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Phylogeography of the Qinghai-Tibetan Plateau endemic *Juniperus przewalskii* (Cupressaceae) inferred from chloroplast DNA sequence variation

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Abstract

The vegetation of the northeast Qinghai-Tibetan Plateau is dominated by alpine meadow and desert-steppe with sparse forests scattered within it. To obtain a better understanding of the phylogeography of one constituent species of the forests in this region, we examined chloroplast *trn*T-*trn*F and *trn*S-*trn*G sequence variation within *Juniperus przewalskii*, a key endemic tree species. Sequence data were obtained from 392 trees in 20 populations covering the entire distribution range of the species. Six cpDNA haplotypes were identified. Significant population subdivision was detected (G_{ST} = 0.772, N_{ST} = 0.834), suggesting low levels of recurrent gene flow among populations and significant phylogeographic structure ($N_{ST} > G_{ST}$, $P < 0.05$). Eight of the nine disjunct populations surveyed on the high-elevation northeast plateau were fixed for a single haplotype (A), while the remaining, more westerly population, contained the same haplotype at high frequency together with two low frequency haplotypes (C and F). In contrast, most populations that occurred at lower altitudes at the plateau edge were fixed or nearly fixed for one of two haplotypes, A or E. However, two plateau edge populations had haplotype compositions different from the rest. In one, four haplotypes (A, B, D and E) were present at approximately equivalent frequencies, which might reflect a larger refugium in the area of this population during the last glacial period. Phylogenetic analysis indicated that the most widely distributed haplotype A is not ancestral to other haplotypes. The contrasting phylogeographic structures of the haplotype-rich plateau edge area and the almost haplotype-uniform plateau platform region indicate that the plateau platform was recolonized by *J. przewalskii* during the most recent postglacial period. This is supported by the findings of a nested clade analysis, which inferred that postglacial range expansion from the plateau edge followed by recent fragmentation is largely responsible for the present-day spatial distribution of cpDNA haplotypes within the species.

Keywords: cpDNA *trn*T-*trn*F and *trn*S-*trn*G, forest invasion, historical biogeography, *Juniperus przewalskii*, phylogeography, postglacial range expansion, Qinghai-Tibetan Plateau , Quaternary

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Introduction

The Qinghai-Tibetan (Q-T) Plateau is the highest and largest plateau in the world, with an average elevation of ~4000 m above sea level and an area of 2.5×10^6 km^2 (Zheng 1996). The plateau dramatically affects terrestrial ecosystems in western China and neighbouring areas because of its high elevation (topography) and specific climate (Zhang 1983). Its vegetation is considered to be highly sensitive and vulnerable to global climate change because plant growth and distribution in the region depend greatly on survivable temperatures (Zheng 1996). Biotic responses in the Q-T Plateau have therefore been used as sensitive indicators for monitoring global climate change (Zhang *et al*. 1996; Ni 2000; Thompson *et al*. 2000).

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The climatic oscillations of the Quaternary since ~2 million years ago (Ma) resulted in several glacial and interglacial cycles during which glaciers developed, expanded and receded in circumpolar and mountainous regions (Shackleton & Opdyke 1973). Glaciation resulted in either the extinction of species or their survival in areas (refugia) with more favourable climate conditions, often following a severe range contraction (Webb & Bartlein 1992; Hewitt 1996, 2000; Gugerli *et al*. 2001; Abbott & Brochmann 2003; Petit *et al*. 2003; Abbott & Comes 2004). During interglacials, significant range expansions took place from such refugia when species colonized/recolonized areas exposed by retreating glaciers. Although no massive ice sheet developed in the Q-T Plateau during glacial periods (Shi *et al*. 1998), pollen fossil records suggest that vegetation of the central Q-T Plateau shifted alternately between permafrost-steppe and forest in response to the Quaternary glaciations and interglaciations, respectively (Tang & Shen 1996). For example, the pollen fossil records indicate that during the period between 53 000 and 23 000 years before present (BP), most of the plateau was covered with forests except for shrub-grasslands in the northwest. However, during the last glacial maximum approximately 18 000 BP permafrost and desert-steppe occupied most of the plateau and forests were restricted to a southeast refugium (Tang *et al*. 1998).

The northeast plateau platform is now dominated by alpine meadow and desert-steppe, but with sparsely disjunct forests scattered within it, the history of which remains in doubt. According to a palaeovegetation reconstruction based on fossil pollen evidence (Tang & Shen 1996), the northeast was invaded by forests most recently in the middle Holocene, and the current dominant alpine meadow and desert-steppe developed during the late Holocene when the climate became cold and dry again. However, some other researchers suggest that the alpine meadow ecosystem developed and replaced the original forest vegetation gradually from the late Pliocene onwards, i.e. following the large-scale uplift of the plateau to an average altitude of 4500 m (Wu 1980; Zhang 1983; Shi *et al*. 1998), and that present-day island-like forests on the plateau platform are relicts of a formerly widespread Pliocene forest. The demographic history of a species undoubtedly affects the geographical patterns of genetic variation within and among populations (Hewitt 1996). Historical events such as range expansion and contraction, founder events, and habitat fragmentation due to vicariance, all leave an imprint on contemporary levels of genetic variation (Hewitt 2000, 2004). Consequently, it might be expected that a forest species occurring on the Q-T plateau platform will contain low levels of genetic diversity both within and between populations if these populations are derived from a recent range expansion from a plateau edge refugium. If, on the other hand, platform forests are relicts of a formerly widespread Pliocene forest, it is feasible that present-day populations would be characterized by low within-population diversity, but possibly high diversity between populations due to genetic drift favouring/fixing different alleles and haplotypes in different populations of reduced size. The purpose of the present study was to determine genetic relationships between individuals of one forest tree species, *Juniperus przewalskii* Kom. (Cupressaceae), which is a constituent of both the platform plateau forests and also the disjunctly distributed forests that occur at lower altitudes along the northeast edge of the plateau platform.

Juniperus przewalskii is a tree species endemic to the Qinghai-Tibet Plateau region. It is mainly distributed in the northeast plateau and forms pure stands on southern slopes in areas where forest occurs. The distribution of the species ranges between 2900 and 3760 m in altitude with some populations extending to the southeast plateau with an altitude between 3200 and 3300 m, but never reaching the moist valleys below 2800 m as do *Picea* forests. Populations of *J. przewalskii* are scattered disjunctly among the dominant alpine meadow and desert-steppe on the plateau platform, and also along the plateau edge at lower altitudes (Fig. 1). The species appears to reproduce completely by seed, as clonal reproduction via root suckers or other means has never been observed in the field. Most established trees have an extremely long generation time, with some older than 1000 years occurring in populations 3 and 7 (Fig. 1).

Here, we present the results of a survey of chloroplast *trn*T-*trn*F and *trn*S-*trn*G sequence variation within and between populations of *J. przewalskii* that was conducted to obtain an improved understanding of the phylogeography of the species. Our survey involved the analysis of 392 trees from 20 populations sampled throughout the entire geographical distribution of *J. przewalskii*. Previous studies of other species of Cupressaceae (Neale *et al*. 1989, 1991; Mogensen 1996; Kondo *et al*. 1998; Hwang *et al*. 2003), show that cpDNA is paternally inherited in members of this family. If the same is true for *J. przewalskii*, it would be expected that population differentiation for cpDNA would be less marked than if the genome were maternally inherited, assuming that pollen in the species is dispersed more widely than seed (Ennos *et al*. 1999). Nevertheless, we recorded a marked pattern of population differentiation within the Q-T Plateau edge area and mainly genetic uniformity in the plateau platform region that is indicative of a postglacial expansion of the species on the plateau platform followed by recent fragmentation.

Materials and methods

Population sampling

Needles were collected from trees in nine populations (1–9) of *Juniperus przewalskii* from the northeast platform of

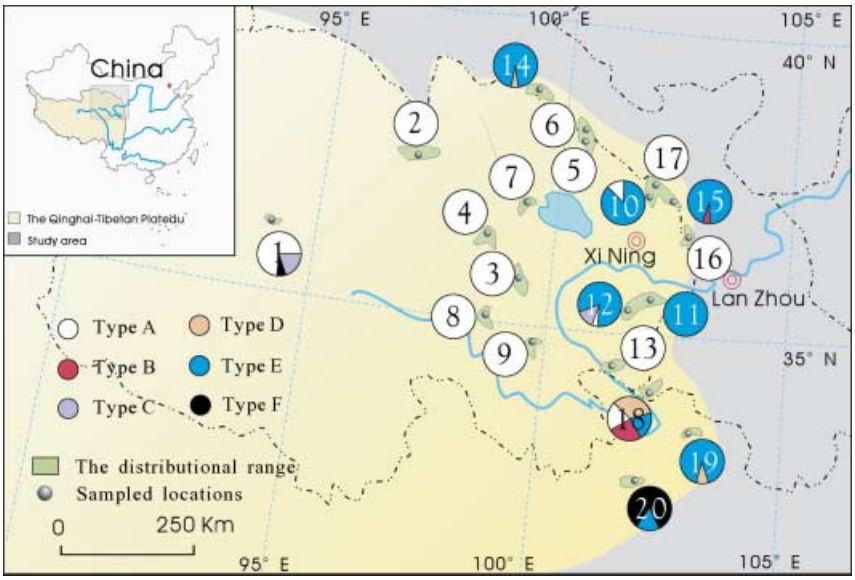

Fig. 1 The distribution of cpDNA haplotypes within and among populations of *Juniperus przewalskii*. Dotted lines indicate the different provinces in west China and the Qinghai-Tibetan Plateau region (in white).

the Q-T Plateau and also from 11 populations of the species occurring along the plateau edge (Table 1, Fig. 1). These populations covered the entire geographical distribution of the species. About 12–24 trees were sampled from each population, with samples taken from trees at least 100 m apart in each population. In each fragmented island-forest, sampled trees were usually collected along a transect across the total forest distribution at each location. The latitude, longitude and altitude at each collection centre were measured using an Etrex GIS monitor (Garmin, Taiwan). Populations from the plateau edge occurred on the southern slopes of mainly deep valleys adjacent to the nearby nonplateau platform area. Populations from the flatter plateau platform were located on the southern slopes of hills. Along the northern edge of the plateau are the Qilian Mountains and several populations were sampled from this area (populations 5, 6, 14–17). Populations 5 and 6 occurred on the southern side of these mountains and were considered to represent platform rather than plateau edge populations. In total, 392 trees were sampled. Following collection, needles were dried and stored in silica gel.

DNA extraction, amplification and sequencing

Total DNAs were extracted from silica gel-dried needles using the CTAB method (Doyle & Doyle 1987). A preliminary universal primer scanning of the chloroplast DNA genome using five different pairs of primers was conducted on 10 individuals sampled from 10 different populations. The *trn*T-*trn*F region was amplified with primers **a** and **f** and sequenced with primers **a**, **c** and **f** of Taberlet *et al*. (1991), while four other regions, *psb*B-*psb*H, *rpl*20–5′*rps*12, *trn*S-*trn*G and *psb*A-*trn*H, were amplified and sequenced using primers described in Hamilton (1999). Two pairs of primers used to amplify *trn*T-*trn*F and *trn*S-*trn*G, respectively, revealed different sequences within the 10 individuals examined, and were used thereafter for the large-scale survey of haplotype variation within *J. przewalskii*. 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 unit of *Taq* polymerase. Initial template denaturation was programmed at 94 °C for 3 min, followed by 32 cycles of 94 °C for 1 min, 50 °C for 1 min, and 72 °C for 1.75 min plus a final extension of 72 °C for 7 min.

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.). We initially selected 10–15 individuals for sequencing from each of the 20 populations. When we found more than one sequence in a particular population sample, we sequenced the remaining individuals sampled from this population and adjacent populations. For those populations where 10 or more trees had been sequenced and found to share a sequence that could be distinguished by an indel difference from those found in adjacent populations, the remaining trees in a sample were checked for sequence according to differences in PCR fragment length in 2% agarose gels.

The sequences of the two regions examined were combined and aligned by CLUSTAL W (Thompson *et al*. 1994). A matrix of combined sequences was constructed for 392

Table 1 Origin of materials, genetic diversity estimates, alleles for two cpDNA regions, and cpDNA haplotype frequencies in 20 populations of *Juniperus przewalskii*

								Allele frequency												
								*trn*T-*trn*F			*trn* S-*trn*G				cpDNA Haplotype					
P.	Population/ location	Latitute	Longitude	Alt. (m)	nh	*N*	H_E	I	II	III	a	b	c	d	A	B	C	D	E	F
1	Gerrmu, QH	36°13.2′	094°17.1′	3760	3	15	0.418	0.933	0.067	0	0.733	0.267	0	0	0.733	0	0.200	0	0	0.067
2	Delingha, QH	37°26.4′	097°45.7′	3340	1	14	0	1.000	0	0	1.000	0	0	0	1.000	0	0	0	0	0
3	Dulan, QH	36°20.3′	098°14.8′	3700	1	21	0	1.000	0	0	1.000	0	0	0	1.000	0	0	0	0	0
4	Xiangride, QH	35°55.1′	097°46.0′	3750	1	20	0	1.000	0	0	1.000	0	0	0	1.000	0	0	0	0	0
5	Qilian, QH	38°13.6′	100°15.2′	3200	1	16	0	1.000	0	0	1.000	0	0	0	1.000	0	0	0	0	0
6	Qilian, QH	38°09.0′	100°55.4′	3040	1	20	0	1.000	0	0	1.000	0	0	0	1.000	0	0	0	0	0
7	Gonghe, QH	36°54.9′	099°36.2′	3210	1	22	0	1.000	0	0	1.000	0	0	0	1.000	0	0	0	0	0
8	Xinghai, QH	35°32.3′	099°50.9′	3700	1	17	0	1.000	0	0	1.000	0	0	0	1.000	0	0	0	0	0
9	Maqin, QH	34°47.8′	100°14.1′	3520	1	24	0	1.000	0	0	1.000	0	0	0	1.000	0	0	0	0	0
10	Huzhu, QH	37°01.8′	102°15.0′	3010	2	23	0.226	0.130	0	0.870	0.174	0	0	0.826	0.130	0	0	0	0.870	0
11	Tongren, QH	35°31.8′	102°14.6′	3100	1	20	0	0	0	1.000	0	0	0	1.000	0	0	0	0	1.000	0
12	Tongren, QH	35°16.1′	101°53.7′	3120	3	22	0.310	0.045	0	0.955	0	0.091	0	0.909	0.046	0	0.136	0	0.818	0
13	Henan, QH	34°31.2′	101°11.3′	3400	1	18	0	1.000	0	0	1.000	0	0	0	1.000	0	0	0	0	0
14	Sunan, GS	38°41.8′	099°30.8′	3280	2	20	0.095	0	0	1.000	0.100	0	0	0.900	0	0	0	0.050	0.950	0
15	Tianzhu, GS	36°53.1′	101°41.2′	2500	2	22	0.165	0.091	0	0.909	0	0	0	1.000	0	0.091	0	0	0.909	0
16	Yongdeng, GS	36°41.0′	102°45.6′	1900	1	18	0	1.000	0	0	1.000	0	0	0	1.000	0	0	0	0	0
17	Haxi, GS	37°23.2′	102°32.4′	3020	1	21	0	1.000	0	0	1.000	0	0	0	1.000	0	0	0	0	0
18	Luqu, GS	34°04.7′	102°38.0′	3480	4	24	0.740	0.542	0	0.458	0.500	0	0	0.500	0.208	0.250	0	0.333	0.208	0
19	Ruoergai, SC	33°37.8′	103°14.7′	3100	2	23	0.159	0	0	1.000	0.043	0	0	0.957	0	0	0	0.087	0.913	0
20	Songpan, SC	32°23.3′	103°31.4′	2990	2	12	0.278	0	0.750	0.250	0	0	0.833	0.167	0	0	0	0	0.167	0.833

Abbreviations: P., the population code; Alt., altitude; QH, Qinghai; GS, Gansu; SC, Sichuan; *N*, number of trees analysed; nh, number of cpDNA haplotypes; H_E, total cpDNA diversity.

trees from the 20 populations examined and six different sequences (haplotypes) were identified.

Data analysis

Estimates of unbiased genetic diversity (H_E) [equivalent to expected heterozygosity for diploid data (Weir 1996)] were calculated for each population based on haplotype composition (Nei 1987). Estimates of average gene diversity within populations (H_S), total gene diversity (H_T) and the proportion of total diversity due to differences between populations (G_{ST}) were calculated for the plateau edge region and across the total distributional range using the program PERMUT (by R.J. Petit, available at http://www.pierroton.inra.fr/genetics/labo/Software/) (Pons & Petit 1996). Population differentiation (G_{ST}) across the total distribution was tested using an exact test (Raymond & Rousset 1995), while interpopulation differentiation between and within the plateau edge and plateau platform regions was evaluated by AMOVA (Excoffier *et al*. 1992) using ARLEQUIN software version 2.000 (Schneider *et al*. 2000) with significance tested by a nonparametric permutation procedure with 1000 permutations. A comparison was made between G_{ST} and N_{ST} using the *U*-statistic, which is approximated by a Gaussian variable by taking into account the covariance between N_{ST} and G_{ST}, and a one-sided test (Pons & Petit 1996). G_{ST} makes use only of haplotype frequencies while N_{ST} also takes into account differences between haplotypes. A higher N_{ST} than G_{ST} usually indicates the presence of phylogeographic structure (Pons & Petit 1996) with closely related haplotypes being found more often in the same area than less closely related haplotypes.

Phylogenetic relationships between the cpDNA haplotypes were reconstructed by neighbour-joining, maximum-parsimony and maximum-likelihood analyses in PAUP 4.0b10 (Swofford 2000) using one sample of *Juniperus tibetica* as outgroup. In all analyses, gaps were treated as missing and two indels were coded as one or zero relative to the corresponding sequences of *J. tibetica*. We used MODELTEST (Posada & Crandall 1998) to select parameters and assumptions for maximum-likelihood analysis. Both maximum-parsimony and maximum-likelihood heuristic search parameters were random addition of sequence (1000 replicates) with tree-bisection–reconnection (TBR) branch swapping, MULTREES and COLLAPSE options on. Bootstrap values were estimated to assess the relative support for relationships between haplotypes (1000 replicates) (Felsenstein 1985).

In an attempt to separate patterns of population history and recurrent gene flow we further subjected data to nested clade analysis (NCA). In this analysis, a minimum spanning haplotype network was constructed with the aid of MINSPNET (Excoffier & Smouse 1994) and relationships among haplotypes were determined and displayed in a nested manner according to the procedure outlined by Templeton *et al*. (1992). Ambiguities (i.e. closed loops) were removed using the procedures described by Crandall & Templeton (1993). Clade distances (D_c) and nested clade distances (D_n) were defined based on the geographical locations of samples in the nesting cladogram, and were estimated as described in Templeton *et al*. (1995). Differences between interior (ancestral) and tip (recent) clade D_c and D_n distances were calculated to yield $D_{cI} - D_{cT}$ and $D_{nI} - D_{nT}$ values, where I and T were interior and tip clades, respectively. The null hypothesis of no geographical associations of tip clades and interior clades was tested by considering that the dispersion distance of clades was not greater or less than expected by chance, and comparing observed D_c and D_n values with a distribution of such values, calculated for each 10 000 random permutations of clades against sampling locations (Templeton *et al*. 1995). Permutation tests were conducted separately for each level of the nested cladogram using GEODIS version 2.2 (Posada *et al*. 2000). After significance levels for D_c and D_n were determined, inferences about the processes that were likely to be responsible for observed patterns of clade structure were made using the latest inference keys provided at http://darwin.uvigo.es (updated July 2004) (cf. Templeton 1998, 2004; Maskas & Cruzan 2000; Abbott & Comes 2004; Ge *et al*. 2005; Qu *et al*. 2005). We also conducted a permutational contingency analysis based on 10 000 resamples to test for the significant ($P < 0.05$) association between geographical locality and clades detected with the entire cladogram.

Results

Sequencing of the *trn*T-*trn*F region within 200 trees sampled from 20 populations across the entire geographical range of *Juniperus przewalskii* identified three different sequences (Table 2). One sequence (703 bp) differed from the other two (728 bp) in length and for a single nucleotide substitution at site 54 (T→G). The insertion of 25 bp occurred from sites 59–83 and was a tandem repeat of the sequence from sites 34–58. The two longer sequences were distinguished only by a single nucleotide substitution at site 625 (A→T). We further sequenced the *trn*T-*trn*F region in 39 trees from populations found to contain the two longer sequences or were adjacent to populations containing a different one of these long sequences. The remaining 153 trees from populations where only one sequence was present were not sequenced for *trn*T-*trn*F. These trees produced an amplified product of either 703 bp or 728 bp and were assumed to have the same sequences as that previously identified for trees in the corresponding population that possessed the same insertion.

The total alignment of *trn*S-*trn*G sequences obtained for the initial screening of 296 trees in 20 populations (12–15 individuals for each population) was 795 bp in length.

Table 2 Variable sites of the aligned sequences of two chloroplast DNA fragments in six haplotypes of *Juniperus przewalskii* (* and †, two indels). Sequences are numbered from the 5′ to the 3′end in each region

Nucleotide position	*trn*T-*trn*F			*trn*S-*trn*G					
			6	2	3	6	6	7	7
	5	5	2	2	2	4	5	1	6
Haplotype	4	9	4	6	5	0	8	6	2
Type A	T	—	A	G	T	G	—	A	C
Type B	T	—	A	A	G	A	†	G	T
Type C	T	—	A	G	T	G	—	G	C
Type D	G	*	T	G	T	G	—	A	C
Type E	G	*	T	A	G	A	†	G	T
Type F	G	*	A	G	T	A	†	G	T

*, AATTATAGCGAATCGAATTAGAATA;
†,
ATTGAGTTTTCAGGAATAGGAAAATATGATGATCGAAACT.

Table 3 The 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) within the plateau edge region and the total distribution calculated with PERMUT, using a permutation test with 1000 permutations

Region	H_S	H_T	G_{ST}	N_{ST}
The plateau edge	0.195 (0.069)	0.662 (0.080)	0.705 (0.101)	0.742 (0.113)
The total distributional range	0.130 (0.046)	0.568 (0.082)	0.772 (0.062)	0.834 (0.057)

Nucleotide substitutions occurred at five sites (226, G/A; 325, T/G; 640, G/A; 716, G/A; 762, T/C), and an indel of 40 bp was present between sites 658 and 697 (Table 2). In total, four different sequences were recovered incorporating two different lengths. We further sequenced 32 trees from populations in which two different sequences that could not be distinguished by an indel difference were either present or occurred in adjacent populations. Where more than 15 individuals of a given population were surveyed and shown to possess the same sequence, additional individuals of the population were assigned this sequence if they produced a PCR-amplified product of the same length. The three *trn*T-*trn*F sequences and four *trn*S-*trn*G sequences recorded in *J. przewalskii*, together with the *trn*T-*trn*F and *trn*S-*trn*G sequences obtained from one individual of *Juniperus tibetica*, have been deposited in the EMBL GenBank databases under accession numbers AY730341–AY730349.

The combined data of both *trn*T-*trn*F and *trn*S-*trn*G sequences identified six different cpDNA haplotypes: A, B, C, D, E and F (Table 2), among the total complement of trees examined. Haplotype frequencies in each population are presented in Table 1 with geographical distributions illustrated in Fig. 1. The distribution of haplotypes was structured into two distinct geographical regions: the plateau platform and the plateau edge. All of the populations (2–8) from the eastern part of the plateau platform were fixed for haplotype A ($H_E = 0$), while a more westerly population (1) contained three haplotypes (A, C and F; $H_E = 0.418$) with haplotype A present at high frequency (0.733). In populations at the plateau edge, haplotype A was fixed in some populations (13, 16, 17), whereas haplotype E was fixed or occurred at high frequency in other populations (10–12, 14, 15, 19). Two plateau edge populations (18, 20) had a markedly different haplotype composition compared to the rest. Population 20 was polymorphic ($H_E = 0.278$) for haplotype F and haplotype E, occurring at high and low frequency, respectively, while population 18, which contained the greatest gene diversity ($H_E = 0.74$), was polymorphic for four haplotypes (A, B, D and E) that occurred in approximately equivalent frequencies.

Interpopulation differentiation across the total distribution of the species was high, $G_{ST} = 0.772$ (Table 3), and AMOVA revealed that approximately 49% of the total genetic variation was assigned between the plateau platform and the plateau edge populations, while about 34% of variation occurred among populations within these regions (Table 4). The F_{ST} value derived for all sampled populations by combining the variance components of 'among groups' and 'among populations within groups' was 0.825 and highly significant ($P < 0.001$, Table 4). The difference in the two estimates of population differentiation across the species (i.e. $G_{ST} = 0.705$ vs. $F_{ST} = 0.825$) is mainly caused by the different default weights employed with reference to variations in population size during processing estimates by PERMUT and AMOVA. Hierarchical AMOVAS showed that variation among populations was significant in both regions, but contributed to a much greater proportion of total variation within the plateau edge region than in the plateau platform area ($F_{ST} = 0.685$ vs. $F_{ST} = 0.178$, Table 4). A test for phylogeographic structure of haplotype variation across the distribution of the species showed that N_{ST} (0.834) was significantly higher than G_{ST} (0.772) ($P < 0.05$), indicating that closely related haplotypes were more likely to co-occur in the same region. However, within the plateau edge region the difference was not significant ($N_{ST} = 0.742$, $G_{ST} = 0.705$; $P = 0.12$).

Total alignment of *trn*T-*trn*F and *trn*S-*trn*G sequences that included indels covered 1525 positions, of which 1516 were constant, and only nine (seven mutations and two

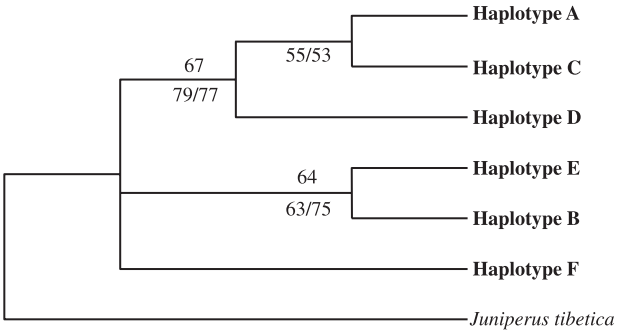

Fig. 2 Phylogenetic relationships of six cpDNA haplotypes resolved in *Juniperus przewalskii*. The single maximum parsimonious tree is presented. This has the same topology as neighbour-joining and maximum-likelihood trees produced from the same data. Bootstrap values (> 50%) are denoted above (maximum-parsimony) and below (neighbour-joining and maximum-likelihood) branches.

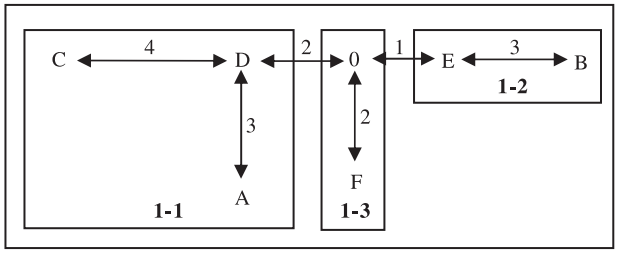

Fig. 3 The nested cladogram of cpDNA haplotypes (A–F) of *Juniperus przewalskii*. Identified haplotypes are in capital letters and '0' represents a hypothetical common ancestor for three 'one-step' clades (1-1, 1-2, and 1-3). The number above or at the right of each connection represents mutational steps.

indels) were phylogenetically informative. The heuristic maximum-parsimony search produced a single most parsimonious tree (length = 13 steps, RI = 0.692 CI = 0.714) (Fig. 2), which was topologically the same as the maximum-likelihood tree (–ln L = 2078.45, the best-fit model F_{81} + I) and the neighbour-joining tree (not shown). Three clades were identified: A/C/D, B/E and F; however, relationships between these clades were not resolved. Clades A/C/D and B/E received low to moderate bootstrap support in all analyses. There was an indication that within the A/C/D clade, haplotypes A and C were more derived, but this relationship had very low bootstrap support.

The nested cladogram constructed for cpDNA haplotypes (Fig. 3) was largely consistent with the most parsimonious tree. Three major haplotype one-step clades (1-1, 1-2 and 1-3) grouped by terminal haplotypes were recovered from a hypothetical common ancestor '0' by nested clade analysis, the first of which contained haplotypes A, C and D, while the second contained haplotypes B and E (Fig. 3). The network indicates that cpDNA haplotypes D and E are likely to represent ancestral types in two clades nested within the network. The statistical nested clade tests of patterns of geographical/genetic association, incorporating estimation of geographical distance parameters (Table 5) suggested a scenario of range expansion as a major process shaping the spatial distribution of haplotypes in clade 1-1 and haplotypes and clades within the entire cladogram (Table 6). In both cases, it is suggested that range expansion occurred either through long-distance colonization possibly coupled with subsequent fragmentation or by past fragmentation followed by range expansion (Templeton 1998). It is not possible to distinguish which of these two historical events was responsible for the present-day distribution of haplotypes and it is feasible that both may have played a role. A failure to identify the cause of the significant geographical structure of haplotypes in clade 1-2 is possibly due to the rarity of tip haplotype B.

Table 4 Analyses of molecular variance (AMOVA) for populations of *Juniperus przewalskii* based on cpDNA *trn*T-F and *trn*S-G sequences

Grouping of regions	Source of variation	d.f.	SS	VC	Variation (%)	Fixation index
Plateau platform	Among populations	8	0.790	0.004	17.8	F_{ST} = 0.178**
	Within populations	160	3.133	0.0196	82.2	
	Total	168	3.923	0.024		
Plateau edge	Among populations	10	44.887	0.217	68.5	F_{ST} = 0.685**
	Within populations	212	21.154	0.010	31.5	
	Total	222	66.040	0.317		
Plateau platform and plateau edge	Among groups	1	37.608	0.182	48.7	F_{SC} = 0.660**
	Among populations within groups	18	45.676	0.126	33.8	F_{ST} = 0.825**
	Within populations	372	24.287	0.065	17.5	F_{CT} = 0.487**
	Total	391	107.57	0.374		

d.f., degrees of freedom; SS, sum of squares; VC, Variance components; **P < 0.001.

Table 5 Results of the nested clade analysis of the geographical distance for cpDNA haplotypes (A–E) of *Juniperus przewalskii* based on the phylogenetic relationships given in Fig. 3

Zero-step						One-step					
Hap	Pos	D_c	*P*	D_n	*P*	Clades	Pos	D_c	*P*	D_n	*P*
H_A	T	211.08s	–0.000	211.60_S	–0	1-1	T	221.65_S	–0.000	231.98_S	–0.022
H_C	T	334.79_L	+0.003	386.95_L	+0						
H_D	I	114.50_S	–0.000	362.48_L	+0						
I-T	—	-99.71_S	–0.000	146.44_L	+0						
H_B	T	126.21	–0.176	187.5109	+0.44	1-2	T	180.37_S	–0.000	226.75_S	–0.045
H_E	I	180.60	–0.533	179.95	+0.554						
I-T	—	54.3855	–0.808	–7.56	+0.56						
						1-3	I	131.08_S	–0.004	495.18_L	+0.000
						I-T	—	-76.16_S	–0.049	256.02_L	+0.000

Hap, haplotypes; 1-1, 1-2, 1-3 (clades) designated in Fig. 3; Pos, position; I, interior; T, tip; I-T, the average difference between interior vs. tip clades for both distance measures. Probabilities for large (+) or smaller (–) than expected values are based on 10 000 randomizations of the data. Significantly small or large values for D_c, D_n (I-T) D_c, and (I-T) D_n are indicated by an 'S' or 'L', respectively, together with their level of significance $P < 0.05$ where *P* is the probability of a randomly generated value being equal to or smaller (larger) than the observed value.

Table 6 Permutational chi-squared probabilities for geographical structure of the clades identified in Fig. 3 from 10 000 resamples. Clades with a probability value less than 0.05 suggest a significant association of haplotypes or subclades with locality. *P* is the probability of a randomly generated chi-squared statistic being greater than or equal to the observed chi-squared. Chain of inference from the nested clade analysis (Table 5 and Fig. 3) following keys given at http://darwin.uvigo.es (Templeton 1998, 2004)

Clades	Chi-squared statistic	*P*	Clade key	Inferences
1-1	291.56	0.000	1-2-11-YES-12-13-YES	Range expansion; long-distance colonization possibly coupled with subsequent fragmentation or past fragmentation followed by range expansion
1-2	52.60	0.000	1-2-11-17-NO	Inconclusive
Entire cladogram	618.78	0.000	1-2-11-YES-12-13-YES	Range expansion; long-distance colonization possibly coupled with subsequent fragmentation or past fragmentation followed by range expansion

Discussion

Population structure

The exact mode of inheritance of cpDNA in *Juniperus przewalskii* remains unknown because no crossing experiments have been conducted; however, other studies of species in the family Cupressaceae indicate that chloroplast transmittance is paternal (Neale *et al*. 1991; Mogensen 1996; Kondo *et al*. 1998; Hwang *et al*. 2003). If this is also the case for *J. przewalskii*, it might be expected that in such a wind-pollinated tree interpopulation differentiation for cpDNA would be reduced relative to what would be expected if inheritance was maternal (Wang & Szmidt 2001). However, because of the marked geographical structure found in the species for cpDNA, a very high estimate of interpopulation differentiation was recorded ($G_{ST} = 0.772$; $F_{ST} = 0.825$). This was largely due to the major differences in haplotype composition between populations on the plateau platform compared to those at the plateau edge, and also to the marked differences between plateau edge populations. The maintenance of large differences between plateau edge populations might be viewed as somewhat surprising, given the potential for high levels of gene flow in a wind-pollinated species. In other conifers with known paternal cpDNA inheritance, low interpopulation differentiation is commonly recorded (Petit *et al*. 2005), e.g. in *Pinus flexilis* (Latta & Mitton 1997), *Pinus banksiana* and *Pinus contorta* (Dong & Wagner 1994), and in *Cunninghamia* spp., Cupressaceae (Hwang *et al*. 2003; $G_{ST} = 0.073$ and 0.017), because of frequent pollen flow, although exceptions have been reported, e.g. in *Pinus muricata* (Hong *et al*. 1993).

The high level of interpopulation differentiation recorded within *J. przewalskii* is most likely due to populations

being separated by high mountains both along the plateau edge and between the plateau platform and plateau edges. These mountains probably impose significant barriers to gene flow between populations. It is also possible that pollen, even in the absence of such barriers, is not naturally dispersed far in junipers. We have monitored pollen dispersion in *Juniperus tibetica* on the Q-T Plateau and found that the maximum distance that pollen was carried by wind did not exceed 2 km (Miehe *et al*., unpublished). However, further studies are required to determine how general low pollen dispersal might be in junipers, and in *J. przewalskii* in particular.

Inference of demographic history

The absence of cpDNA variation within and among eight of the nine plateau platform populations examined, would seem to support the hypothesis that these populations are derived from a recent colonization of the area by *J. przewalskii* rather than them being relicts of a once widespread Pliocene forest on the plateau platform which subsequently became fragmented during the Quaternary. If the latter hypothesis were true, and assuming that *J. przewalskii* was polymorphic for cpDNA haplotypes during the period when it was widespread on the plateau platform, present-day populations would more likely be fixed or nearly fixed for different haplotypes due to the random effects of genetic drift in fragmented populations. In fact, such a pattern of differentiation was found only among plateau edge populations rather than among plateau platform populations. The phylogeographic structure resolved in *J. przewalskii* is similar to that reported for alder buckthorn (*Frangula alnus*), which occurs widely in Europe. This species was also shown to exhibit marked population differentiation in its refuge area, but almost completely genetic uniformity in regions it has recolonized (Hampe *et al*. 2003). To further discriminate between alternative phylogeographic hypotheses, we examined the spatial haplotype structure by nested clade analysis, bearing in mind that conclusions drawn from this form of analysis must be treated with caution (Knowles & Maddison 2002; Petit & Grivet 2002). Range expansion, including bottlenecks and exponential population growth, may allow the replacement of ancestral haplotypes with novel derived haplotypes (Hewitt 1996). If ancestral and derived haplotypes do not overlap and are located in different regions, then ancestral haplotypes should be found close to refugia, while derived haplotypes are more likely to occur at the leading edge of the range expansion (cf. Rowe *et al*. 2004). NCA revealed that haplotype A, which was fixed in eight of nine plateau platform populations examined, is at a tip (derived) position within the haplotype network, whereas the internal (ancestral) haplotype D within the same clade (1-1) is restricted to the southeastern edge of the plateau (Fig. 1), a possible refugium for *J. przewalskii* during the last glacial maximum. Range expansion was inferred as the major process influencing the spatial distribution of haplotypes within this clade and the entire cladogram. In both cases, it was not possible to establish whether a range expansion occurred through long-distance colonization possibly followed by subsequent fragmentation or by past fragmentation followed by range expansion. The presence of haplotypes C and F in both the most westerly plateau platform population (1) and in certain plateau edge populations, might suggest that long-distance colonization was responsible for this particular marked disjunction, although the alternative explanation of past fragmentation cannot be ruled out. The inference that range expansion was the major process influencing the present-day spatial distribution of haplotypes within *J. przewalskii* supports the hypothesis that the plateau platform populations of this species originated following a postglacial invasion of the platform from the eastern margin. This expansion is most 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 evidence from the northeast Q-T Plateau region revealed that the Holocene forest invasion began approximately 8000 BP and that forests were predominant until 3000 BP when vegetation replacement with alpine meadow and desert-steppe vegetation occurred (Tang & Shen 1996). A relatively high level of haplotypic diversity in the southeast plateau edge populations (18–20) is further evidence that a glacial refugium could have existed for the species in this area, which is in keeping with the proposal that forests were restricted to a southeast refugium at the margin of the Q-T Plateau during the last glacial maximum (Tang *et al*. 1998).

It might be argued that interspecific hybridization and the transfer of cpDNA haplotypes from other species of *Juniperus* could also have contributed to the current geographical pattern of cpDNA variation in *J. przewalskii*. In a study of other *Juniperus* species, one of two cp *trn*L-*trn*F haplotypes in *Juniperus osteosperma* was shared with *Juniperus occidentalis* and occurred only in populations sympatric with the latter species. It was suggested that this haplotype had been captured through cytoplasmic gene flow from *J. occidentalis* (Terry *et al*. 2000). Although we cannot refute the possibility of ancient hybridization and haplotype introgression between *J. przewalskii* and other junipers distributed in the Q-T Plateau, there is no evidence that such introgression has occurred in recent times. We have never found *J. przewalskii* in sympatry with other junipers in the wild. Some junipers, such as *J. tibetica* and *Juniperus convallium*, have distributions that come close to certain southern platform populations of *J. przewalskii* (e.g. populations 8 and 9) that are fixed for haplotype A, but this haplotype is also fixed in plateau platform populations distant from these two congenerics. Similarly, *J. tibetica* and *J. convallium*

are found in valleys near to those occupied by southeastern plateau edge populations of *J. przewalskii* (18, 19 and 20), but once again the haplotypes that occur in these populations (B, C, D and F) also occur in populations (1, 14 and 15) distant from these two congenerics. Thus the presence of any particular haplotype in a population of *J. przewalskii* is not correlated with the presence nearby of *J. tibetica* and *J. convallium*, which argues against the possibility that introgression has been a factor affecting the geographical pattern of cpDNA variation in *J. przewalskii*. Furthermore, a limited survey of cpDNA variation in *J. tibetica* and *J. convallium* failed to detect any of the six haplotypes resolved in *J. przewalskii* (Zhang *et al*., unpublished).

The results of our phylogeographic study of *J. przewalskii* taken together with the available fossil pollen evidence support the hypothesis that forest recolonized the plateau platform in the early Holocene period, but was subsequently replaced by vegetation in which forest is distributed disjunctly. It is of interest therefore to consider which factors brought about the recent forest fragmentation. One possibility is that drier and colder conditions from the late Holocene onwards combined to cause such fragmentation. Another possibility, however, is that forest was destroyed by fire caused by natural means or intentionally by humans for raising yaks or sheep. This alternative explanation is supported by our recent finding that a quantity of juniper and *Picea* charcoals occurs in the soil layers under alpine meadow vegetation located, respectively, between populations 4 and 8, and 2 and 7. These charcoals have been dated to between 4000 and 8900 BP (Knut & Miehe, unpublished), providing evidence both that the eastern plateau platform had supported a continuous forest shortly before this time and that fire was one factor causing the current disjunct distribution of forest.

In a recent study of the phylogeography of the red-necked snow finch, *Pyrgilauda ruficollis*, which is endemic to the Tibetan Plateau, evidence was obtained that suggested this species also colonized the plateau from the eastern margin after the last extensive glacial period (Qu *et al*. 2005). A high level of mitochondrial DNA haplotypic diversity coupled with low nucleotide diversity within the species indicated a rapid range expansion following a population bottleneck. In this regard therefore the recent history of *P. ruficolis* and *J. przewalskii* appears to be similar. However, an absence of geographical structure in the distribution of haplotypic variation within *P. ruficolis* suggests that considerable gene flow occurred between populations during the colonization process. In contrast, our findings of a high level of population differentiation and significant phylogeographic structure across the present–day geographical range of *J. przewalskii* imply that gene flow between populations has been far more restricted in this species. More phylogeographic studies are now required on a wide range of different species endemic to the Tibetan Plateau to obtain a better understanding of the factors that have influenced the evolutionary history of this region's flora and fauna.

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