

Research Article

Phylogeography of *Spiraea alpina* (Rosaceae) in the Qinghai–Tibetan Plateau inferred from chloroplast DNA sequence variations

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Abstract The aim of the present study was to investigate the phylogeographic patterns of *Spiraea alpina* (Rosaceae) and clarify its response to past climatic changes in the climate-sensitive Qinghai–Tibetan Plateau (QTP). We sequenced a chloroplast DNA fragment (*trnL–trnF*) from 528 individuals representing 43 populations. We identified 10 haplotypes, which were tentatively divided into three groups. These haplotypes or groups were distributed in the different regions of the QTP. Only half the populations were fixed by a single haplotype, whereas the others contained two or more. In the central and eastern regions, adjacent populations at the local scale shared the same haplotype. Our phylogeographic analyses suggest that this alpine shrub survived in multiple refugia during the Last Glacial Maximum and that earlier glaciations may have triggered deep intraspecific divergences. Post-glacial expansions occurred only within populations or across multiple populations within a local range. The findings of the present study together with previous phylogeographic reports suggest that evolutionary histories of plants in the QTP are complex and variable depending on the species investigated.

Key words chloroplast DNA, haplotype, phylogeography, Qinghai–Tibetan Plateau, refugia, *Spiraea alpina*, *trnL–trnF*.

As the largest and highest region, the Qinghai–Tibetan Plateau (QTP) has been considered the most sensitive to historical climate changes (Zheng, 1996; Zheng & Yao, 2004). Thus, it should be possible to trace climate changes as shifts in the distributional range of both the plants and animals that occur there (Zheng, 1996). These shifts can be detected from the genetic structure of current populations, especially with regard to glacial retreat (into refugia) and post-glacial recolonization since the Last Glacial Maximum (LGM; Hewitt, 1996, 2000, 2004; Avise, 2000, 2004). Such a pattern of retreat and recolonization has been found for several alpine species in the QTP (Zhang et al., 2005; Meng et al., 2007; Chen et al., 2008; Yang et al., 2008; Wu et al., 2010). These species retreated into the south-eastern refugia and recolonized the platform during the interglacial ages or at the end of the LGM. However, others may have survived through the Quaternary glacial

ages at high altitude (Wang et al., 2009a; Jia et al., 2011, 2012). These studies further suggest that although the LGM did not seriously affect the distributional range of the species and that they survived in multiple refugia, previous climatic changes may have led to deep intraspecific divergences (Gao et al., 2007, 2009; Wang et al., 2008a, 2008b, 2009a, 2009b; Opgenoorth et al., 2010; Sun et al., 2010; Wu et al., 2010; Jia et al., 2011). This is understandable given the fact that the massive ice sheet never developed on the QTP and that the coldest climate occurred between 1.2 and 0.4 Ma when the largest glaciation developed, rather than at the time of the LGM (Shi et al., 1998; Zhou et al., 2006). The available data also suggest that plant species with different habits or traits may show contrasting patterns of responses to Quaternary climatic oscillations. In the QTP, more than 1800 alpine species have been recorded at high altitude (i.e. >4500 m asl; Wu et al., 1995). However, the phylogeographic patterns of most species remain unknown.

Herein we report on the phylogeographic structure of *Spiraea alpina* Pall. (Rosaceae). This shrub is widely distributed at altitudes between 2000 and 4500 m asl

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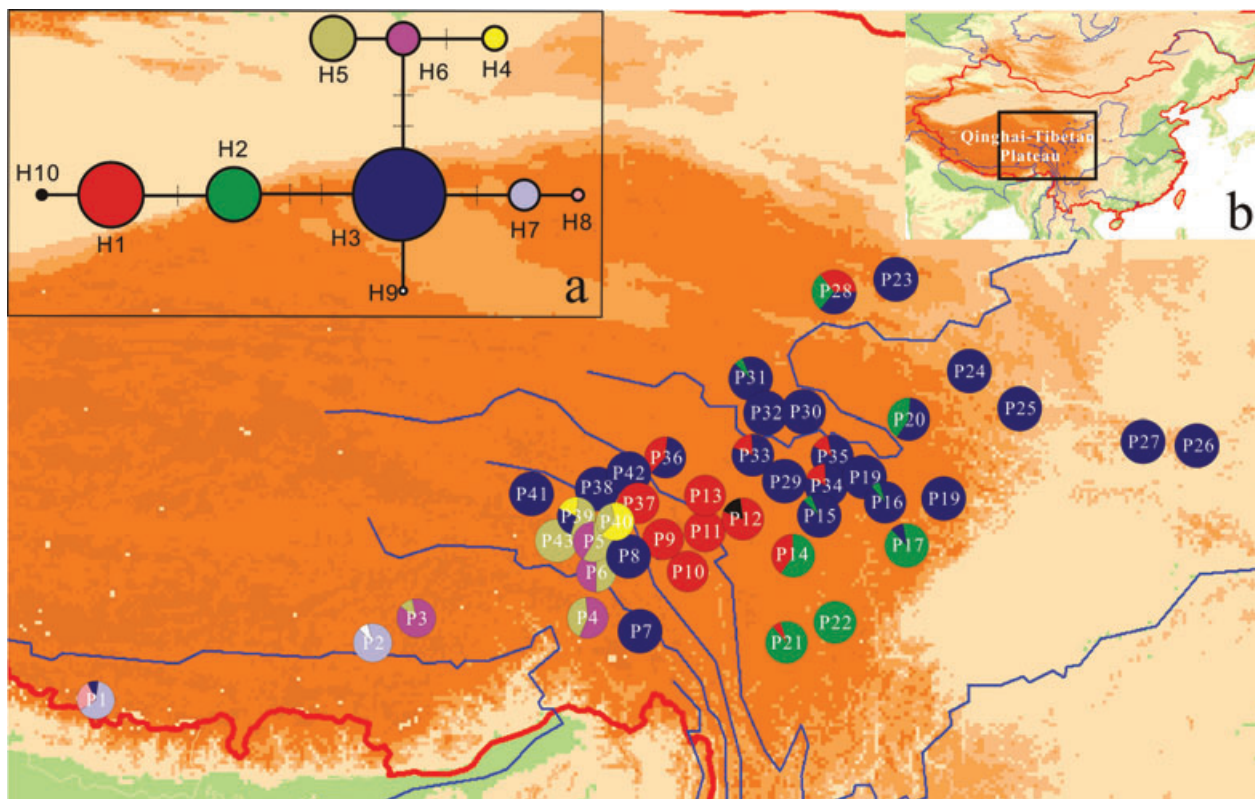


Fig. 1. Map showing locations of the sampled populations of *Spiraea alpina* and haplotype distribution. **a**, Genealogical relationships of haplotypes based on the *trnL-trnF* intergenic spacer of the chloroplast (cp) DNA genome. The diameter of the circles is proportional to haplotype frequency; vertical dashed lines represent missing intermediate haplotypes. Different shading of circles indicates different haplotypes and these correspond to the shaded circles that appear on the map. **b**, Map of China showing the Qinghai–Tibetan Plateau. The map is reproduced with permission from the Data-Sharing Network of Earth System Science (<http://www.geodata.cn>, accessed 20 May 2009).

in the QTP, with partial extensions to adjacent regions (Lu et al., 2003; Zhang et al., 2006; Potter et al., 2007; Fig. 1). Chloroplast (cp) DNA is maternally inherited in Rosaceae (Soltis & Soltis, 1998) and is therefore a good marker for tracing population or range expansion of the species through seed dispersal (e.g. Meng et al., 2008; Wang et al., 2008a; Weeks, 2008; Pan et al., 2009). In the present study, we examined the sequence variation of all samples using the cpDNA fragment *trnL-trnF* because this intergenic spacer region has been found to be highly variable within and between the other congeners (Zhang et al., 2006; Potter et al., 2007). We used these population genetic data to trace shifts in the range of this alpine shrub in response to past climatic changes. We were particularly interested in whether this shrub species survived in multiple refugia during the LGM as did other alpine shrubs that occur in the QTP (e.g. Wang et al., 2009b; Sun et al., 2010; Jia et al., 2011).

1 Material and methods

1.1 Population sampling

During the summers of 2006–2009, 528 individuals from 43 populations of *S. alpina* were collected from the QTP and adjacent regions (Table 1; Fig. 1). For each population, between six and 15 individuals (spaced at least 100 m apart) were sampled and voucher specimens were deposited in the archives of the Northwest Institute of Plateau Biology (HNWP), Chinese Academy of Sciences.

1.2 DNA extraction, PCR amplification and sequencing

Total genomic DNA was extracted from silica gel-dried leaves following the cetyltrimethyl ammonium bromide (CTAB) method described by Doyle & Doyle (1987) and used as the template DNA for PCR amplification of the *trnL-trnF* intergenic spacer (Taberlet et al.,

Table 1 Geographic locations and sample sizes of the 43 *Spiraea alpina* populations sampled in the present study and their chloroplast (cp) DNA haplotypes and frequencies

Population	Locality (All in China)	Voucher No.	Latitude	Longitude	Altitude (m asl)	No. individuals sampled	Haplotype (frequency, %)	<i>He</i>	<i>PD</i>	π
P1	Nyalam, T	Chen2007103	28°08'	85°58'	3750	11	H3 (9.1), H7 (63.6), H8 (27.3)	0.5636 ± 0.1340	0.6181 ± 0.5255	0.000 67 ± 0.000 64
P2	Mozhugongka, T	Chen2007110	29°42'	92°04'	4150	12	H7 (91.7), H9 (8.3)	0.1667 ± 0.1343	0.3333 ± 0.3564	0.000 36 ± 0.000 43
P3	Gongjujiangda, T	Chen2007126	29°54'	92°26'	4340	9	H5 (11.1), H6 (88.9)	0.2222 ± 0.1662	0.2222 ± 0.2880	0.000 24 ± 0.000 35
P4	Leiwuqi, T	Chen2007035	30°21'	96°27'	4290	12	H5 (41.7), H6 (58.3)	0.5303 ± 0.0764	0.5303 ± 0.4734	0.000 57 ± 0.000 58
P5	Leiwuqi, T	Chen2007043	31°32'	96°22'	4210	12	H5 (58.3), H6 (41.7)	0.5303 ± 0.0764	0.5303 ± 0.4734	0.000 57 ± 0.000 58
P6	Dingqing, T	Chen2007054	31°05'	96°24'	4410	12	H5 (50), H6 (50)	0.5455 ± 0.0615	0.5454 ± 0.4820	0.000 59 ± 0.000 59
P7	Basu, T	Chen2007179	30°07'	97°17'	4320	14	H3 (100)	0.0000	0.0000	0.000 00
P8	Changdu, T	Chen2007205	31°11'	97°02'	3380	9	H3 (100)	0.0000	0.0000	0.000 00
P9	Jiangda, T	Chen2007210	31°21'	97°42'	4490	6	H1 (100)	0.0000	0.0000	0.000 00
P10	Jiangda, T	Chen2007218	31°20'	98°03'	4360	10	H1 (100)	0.0000	0.0000	0.000 00
P11	Dege, T	Chen2007239	31°57'	98°54'	4410	7	H1 (100)	0.0000	0.0000	0.000 00
P12	Dege, T	Chen2007244	32°03'	99°01'	4570	12	H1 (83.3), H10 (16.7)	0.3030 ± 0.1475	0.3030 ± 0.3370	0.000 33 ± 0.000 41
P13	Shiqu, SC	Chen2007251	32°30'	98°27'	4380	13	H1 (100)	0.0000	0.000 00	0.000 00
P14	Luhuo, SC	Chen06318	31°37'	100°43'	3460	15	H1 (40), H2 (60)	0.5143 ± 0.0690	0.5143 ± 0.4582	0.000 56 ± 0.000 56
P15	Rangtang, SC	Chen06321	32°18'	101°03'	3820	15	H2 (6.7), H3 (93.3)	0.1333 ± 0.1123	0.1333 ± 0.2099	0.000 14 ± 0.000 25
P16	Hongyuan, SC	Chen06099	32°46'	102°21'	3654	15	H2 (6.7), H3 (93.3)	0.1333 ± 0.1123	0.1333 ± 0.2099	0.000 14 ± 0.000 25
P17	Hongyuan, SC	Chen06108	31°53'	102°40'	3970	11	H2 (90.9), H3 (9.1)	0.1818 ± 0.1436	0.1818 ± 0.2534	0.000 20 ± 0.000 31
P18	Songpan, SC	Chen06078	32°35'	103°37'	2830	14	H3 (100)	0.0000	0.0000	0.000 00
P19	Aba, SC	Chensl-0455	32°55'	101°49'	3490	9	H3 (100)	0.0000	0.0000	0.000 00
P20	Zoige, SC	Chensl-0464	34°07'	102°39'	3270	12	H2 (41.7), H3 (58.3)	0.5303 ± 0.0764	0.5303 ± 0.4747	0.000 57 ± 0.000 58
P21	Litang, SC	Chen06280	29°38'	100°21'	3891	15	H1 (6.7), H2 (93.3)	0.1333 ± 0.1123	0.1333 ± 0.2099	0.000 14 ± 0.000 25
P22	Yajiang, SC	Chen06307	30°04'	101°20'	4280	15	H2 (100)	0.0000	0.00 00	0.000 00
P23	Tianzhu, GS	Chensl-0473	37°24'	102°34'	2670	9	H3 (100)	0.0000	0.00 00	0.000 00
P24	Weyuan, GS	Chen06063	35°00'	103°59'	2530	13	H3 (100)	0.0000	0.0000	0.000 00
P25	Gangu, GS	Chen06069	34°30'	105°07'	2424	12	H3 (100)	0.0000	0.0000	0.000 00
P26	Shangluo, SX	Chen2009013	33°53'	108°50'	2426	11	H3 (100)	0.0000	0.0000	0.000 00
P27	Baoji, SX	Chen2009016	33°56'	107°44'	3355	11	H3 (100)	0.0000	0.0000	0.000 00
P28	Datong, QH	Chen06001	36°59'	101°25'	3210	14	H1 (35.7), H2 (28.6), H3 (35.7)	0.7143 ± 0.0522	0.9890 ± 0.7098	0.001 04 ± 0.000 86
P29	Dari, QH	Chen06326	33°17'	100°23'	4370	15	H3 (100)	0.0000	0.00000	0.000 00
P30	Maqin, QH	Chen06330	34°36'	100°34'	3380	15	H3 (100)	0.0000	0.00000	0.000 00
P31	Maqin, QH	Chensl-0162	34°44'	99°41'	3780	15	H3 (6.7), H3 (93.3)	0.1333 ± 0.1123	0.1333 ± 0.2099	0.000 14 ± 0.000 25
P32	Gande, QH	Chensl-0190	34°13'	100°14'	4310	12	H3 (100)	0.0000	0.0000	0.000 00
P33	Dari, QH	Chensl-0295	33°44'	99°53'	4010	11	H1 (18.2), H3 (81.8)	0.3273 ± 0.1533	0.6546 ± 0.5455	0.000 71 ± 0.000 67
P34	Banma, QH	Chensl-0347	32°50'	100°52'	3590	11	H1 (18.2), H3 (81.8)	0.3273 ± 0.1533	0.6546 ± 0.5455	0.000 71 ± 0.000 67
P35	Jiuzhi, QH	Chensl-0425	33°24'	101°15'	4210	15	H1 (13.3), H3 (86.7)	0.2476 ± 0.1307	0.4952 ± 0.4475	0.000 54 ± 0.000 54
P36	Chenduo, QH	Chen2007025	33°11'	97°24'	4040	15	H1 (40), H3 (60)	0.5143 ± 0.0690	1.0286 ± 0.7267	0.001 11 ± 0.000 88
P37	Shanglaxiu, QH	Chen06031	32°46'	96°39'	4090	8	H1 (100)	0.0000	0.0000	0.000 00
P38	Xialaxiu, QH	Chen06032	32°45'	96°34'	3900	14	H3 (100)	0.0000	0.0000	0.000 00
P39	Nangqian, QH	Chen06034	32°15'	96°19'	4030	15	H1 (26.7), H4 (20), H5 (53.3)	0.6476 ± 0.0882	2.5524 ± 1.4512	0.002 76 ± 0.001 76
P40	Nangqian, QH	Chen06037	31°58'	96°30'	4320	15	H4 (66.7), H5 (33.3)	0.4762 ± 0.0920	0.9524 ± 0.6886	0.001 03 ± 0.000 84
P41	Zaduo, QH	Chen06055	32°52'	95°19'	4030	11	H3 (100)	0.0000	0.0000	0.000 00
P42	Yushu, QH	Chen06061	32°52'	97°04'	3810	15	H3 (100)	0.0000	0.0000	0.000 00
P43	Angqian, QH	Chen2007034	31°58'	96°25'	4290	11	H5 (100)	0.0000	0.0000	0.000 00
Total						528	H1-H10	0.6721 ± 0.0188	1.4885 ± 0.9007	0.001 61 ± 0.001 08

Where appropriate, data are given as the mean ± SD.

Note: voucher specimens have been deposited in the archives of the Northwest Institute of Plateau Biology (HNWP), Chinese Academy of Sciences. GS, Gansu Province; He, haplotype diversity; PD, mean number of pairwise differences; QH, Qinghai Province; SC, Sichuan Province; SX, Shanxi Province; T, Tibet Autonomous Region; π , nucleotide diversity.

1991) (Table 1). The PCR reactions were performed in 25- μ L mixtures, containing 0.8 μ L (10–30 ng) template DNA, 2.5 μ L of 10 \times PCR buffer (15 mmol/L MgCl₂), 0.2 μ L dNTP mix (10 mmol/L), 0.8 μ L each primer (5 pmol/L), and 0.2 μ L (1 unit) *Taq* DNA polymerase (CASarray, Shanghai, China). The reaction conditions were as follows: 4 min at 94°C, followed by 30 cycles of 1 min at 94°C, 1 min at 54°C, and 2 min at 72°C, with a final extension for 7 min at 72°C. Amplification products were visualized on 0.7% agarose gels stained with ethidium bromide and purified using a CASpure PCR Purification Kit (CASarray) according to the manufacturer's instructions. Sequencing reactions were performed using a Biometra thermocycler and a DYEnamic Dye Terminator Cycle Sequencing Kit (Amersham Pharmacia Biotech, Piscataway, NJ, USA) according to the manufacturer's instructions. Sequencing products were separated and analyzed using a MegaBACE 500 Automated Sequencer (Amersham Pharmacia Biotech).

1.3 Data analysis

Sequences were checked manually and aligned using CLUSTAL X (Thompson et al., 1997). All sequences have been deposited in GenBank under accession numbers JQ765640–JQ765649. Arlequin version 3.01 (Excoffier et al., 2006) was used to calculate haplotype diversity (H_e), mean pairwise differences (PD), and nucleotide diversity (π), as well as for analyses of molecular variance (AMOVA; Excoffier et al., 1992). Measures of DNA divergence between populations and groups (F_{ST} ; Excoffier et al., 2006) were calculated and the significance level was determined using 10 000 permutations. 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} and N_{ST} ; see below) were calculated using PERMUT software (Pons & Petit 1996; <http://www.pierrton.intra.fr/genetics/labo/software/permut>, accessed 12 May 2009) with 1000 permutations. The term G_{ST} only considers haplotype frequencies, whereas N_{ST} considers both haplotype frequencies and their genetic divergence.

Relationships between cpDNA haplotypes were constructed using NETWORK ver. 4.2.0.1 (Bandelt et al., 1999). In this analysis, both site mutations and indels were hypothesized to evolve with equal likelihood and each indel was assumed to have originated independently of all other indels. Phylogenetic relationships among the cpDNA haplotypes were evaluated by Maximum Parsimony (MP) and Maximum Likelihood (ML) analyses using PAUP* 4.0b10 (Swofford, 2003),

Table 2 Variable nucleotide sites of the aligned sequences of the *trnL*–*trnF* genetic spacer allowing the identification of 10 chlorotypes in *Spiraea alpina* (sequences are numbered from the 5'-end to the 3'-end in the region)

Haplotype	No. individuals sampled	Nucleotide position									
		<i>trnL</i> _____ <i>trnF</i>									
		280	348	487	605	730	743	751	797	827	859
H1	82	C	G	A	C	G	T	T	T	A	C
H2	60	C	G	A	C	G	A	T	T	A	C
H3	280	C	G	A	C	G	A	G	T	A	C
H4	13	C	T	A	C	A	A	G	T	A	A
H5	43	C	T	T	C	A	A	G	T	A	C
H6	26	C	T	A	C	A	A	G	T	A	C
H7	18	C	G	A	C	G	A	G	T	G	C
H8	3	C	G	A	C	G	A	G	T	G	C
H9	1	T	G	A	C	G	A	G	T	A	C
H10	2	C	G	A	C	G	T	T	A	A	C

with *Spiraea betulifolia* (AJ390368; Richardson et al., 2000), *Rosa californica* (AF348567; Potter et al., 2002), and *Sorbaria sorbifolia* (AF348569; Potter et al., 2002) as outgroups. In the analysis, all characters were equally weighted and treated as unordered. In addition, heuristic search parameters were used with the random addition sequence (1000 replicates), tree bisection–reconnection (TBR) branch swapping, and the MULTREES options selected. We chose the K81uf model (Kimura, 1981), which was determined to be the best evolutionary model for the *trnL*–*trnF* dataset by the hierarchical likelihood ratio test (LRT) in MODELTEST 3.06 (Posada & Crandall, 1998). Bootstrap values (BS) were estimated (1000 replicates) to assess the robustness of the groups identified in the MP and ML trees (Felsenstein, 1985).

2 Results

2.1 Sequence variations

The total alignment length of the sequences was 925 bp across the 528 individuals from 43 populations. These sequences included 10 polymorphic sites (1.08%), five of which were parsimony informative, showing variations apparently arising from point mutations (Table 2). Nucleotide diversity (π) for the sampled populations ranged from 0 (numerous populations without variation, Fig. 1) to 0.002 76 (P39) on the southern QTP, whereas haplotype diversity (H_e) ranged from 0 to 0.7143 (P28). We identified 10 haplotypes (H1–H10) at these 10 polymorphic sites (Table 1; Fig. 1). The H3 haplotype was the most geographically widespread, occurring in 280 individuals from 27 populations (Table 2). It was exclusively fixed in 15 populations and also occurred in the other 12 populations with other low-frequency haplotypes (Fig. 1). The H1 haplotype occurred mainly in central regions of the QTP at high

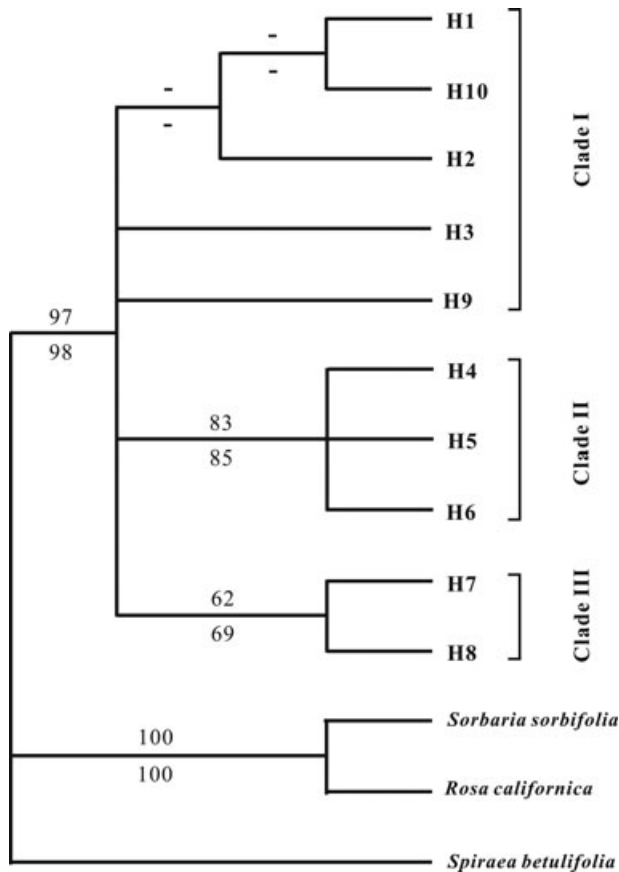


Fig. 2. The most parsimonious tree (tree length = 185; cluster index (CI) = 0.9784; retention index (RI) = 0.9167; rescaled consistency index (RC) = 0.8968) based on 10 chloroplast (cp) DNA haplotypes of *Spiraea alpina* with three outgroups. Bootstrap supports over 1000 pseudoreplicates are given at the nodes for maximum parsimony (numbers above the branch) and maximum likelihood (numbers below the branch). –, support value < 50; H, haplotype.

frequency (Populations 9–13 and 37), but appeared at low frequency in the northeastern region. The H2 haplotype was mainly distributed in the southeastern region (Populations 21, 22, 17, and 14), although it was also seen in the northeastern region (e.g. Population 28) at low frequency. It is interesting that three closely related haplotypes (i.e. H4, H5, and H6) occurred together in the central QTP westward, whereas two closely related haplotypes (i.e. H7 and H8) were distributed more westward. Two low-frequency haplotypes also occurred in the western region.

2.2 Relationships between haplotypes

Both MP and ML ($-\ln L = 2242.120\ 59$) analyses suggested that 10 cpDNA haplotypes clustered into three tentative groups: Group I, haplotypes H1, H2, H3, H9, and H10; Group II, haplotypes H4, H5, and H6; and Group III, haplotypes H7 and H8 (Fig. 2). Groups

Table 3 Results of analysis of molecular variance of chloroplast DNA sequence data from populations of *Spiraea alpina*

Source of variation	d.f.	SS	VC	Variation (%)	Fixation index
Among populations	42	316.564	0.60169	79.41	$F_{ST} = 0.79414^*$
Within populations	485	75.646	0.15597	20.59	
Total	527	392.210	0.75766		

* $P < 0.001$, 1000 permutations.

d.f., degrees of freedom; SS, sum of squares; VC, variance components; F_{ST} , correlation within populations relative to total.

II and III received moderate support in both analyses, whereas such support was very weak for Group I (<50). The haplotype network obtained from the NETWORK 4.5.0.0 analysis (Fig. 1: a) was largely consistent with these analyses (Fig. 2). Given its distribution and frequency, the H3 haplotype appears to be the ancestral haplotype that gave rise to all other haplotypes.

2.3 Phylogeographic structure

The genetic differentiation between populations is estimated to be high ($G_{ST} = 0.737$). The value for N_{ST} (0.819) was significantly higher than that for G_{ST} ($P < 0.05$), indicating significant phylogeographic structure across the entire distribution of the species. Analysis of molecular variance revealed that 79.41% of the total genetic variation occurred among populations and 20.59% within populations (Table 3).

3 Discussion

The present study revealed a high level of population differentiation with $G_{ST} = 0.737$ and low genetic diversity within populations of *S. alpina* (Table 1). This was confirmed by AMOVA analyses, which indicated that 79.41% of the total genetic variation occurred among populations (Table 1). This high between-population differentiation has also been found for numerous other alpine species in the QTP (e.g. Zhang et al., 2005; Chen et al., 2008; Wang et al., 2008a, 2008b; Yang et al., 2008; Zeng et al., 2010). Such genetic structure may arise from strong bottlenecks and founder effects in favoring and/or fixing different alleles in isolated regions (Birky et al., 1989). In fact, the geographical distribution of the three tentative haplotype groups and each of the 10 haplotypes supports this hypothesis (Fig. 1). For example, one clade comprising three haplotypes (H4, H5, and H6) occurred exclusively in the central region westward, whereas another clade consisting of the H7 and H8 haplotypes was distributed more westward. The remaining five haplotypes occurred in the eastern, southern, or western regions. As suggested by Avise (2004), such a high genetic

differentiation between populations is usually coupled with distinct phylogeographic structure. Our PERMUT analyses did suggest a distinct phylogeographic structure ($G_{ST} = 0.737 < N_{ST} = 0.819$; $P < 0.05$). This is also similar to that found in other alpine species (e.g. Zhang et al., 2005; Meng et al., 2007; Chen et al., 2008; Wang et al., 2008a, 2008b; Yang et al., 2008; Zeng et al., 2010). In some of these species, such as *Juniperus przewalskii* (Zhang et al., 2005), *Picea crassifolia* (Meng et al., 2007), and *Pedicularis longiflora* (Yang et al., 2008), this pattern resulted from founder effects due to the large-scale range recolonization from the edge refugia. However, for *S. alpina* (present study), *Potentilla fruticosa* (Sun et al., 2010), *Potentilla glabra* (Wang et al., 2009b), and *Hippophae tibetana* (Jia et al., 2011), the bottlenecks and small-scale range expansions within the local regions may have contributed more to such a high between-population differentiation and distinct phylogeographic structure.

The accurate mutation rate of the cpDNA in *S. alpina* or congeners remains unknown. However, cpDNA mutation rates in most plants are very low, varying between 1×10^{-9} and 3×10^{-9} substitutions per site per year (Wolfe et al., 1987; Demesure et al., 1996; Posada & Crandall, 2001). Even if the fast rate is assumed, each mutation that resulted in the haplotypes identified within our sequenced *trnL-trnF* intergenic spacer should have occurred before the LGM (approximately 16 000 years ago; Petit et al., 1997, 2004; Newton et al., 1999). Therefore, at least one refugium was maintained within the current distribution of each haplotype recovered during the LGM. Because some haplotypes (e.g. H9, H10, and H4) were restricted into one or two adjacent populations (Fig. 1), these populations can be considered as independent refugia during the LGM. In addition, some haplotypes (e.g. H1 and H2) were mainly fixed in adjacent populations of one region, but also disjunctly distributed in some population of another region (Fig. 1). The disjunct distributions of the same haplotype in different regions may also represent independent refugia, although we cannot rule out the possibility that long-distance dispersal may have also contributed to such a distribution pattern. However, in most populations recent expansion mainly occurred (monotypic in haplotype fixing, as shown in Fig. 1) or at the local scale (the same haplotype fixed in adjacent populations; Fig. 1). This pattern differs from that seen with large-scale range expansion (Zhang et al., 2005; Meng et al., 2007; Yang et al., 2008), in which the genetic diversity and the number of haplotypes gradually decrease with increasing distance to the recolonization region from the edge refugia (Hewitt, 2000; Heuertz et al., 2004; Petit et al., 2005; Latch et al., 2009). Instead, our results are largely

consistent with phylogeographic patterns reported for other species (Wang et al., 2009b; Opgenoorth et al., 2010; Sun et al., 2010; Wu et al., 2010; Jia et al., 2011; Li et al., 2011). Similarly, these species survived the LGM in multiple refugia in the QTP and the post-glacial expansions occurred mostly within populations or across adjacent populations at the local scale.

It is interesting that the H4, H5, and H6 haplotypes comprised an independent group with two mutations from the H3 haplotype. This group may have originated earlier before the LGM; for example, due to earlier glaciations or climatic changes, as found in other alpine species occurring there (Wang et al., 2009a; Jia et al., 2011, 2012). This haplotype group was exclusively distributed in the high-altitude region, suggesting that *S. alpina* may have survived there even during the early glaciations. However, the early climatic changes may have caused deep intraspecific divergences in this species. In fact, these findings agree well with recent geological and climatic studies of the QTP (Shi et al., 1998). The largest glaciation in the QTP occurred between 1.2 and 0.4 Ma, and even during this stage the total plateau was not covered by the ice sheet. It is likely that a limited number of species may have survived this glaciation at high altitude, but developed the deeply diverged lineages in response to such a climatic change. However, the climatic changes of the LGM were much weaker and therefore had smaller effects on plant shifts.

In conclusion, we found that *S. alpina* may have survived in multiple refugia and been subjected to deep intraspecific divergences, while the recent expansions occurred mainly within populations or at a local scale if compared with phylogeographic patterns of other shrub or herb species (e.g. Yang et al., 2008; Wang et al., 2009a; Jia et al., 2011, 2012). Together, the results of the present study and these previous reports suggest that evolutionary histories of plants in the QTP are more complex than expected and are highly variable depending on the species studied.

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References

- Avice JC. 2000. Phylogeography: the history and formation of species. Cambridge: Harvard University Press.
- Avice JC. 2004. Molecular markers, natural history, and evolution. 2nd edition. Cambridge: Harvard University Press.
- Bandelt HJ, Forster P, Röhl A. 1999. Median-joining networks for inferring intraspecific phylogenies. *Molecular Biology and Evolution* 16: 37–48.

- Birky CM, Fuerst P, Maruyama T. 1989. Organelle gene diversity under migration, mutation, and drift: equilibrium expectations, approach to equilibrium, effects of heteroplasmic cells, and comparison to nuclear genes. *Genetics* 121: 613–627.
- Chen SY, Wu GL, Zhang DJ, Gao QB, Duan YZ, Zhang FQ, Chen SL. 2008. Potential refugium on the Qinghai–Tibet Plateau revealed by the chloroplast DNA phylogeography of the alpine species *Metagentiana striata* (Gentianaceae). *Botanical Journal of the Linnean Society* 157: 125–140.
- Demesure B, Comps B, Petit RJ. 1996. Chloroplast DNA phylogeography of the common beech (*Fagus sylvatica* L.) in Europe. *Evolution* 50: 2515–2520.
- Doyle JJ, Doyle JL. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf material. *Phytochemical Bulletin* 19: 11–15.
- Excoffier L, Smouse PE, Quattro JM. 1992. Analysis of molecular variance inferred from metric distances among DNA haplotype: application to human mitochondrial DNA restriction data. *Genetics* 131: 479–491.
- Excoffier L, Laval G, Schneider S. 2006. Arlequin v.3.01. An integrated software package for population genetics data analysis. Berne: Computational and Molecular Population Genetics Lab, University of Berne.
- Felsenstein J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* 39: 783–791.
- Gao LM, Moller M, Zhang XM. 2007. High variation and strong phylogeographic pattern among cpDNA haplotypes in *Taxus wallichiana* (Taxaceae) in China and North Vietnam. *Molecular Ecology* 16: 4684–4698.
- Gao QB, Zhang DJ, Chen SY, Duan YZ, Zhang FQ, Li YH, Chen SL. 2009. Chloroplast DNA phylogeography of *Rhodiola alsia* (Crassulaceae) in the Qinghai–Tibet Plateau. *Botany* 87: 1077–1088.
- Heuertz M, Fineschi S, Anzidei M. 2004. Chloroplast DNA variation and postglacial recolonization of common ash (*Fraxinus excelsior* L.) in Europe. *Molecular Ecology* 13: 3437–3452.
- Hewitt GM. 1996. Some genetic consequences of ice ages, and their role, in divergence and speciation. *Biological Journal of the Linnean Society* 58: 247–276.
- Hewitt GM. 2000. The genetic legacy of the Quaternary ice ages. *Nature* 405: 907–913.
- Hewitt GM. 2004. Genetic consequences of climatic oscillations in the Quaternary. *Philosophical Transactions of the Royal Society of London, Series B: Biological Sciences* 359: 183–195.
- Jia DR, Liu TL, Wang LY, Zhou DW, Liu JQ. 2011. Evolutionary history of an alpine shrub *Hippophae tibetana* (Elaeagnaceae): allopatric divergence and regional expansion. *Biological Journal of the Linnean Society* 102: 37–50.
- Jia DR, Abbott RJ, Liu TL, Mao KS, Bartish IV, Liu JQ. 2012. Out of the Qinghai–Tibet Plateau: evidence for the origin and dispersal of Eurasian temperate plants from a phylogeographic study of *Hippophaë rhamnoides* (Elaeagnaceae). *New Phytologist*. Epub 20 March 2012; doi:10.1111/j.1469-8137.2012.04115.x.
- Kimura M. 1981. Estimation of evolutionary distances between homologous nucleotide sequences. *Proceedings of the National Academy of Sciences USA* 78: 454–458.
- Latch EK, Heffelfinger JR, Fike JA, Rhodes OE. 2009. Species-wide phylogeography of North American mule deer (*Odocoileus hemionus*): cryptic glacial refugia and post-glacial recolonization. *Molecular Ecology* 18: 1730–1745.
- Li Y, Zhai SN, Qiu YX, Guo YP, Ge XH, Comes HP. 2011. Glacial survival east and west of the ‘Mekong–Salween Divide’ in the Himalaya–Hengduan Mountains region as revealed by AFLPs and cpDNA sequence variation in *Sinopodophyllum hexandrum* (Berberidaceae). *Molecular Phylogenetics and Evolution* 59: 412–424.
- Lu LD, Gu CZ, Li CL, Alexander C, Bruce B, Anthony RB, Davide EB, Hiroshi I, Hideaki O, Kenneth RR, Steven AS. 2003. Rosaceae. In: Wu ZY, Raven PH eds. *Flora of China*. Beijing: Science Press; St. Louis: Missouri Botanical Garden Press. 9: 46–434.
- Meng LH, Yang R, Abbott RJ, Miede G, Hu TH, Liu JQ. 2007. Mitochondrial and chloroplast phylogeography of *Picea crassifolia* Kom. (Pinaceae) in the Qinghai–Tibetan Plateau and adjacent highlands. *Molecular Ecology* 16: 4128–4137.
- Meng LH, Yang HL, Wu GL, Wang YJ. 2008. Phylogeography of *Hippophae neurocarpa* (Elaeagnaceae) inferred from the chloroplast DNA *trnL-F* sequence variation. *Journal of Systematics and Evolution* 46: 32–40.
- Newton AC, Allnutt TR, Gillies ACM, Lowe AJ, Ennos RA. 1999. Molecular phylogeography, intraspecific variation and conservation of tree species. *Trends in Ecology & Evolution* 14: 140–145.
- Opgenoorth L, Vendramin GG, Mao KS, Miede G, Miede S, Liepelt S, Liu JQ, Ziegenhagen B. 2010. Tree endurance on the Tibetan Plateau marks the world’s highest known tree line of the Last Glacial Maximum. *New Phytologist* 185: 332–342.
- Pan YZ, Fang LQ, Hao G, Cai J, Gong X. 2009. Systematic positions of *Lamiophlomis* and *Paraphlomis* (Lamiaceae) based on nuclear and chloroplast sequences. *Journal of Systematics and Evolution* 47: 535–542.
- Petit RJ, Pineau E, Demesure B, Basilieri R, Ducousso A, Kremer A. 1997. Chloroplast DNA footprints of postglacial recolonization by oaks. *Proceedings of the National Academy of Sciences USA* 94: 9996–10001.
- Petit RJ, Bialozyt R, Garnier-Gere P, Hampe A. 2004. Ecology and genetics of tree invasions: from recent introductions to Quaternary migrations. *Forest Ecology and Management* 197: 117–137.
- Petit RJ, Duminiel J, Fineschi S, Hampe A, Salvini D, Vendramin GG. 2005. Comparative organization of chloroplast, mitochondrial and nuclear diversity in plant populations. *Molecular Ecology* 14: 689–701.
- Pons O, Petit RJ. 1996. Measuring and testing genetic differentiation with ordered versus unordered alleles. *Genetics* 144: 1237–1245.
- Posada D, Crandall KA. 1998. MODELTEST: testing the model of DNA substitution. *Bioinformatics* 14: 817–818.
- Posada D, Crandall KA. 2001. Intraspecific gene genealogies: trees grafting into networks. *Trends in Ecology and Evolution* 16: 37–45.
- Potter D, Gao F, Bortiri PE, Oh SH, Baggett S. 2002. Phylogenetic relationships in Rosaceae inferred from chloroplast *matK* and *trnL-trnF* nucleotide sequence data. *Plant Systematics and Evolution* 231: 77–89.
- Potter D, Still SM, Grebenc T, Ballian D, Bozic G, Franjia J, Kraigher H. 2007. Phylogenetic relationships in tribe

- Spiraea* (Rosaceae) inferred from nucleotide sequence data. *Plant Systematics and Evolution* 266: 105–118.
- Richardson JE, Fay MF, Cronk QCB, Bowman D, Chase MW. 2000. A phylogenetic analysis of Rhamnaceae using *rbcL* and *trnL-F* plastid DNA sequences. *American Journal of Botany* 87: 1309–1324.
- Shi YF, Li JJ, Li BY. 1998. Uplift and environmental changes of Qinghai–Tibetan Plateau in the late Cenozoic. Guangzhou: Guangdong Science and Technology Press.
- Soltis DE, Soltis PS. 1998. Choosing an approach and appropriate gene for phylogenetic analysis. In: Soltis DE, Doyle JJ eds. *Molecular systematics of plant II: DNA sequencing*. Norwell, MA: Kluwer Academic. 1–42.
- Sun YS, Ikeda H, Wang YJ, Liu JQ. 2010. Phylogeography of *Potentilla fruticosa* (Rosaceae) in the Qinghai–Tibetan Plateau revisited: a reappraisal and new insights. *Plant Ecology & Diversity* 3: 249–257.
- Swofford DL. 2003. PAUP*: phylogenetic analysis using parsimony (*and other methods), version 4.0b10. Sunderland: Sinauer Associates.
- Taberlet P, Gielly L, Pautou G, Bouvet J. 1991. Universal primers for amplification of three non-coding regions of chloroplast DNA. *Plant Molecular Biology* 17: 1105–1109.
- Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG. 1997. The Clustal_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research* 25: 4876–4882.
- Wang AL, Schluetz F, Liu JQ. 2008a. Molecular evidence for double maternal origins of the diploid hybrid *Hippophae goniocarpa* (Elaeagnaceae). *Botanical Journal of the Linnean Society* 156: 111–118.
- Wang FY, Gong X, Hu CM, Hao G. 2008b. Phylogeography of an alpine species *Primula secundiflora* inferred from the chloroplast DNA sequence variation. *Journal of Systematics and Evolution* 46: 13–22.
- Wang LY, Abbott RJ, Zheng W, Chen P, Wang Y, Liu JQ. 2009a. History and evolution of alpine plants endemic to the Qinghai–Tibetan Plateau: *Aconitum gymnanthum* (Ranunculaceae). *Molecular Ecology* 18: 709–721.
- Wang LY, Hiroshi I, Liu TL, Wang YJ, Liu JQ. 2009b. Repeated range expansion and glacial endurance of *Potentilla glabra* (Rosaceae) in the Qinghai–Tibetan Plateau. *Journal of Integrative Plant Biology* 51: 698–706.
- Weeks A. 2008. Evolution of the pili nut genus (*Canarium* L., Burseraceae) and its cultivated species. *Genetic Resources and Crop Evolution* 56: 765–781.
- Wolfe KH, Li WH, Sharp PM. 1987. Rates of nucleotide substitution vary greatly among plant mitochondrial, chloroplast, and nuclear DNAs. *Proceedings of the National Academy of Sciences USA* 84: 9054–9058.
- Wu LL, Cui XK, Milne RI, Sun, YS, Liu JQ. 2010. Multiple autopolyploidizations and range expansion of *Allium przewalskianum* Regel. (Alliaceae) in the Qinghai–Tibetan Plateau. *Molecular Ecology* 19: 1691–1704.
- Wu SG, Yang YP, Fei Y. 1995. On the flora of the alpine region in the Qinghai–Xizang (Tibet) plateau. *Acta Botanica Yunnan* 17: 233–250 (in Chinese).
- Yang FS, Li YL, Ding X, Wang XQ. 2008. Extensive population expansion of *Pedicularis longiflora* (Orobanchaceae) on the Qinghai–Tibetan Plateau and its correlation with the Quaternary climate change. *Molecular Ecology* 17: 5135–5145.
- Zeng LY, Xu LL, Tang SQ, Tersing T, Geng YP, Zhong Y. 2010. Effect of sampling strategy on estimation of fine-scale spatial genetic structure in *Androsace tapete* (Primulaceae), an alpine plant endemic to Qinghai–Tibetan Plateau. *Journal of Systematics and Evolution* 48: 257–264.
- Zhang Q, Chiang TY, George M, Liu JQ, Abbott RJ. 2005. Phylogeography of the Qinghai–Tibetan Plateau endemic *Juniperus przewalskii* (Cupressaceae) inferred from chloroplast DNA sequence variation. *Molecular Ecology* 14: 3513–3524.
- Zhang ZY, Fan LM, Yang JB, Hao XJ, Gu ZJ. 2006. Alkaloid polymorphism and ITS sequence variation in the *Spiraea japonica* complex (Rosaceae) in China: traces of the biological effects of the Himalaya–Tibet Plateau uplift. *American Journal of Botany* 93: 762–769.
- Zheng D. 1996. The system of physico-geographical regions of the Qinghai–Tibet (Xizang) Plateau. *Science in China, Series D: Earth Sciences* 39: 410–417.
- Zheng D, Yao T. 2004. Uplifting of Tibetan Plateau with its Environmental Effects. Beijing: Science Press (in Chinese).
- Zhou S, Wang X, Wang J, Xu L. 2006. A preliminary study on timing of the oldest Pleistocene glaciation in Qinghai–Tibetan plateau. *Quaternary International* 154–155: 44–51.