holocene period as suggested by the pollen fossil record for the region (Tang & Shen 1996). Well-preserved fossil pollen from the northeast QTP region indicates that the Holocene forest invasion began approximately 8000 years BP and that forests were the predominant vegetation until 3000 BP when they were largely replaced by alpine meadow and desert-steppe (Tang & Shen 1996). The factors that brought about such recent forest fragmentation remain uncertain. One possibility is that drier and colder conditions from the late Holocene onwards combined to cause such fragmentation, while another possibility is that forest was destroyed by fire caused by natural means or intentionally by humans for raising yaks or sheep. The latter explanation is supported by our recent finding that a quantity of juniper and *Picea* charcoals occurs in the soil layers under the present alpine meadow vegetation. These charcoals have been dated to between 4000 BP and 8900 BP (Kaiser *et al*. 2007), providing evidence that the eastern plateau platform supported a more extensive forest shortly before this time and that fire might have subsequently led to the development of the current disjunct distribution of forest. In addition, the domestication of yaks and the development of husbandry in the QTP during the Holocene may be another factor that prevented the restoration of forests and promoted the large-scale persistence of steppe and alpine meadow through top-down controls of the alpine ecosystem (Guo *et al*. 2006).

The high level of mitotype diversity recorded within the Helan Shan populations and absence of such diversity from the Daqing population examined raises the issue as to whether the forests in Daqing may have been recently founded from material dispersed from Helan Shan. However, more populations of *P. crassifolia* need to be surveyed from Daqing Shan before considering whether material in this region could have also passed through a recent genetic bottleneck.

Pollen-mediated gene flow across the distribution of fragmented forests

In contrast to the significant phylogeographical structure obtained with mtDNA, there was an extremely low level of

genetic differentiation and phylogeographical structure for chlorotype variation when examined over all populations ($G_{ST} = N_{ST} = 0.093$). Only two chlorotypes were resolved, C1 and C2, and both were present in most populations surveyed in the Helan/Daqing and QTP regions. A large level of pollen-mediated gene flow between the two regions is therefore suggested by our results.

Although the spatial pattern of paternally inherited chloroplast differentiation has indicated the presence of glacial refugia in some conifers (e.g. Anderson *et al*. 2006), gene flow through long-distance dispersed pollen in wind-pollinated plants usually erodes the genetic signature of refugial isolation (Liepelt *et al*. 2002). Our results therefore represent another example of this situation, and provide evidence for efficient pollen-mediated gene flow among the isolated forest patches within and between the QTP region and the adjacent Helan and Daqing Shan mountains. Because of the effects of such long-distance, pollen-mediated gene flow, forest fragmentation and habitat isolation between the QTP and adjacent regions may not have played an important role in nuclear genomic diversification and speciation at least in wind-pollinated taxa (Wu & Wu 1996).

In conclusion, we have shown that the pattern of mtDNA and cpDNA variation resolved in the present study provides different insights into the phylogeographic structure and gene flow in *P. crassifolia*. A high level of population differentiation for maternally inherited mtDNA indicates that gene flow through seeds between populations in the QTP and Helan/Daqing regions is limited in the species, and that throughout the Quaternary, there were independent refugia for the species in these two regions. Moreover, in the former region the species most likely experienced contractions and a common expansion in its distribution in response to climatic oscillations. In contrast, our cpDNA results indicate that long-distance pollen flow occurs in the species and would therefore likely erode the nuclear genetic signature of past historical events, even the long period of disjunction experienced by populations occurring in the QTP and adjacent highland regions, respectively.

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The authors collaborate on projects on the molecular systematics, population dynamics and evolution of alpine and forest plants in the Qinghai-Tibetan Plateau (QTP). Meng Lihua and Yang Rui are interested in population genetics and the historical distribution of tree species in the QTP. Jianquan Liu and his laboratory are studying evolution and adaptation of various organisms in the QTP. Georg Miehe has worked on the evolutionary history of alpine ecosystems of the QTP. Richard Abbott conducts research on various aspects of plant variation and evolution.
