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Molecular phylogeography and intraspecific divergence of *Spiraea alpina* (Rosaceae) distributed in the Qinghai-Tibetan Plateau and adjacent regions inferred from nrDNA

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ABSTRACT

The Qinghai-Tibetan plateau (QTP) uplift had a decisive effect on climatic and eco-environmental evolution in East Asia during the Quaternary. In the current study phylogeographic structure and diversification history of *Spiraea alpina* across the QTP were investigated for the first time based on nuclear internal transcribed spacer. The nuclear internal transcribed spacers (ITS_{1a}–ITS_4) were generated for a total of 284 individuals distributed within 31 natural populations. A clear phylogeographic structure was found for *S. alpina*. The results showed that this species colonized in three different glacial refugia during the Quaternary extensive glaciation and expanded during the Interglacial period. Analysis of molecular variance (AMOVA) showed 74.13% genetic diversity among populations and 25.87% genetic variation within populations with distinct phylogeographic structure ($F_{ST} = 0.741^*$). The estimated divergence time revealed that the main lineages of *S. alpina* diversified during the Quaternary 1.2–0.6 million years ago. The study concluded that severe climatic oscillations during Quaternary and the uplift of QTP had a profound effect on intraspecific divergence of *S. alpina*.

1. Introduction

The response of species towards eco-environmental changes has been considered a highly dynamic process consisting of repeated retreats into refugia during glacial periods and range expansions from the refugia during interglacial periods. The Qinghai-Tibetan Plateau (QTP) is considered as the most sensitive region for historical climatic changes. The Flora and fauna

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found at QTP should have traced the climatic variations to shift their distributional ranges (Zheng and Rutter, 1998). These range shifts can be detected from the genetic structure of current populations, especially glacial retreats (into refugia) and postglacial re-colonization (Avise, 2000; Hewitt, 2004). Such types of retreat and re-colonization patterns have been identified in some alpine species distributed in the QTP (Meng et al., 2007; Chen et al., 2008). Different studies have suggested that the last glacial maximum (LGM) did not seriously affect species ranges shift and the species survived in multiple refugia, while the largest glaciation occurred at the QTP may have had a great impact on the distribution or evolution of species (Wang et al., 2009a; Opgenoorth et al., 2010). The cold age occurred between 1.2 MYA (million years ago) and 0.4 MYA when the largest glaciation developed at QTP, however this glaciation did not result in a massive ice sheet (Shi et al., 1998; Zhou et al., 2006). Some of the previous studies showed that plants species with different habitats and morphologies responded differently to Quaternary severe climatic episodes. The Qinghai-Tibetan Plateau has more than 1800 alpine species at high-altitude (Wu et al., 1995). However, the phylogeographic patterns of most species remain unknown. In the current study, the phylogeographic structure and intraspecific divergence of *Spiraea alpina* (Rosaceae) based on nuclear DNA is reported. This alpine shrub is endemic to the QTP, with wide distribution at high altitudes between 2000 and 4500 m; partly extended to adjacent regions (Lu et al., 2003). The nuclear ribosomal internal transcribed spacer (ITS) is a suitable nuclear marker to trace population range shifts of species, dispersed through seeds (Gao et al., 2012; Wang et al., 2009b). In this study the nuclear internal transcribed spacers (ITS_{1a}-ITS_4) were used to trace the range shifts of this alpine shrub in response to the past eco-environmental changes. The main objectives were (I) to investigate whether this species survived in multiple refugia during the LGM, as other alpine shrubs found on the QTP, or underwent through range expansion, (II) to find out the intraspecific diversification in *S. alpina* based on nrDNA, and (III) to compare genetic diversity of biparentally inherited nrDNA with that of maternally inherited plastid cpDNA in *S. alpina*.

2. Materials and methods

2.1. Sampling

A total of 284 individuals, distributed in 31 natural populations of *S. alpina* were collected from the QTP and adjacent regions (Table 1; Fig. 1). Three to nineteen individuals (spaced at least 100 m apart) were collected for each population. Voucher specimens were deposited in the herbarium of the Northwest Institute of Plateau Biology (HNWP) at the Chinese Academy of Sciences.

Table 1
Population codes, locations, geographical coordinates, No of individuals used for nrDNA (each population column shows the number of individuals sampled for that population, followed by the number of haplotypes in parentheses), estimates of gene diversity (h), nucleotide diversity (π), mean number of pairwise differences (P.D), and haplotype composition for 31 *Spiraea alpina* populations. YN = Yunnan, SC = Sichuan, QH = Qinghai, T = Tibet, GS = Gansu.

POP	Locality	Latitude (N)	Longitude (E)	Altitude (m)	No. of individuals used for nr(DNA)	Nuclear DNA haplotypes(N)	Gene diversity(h)	Nucleotide diversity (π)	P.D
P1	Datong, QH	36°59′	101°25′	3210	3(3)	A1,A2,A5	1.000 ± 0.272	0.002 ± 0.002	1.333 ± 1.098
P2	Shanglaxiu,QH	32°46′	96°39′	4090	6(2)	A12,A21	0.333 ± 0.215	0.0006 ± 0.001	0.333 ± 0.380
P3	Xialaxiu, QH	32°45′	96°34′	3900	4(1)	A13	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000
P4	Zaduo, QH	32°52′	95°19′	4030	11(3)	A1,A11,A13	0.473 ± 0.161	0.009 ± 0.005	5.127 ± 2.691
P5	Yushu, QH	32°52′	97°04′	3810	17(6)	A13,A21,A15,A17,A12,A9	0.654 ± 0.122	0.009 ± 0.005	5.103 ± 2.603
P6	Hongyuan, SC	32°46′	102°21′	3654	7(3)	A2,A3,A5	0.523 ± 0.208	0.001 ± 0.001	0.762 ± 0.629
P7	Litang, SC	29°38′	100°21′	3891	16(5)	A5,A8,A19,A15,A10	0.650 ± 0.108	0.004 ± 0.003	2.575 ± 1.456
P8	Yajiang, SC	30°04′	101°20′	4280	7(4)	A4,A5,A8,A10	0.809 ± 0.129	0.002 ± 0.002	1.428 ± 0.984
P9	Luhuo, SC	31°37′	100°43′	3460	11(4)	A2,A3,A5,A10	0.690 ± 0.127	0.002 ± 0.001	1.090 ± 0.773
P10	Rangtang, SC	32°18′	101°03′	3820	10(4)	A2,A4,A5,A10	0.777 ± 0.090	0.002 ± 0.001	1.022 ± 0.744
P11	Dari, QH	33°17′	100°23′	4370	19(5)	A2,A3,A4,A5,A18	0.690 ± 0.094	0.002 ± 0.001	1.169 ± 0.787
P12	Maqin, QH	34°36′	100°34′	3380	9(4)	A2,A3,A4,A17	0.777 ± 0.110	0.004 ± 0.002	2.111 ± 1.295
P13	Angqian, QH	31°58′	96°25′	4290	11(7)	A1,A2,A5,A12,A16,A19,A21	0.909 ± 0.065	0.003 ± 0.002	1.927 ± 1.183
P14	Leiwuqi, T	31°32′	96°22′	4210	10(6)	A1,A5,A10,A16,A17,A21	0.889 ± 0.075	0.004 ± 0.003	2.444 ± 1.443
P15	Mozhugongka, T	29°42′	92°04′	4150	9(1)	A14	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000
P16	Basu, T	30°07′	97°17′	4320	7(2)	A13,A14	0.476 ± 0.171	0.002 ± 0.001	0.952 ± 0.733
P17	Changdu, T	31°11′	97°02′	3380	8(1)	A13	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000
P18	Jiangda, T	31°21′	97°42′	4490	9(5)	A1,A2,A12,A5,A22	0.805 ± 0.119	0.002 ± 0.001	1.333 ± 0.910
P19	Dege, T	31°57′	98°54′	4410	15(5)	A2,A5,A9,A12,A21	0.733 ± 0.089	0.003 ± 0.002	1.714 ± 1.058
P20	Shiqu, SC	32°30′	98°27′	4380	7(3)	A3,A11,A21	0.523 ± 0.208	0.002 ± 0.002	1.142 ± 0.835
P21	Songpan,SC	32°35′	103°37′	2830	14(1)	A13	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000
P22	Qilian, QH	38°14′	99°57′	2880	8(2)	A17,A19	0.250 ± 0.180	0.0004 ± 0.0006	0.250 ± 0.311
P23	Menyuan, QH	37°24′	101°57′	3150	4(2)	A19,A20	0.500 ± 0.265	0.0009 ± 0.001	0.500 ± 0.519
P24	Huzhu,QH	36°55′	102°22′	2690	15(2)	A19,A20	0.133 ± 0.112	0.0002 ± 0.0004	0.133 ± 0.209
P25	Pingan, QH	36°17 ′	101°58′	3160	11(3)	A11,A16,A19	0.563 ± 0.134	0.002 ± 0.001	1.127 ± 0.791
P26	Lingxia, GS	34°53 ′	102° 49′	3220	11(4)	A5,A11,A19,A20	0.709 ± 0.099	0.003 ± 0.0022	1.781 ± 1.113
P27	Xiahe, GS	34°45′	102° 34′	3210	6(3)	A2,A3,A5	0.600 ± 0.215	0.0015 ± 0.001	0.866 ± 0.700
P28	Zoigè, SC	34° 07′	102° 38′	3280	4(2)	A2,A5	0.500 ± 0.265	0.0008 ± 0.001	0.500 ± 0.519
P29	Shagong, SC	29° 08′	100°02′	4610	4(1)	A7	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000
P30	Ganzi, SC	31° 36′	100° 09′	3830	3(1)	A12	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000
P31	Lhasa, T	31° 50′	102°41′	3370	3(1)	A6	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000

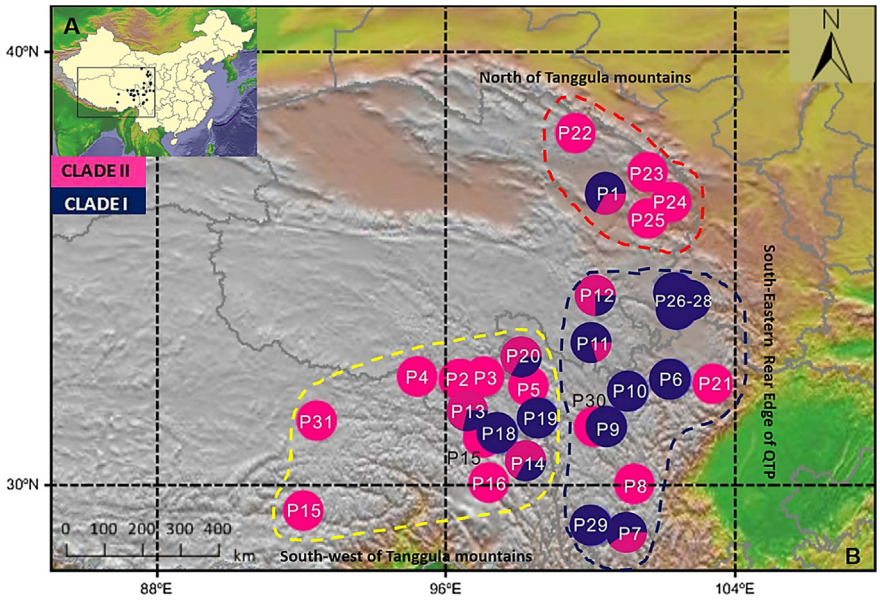

Fig. 1. (A) Geographical distribution of the sampled populations. (B) Pie Diagrams indicating haplogroups composition of each population.

2.2. DNA extraction, amplification and sequencing

Genomic DNA was extracted from silica-gel dried leaves following the 3 × CTAB method (Doyle and Doyle, 1987). Nuclear ribosomal internal transcribed spacers (ITS_{1a}–ITS_4) (White et al., 1990) were used to amplify the template DNA. Polymerase chain reactions were performed in 25 μL volume containing 0.6 μL (approximately 20 ng) template DNA, 2.5 μL of 10 × PCR buffer (without Mg^{2+}), 1.5 μL of 25 mM $MgCl_2$, 0.3 μL of 10 mM dNTPs, 1.0 μL of 5 pM of each primer, and 0.3 μL (1.5 units) of Taq polymerase (Takara, Dalian, China). Amplification conditions were: 5 min at 95 °C; followed by 35 cycles of 50 s at 95 °C, 1 min at 58 °C, and 1 min at 72 °C; with a final extension of 7 min at 72 °C. The PCR products were purified using a CAS pure PCR Purification Kit in accordance with manufacturer's instructions. Sequencing reactions and analysis were performed using an ABI 3730*xl* DNA sequencer (Applied Biosystems, Foster City, CA). Sequences were manually checked and aligned using CLUSTAL X (Thompson et al., 1997).

2.3. Molecular variability and demographic analysis

Arlequin ver. 3.5 (Excoffier and Lischer, 2010) was used to calculate haplotype diversity (h), nucleotide diversity (π), and analysis of molecular variance (AMOVA) (Excoffier et al., 1992). Measurements of genetic divergence between populations and groups (F_{ST}; Excoffier and Lischer, 2010) were calculated, and the significance level was determined using 10,000 permutations. Estimates of average gene diversity within populations (H_D), total gene diversity (H_T), and the proportion of total diversity due to differences between populations (G_{ST} and N_{ST}) were calculated using PERMUT (Pons and Petit, 1996) with 1000 permutations (G_{ST} only considers haplotype frequencies, while N_{ST} considers both haplotype frequencies and their genetic divergence). Spatial analysis of molecular variance (SAMOVA; Dupanloup et al., 2002) was used to define groups of populations that were geographically homogeneous and maximally differentiated. The Sum of squared deviations (*SSD*) between the observed and expected mismatch distributions was calculated. One thousand parametric bootstrap replicates were used to generate an expected distribution under a sudden demographic expansion model. Multimodal or random and rough distributions of pairwise differences were characterized for populations that have not been expanded for a long time, while unimodal and smooth distribution explained populations that experienced recent demographic expansions (Rogers and Harpending, 1992). Raggedness index (*RAG*) and its significance level were calculated to quantify smoothness of the observed mismatch distribution. Additionally, Tajima's D and Fu's Fs were calculated. The parameter value was converted for mode of mismatch distribution (τ) and its 95% confidence interval (CI) at 1000 parametric bootstrap replicates used to estimate the time since sudden range expansion using the equation $t = \tau/2u$ (Rogers and Harpending, 1992). The value of u was calculated as $u = 2\ \mu kg$, where; μ = substitution rate, k = average sequence length used for analysis (571 bp), and g = generation time in years. For μ, we assumed the same average values as 5.69×10^{-9} substitution per site per year (Fan et al., 2013), and 5 years was used as an approximation for g as this is a shrub, which takes 5 years to reach first reproduction.

2.4. Phylogenetic analysis

To study the relationships among haplotypes, a haplotype network was constructed with a statistical parsimony approach (Templeton et al., 1992) using TCS 1.21 (Clement et al., 2000) with a 95% connection limit. Ambiguous connections (loops)

were resolved between haplotypes in accordance with Templeton et al. (1995), and haplotypes were divided into haplogroups using the program AN_ECA (Panchal, 2007; Panchal and Beaumont, 2007) applying the criteria of Templeton (Templeton et al., 1987). Phylogenetic relationships among the nrDNA haplotypes were constructed by Maximum Parsimony (MP) and Maximum Likelihood (ML) analysis, as implemented in PAUP (Swofford, 2003). *Sorbaria kirilowii* (Voucher Number Chen2012034 in HNWP; Accession Number KF983155 in Gene Bank) and *Sorbus koehneana* (Voucher Number Chen201193 in HNWP; Accession Number KF983154 in Gene Bank) were used as outgroup taxa. Heuristic search parameters were used with random addition sequence (1000 replicates), tree-bisection-reconnection (TBR) branch swapping, and MULTREES options. The TrN + G model (Kimura, 1981) was selected as a best evolutionary model for *S. alpina* through a hierarchical likelihood ratio test (LRT) in Modeltest 3.7 (Posada and Buckley, 2004). Bootstrap values (BS) were estimated (1000 replicates) to assess the robustness of each group identified in the MP and ML trees (Felsenstein, 1985). The trees were then displayed using FigTree 1.4 (Rambaut, 2012).

2.5. Estimation of divergence time

The likelihood ratio test (LRT) did not reject the null hypothesis of rate constancy. Despite a higher likelihood for the model of rate constancy, a comparison of phylogenetic reconstruction was carried out by using both strict and relaxed molecular clock models assumed a Yule speciation process as tree prior and general time-reversible models (GTR + I). This substitution model is closest to the one selected by Modeltest 3.7. The strict molecular clock model was used in beast, with an exponential prior distribution for molecular rate; this implies that changes occurred at the nodes, with the size of change is independent of branch length. The tree root prior was set as 65 MY (Million years), which matches the fossil record of *Sorbaria* (Tao and Xiong, 1986; Lu, 1996). The Markov chain Monte Carlo (MCMC) was run for 10,000,000 generations, and sampled every 1000th generation, with removal of the first 25% as burn-in. The Tracer1.5 program (Drummond and Rambaut, 2007) was used to check convergence of the MCMC searches and to check whether effective samples sizes (ESS) of posterior probability were greater than 200 for each estimated parameter or not. Information contained in the sampled 10,001 trees was summarized using Tree Annotator (Drummond and Rambaut, 2007). This algorithm selects the maximum clade credibility tree and annotates it with posterior summaries calculated for each node having a posterior probability >0.95. Finally, the Fig Tree 1.4 program (Rambaut, 2012) was used to view, edit, and export the annotated tree.

3. Results

3.1. Sequence analysis

Sequence length after alignment was 571 bp across all individuals. Twenty-one variable sites (3.7%) and 15 parsimony informative sites were recovered (Supplementary Table S1). Twenty-one haplotypes (A1–A21) were generated at these 21 polymorphic sites (Table 1; Fig. 1). Each Haplotypes A5 and A13 was found in 49 individuals, in a total of 98 individuals, within 14 and 6 populations respectively (Table 1). Haplotype A5 is mainly distributed at the south-east rear edge of the QTP, whereas haplotype A13 at the south west of the Tanggula Mountains, and the north of the Tanggula Mountains (Fig. 1B). The distribution of haplotypes in haplogroups revealed that the first haplogroups of CLADE I distributed at the southeastern rear edge of the QTP, while haplogroups of CLADE II expanded towards the center, north, and west of the QTP. Haplotypes with low frequency (A4, A6, A7, A8, A9, A16, A17, A18, and A20) covered the central, western, and northern parts of the QTP (Fig. 1B). All the haplotypes sequences have been deposited in Gene Bank under Accession numbers KF983262–KF983282. The Nucleotide diversity (π) ranged from 0 (no variation) to 0.009 in populations P4 and P5 in the southwestern region of the Tanggula Mountains; the haplotypes or gene diversity (h) and pairwise difference (P.D), ranged from 0 to 1.000 (P1) and 0 to 5.127, respectively.

3.2. Phylogeographic structure

Genetic differentiation between population (G_{ST}) and the number of substitution types (N_{ST}) values were significantly different. The N_{ST} value was 0.693 while the G_{ST} value was 0.473; $N_{ST} > G_{ST}$ $P < 0.01$. Analysis of molecular variance revealed that 74.13% of total genetic variation occurred among populations, and 25.87% within populations; the pairwise F_{ST} value was 0.741*. Both permute results with 1000 replicates, and AMOVA implemented in Arliquin confirmed a clear phylogeographic structure for the species. Spatial analysis of molecular variance suggested that the genetic differentiation was $F_{CT} = 0.226^*$ ($P < 0.001$) when all the populations were pooled into three groups (Table 2).

3.3. Phylogenetic analysis

Maximum likelihood and maximum parsimony trees implemented in PAUP* clustered the 21 haplotypes into two haplogroups or clades. Clade I contained eight haplotypes (A2, A3, A4, A5, A6, A7, A8, and A10), while the remaining haplotypes clustered into Clade II (Fig. 2A). Network results obtained through NETWORK 4.5.0.0 analysis had congruent topology (Fig. 2B). The A7 haplotype appeared to be the ancestral haplotype.

Table 2
Analysis of molecular variance for 31 populations of *Spiraea alpina* based on nuclear DNA sequence data. F_{ST}, correlation within populations relative to total; F_{SC}, correlation within populations relative to groups; F_{CT}, correlation of haplotypes within groups relative to total; $^{*}P < 0.001$, 10,000 permutations.

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation	Fixation index
Among populations	30	604.117	2.17 Va	74.13	$F_{ST} = 0.741^{*}$
Within populations	248	187.812	0.75 Vb	25.87	
Total	278	791.928	2.93		
(P24), (P6) and (The Remaining Populations) Among groups	3	125.096	0.7967 Va	22.58	$F_{SC} = 0.722^{*}$
Among populations within groups	27	479.021	1.9746 Vb	55.96	$F_{ST} = 0.785^{*}$
Within population	248	187.812	0.7573 Vc	21.46	$F_{CT} = 0.226^{*}$
Total	278	791.928	3.5286		

3.4. Demographic history

Mismatch distribution for total haplotypes of *S. alpina* was non-unimodal (Fig. 3). Statistical assessments between observed and simulated distributions under a sudden demographic expansion model significantly rejected the expansion model (P values > 0.05) based on SSD and Harpending RAG. These results were not considered as evidence towards the demographic expansion, as sometimes multimodal distributions generally indicate either populations stability, shrinkage in size, or structured populations (Rogers and Harpending, 1992; Ray et al., 2003). Values for Tajima's D and Fu's Fs were also not significantly negative. This investigation suggests that *S. alpina* followed a bottleneck and retreat phenomeona rather than expansion. The total expansion time for *S. alpina* was 3.27 MY, while for clades I and II the expansion time were 0.02 MY and 0.07 MY, respectively (Supplementary Table S2).

3.5. Divergence time

BEAST 1.7.5 was used to estimate the divergence time for all *S. alpina* haplotypes. The tree obtained showed congruent topology with the ML and MP trees. All the haplotypes coalesced at about 5.54 MYA (Highest Posterior Density [HPD] at 95%;

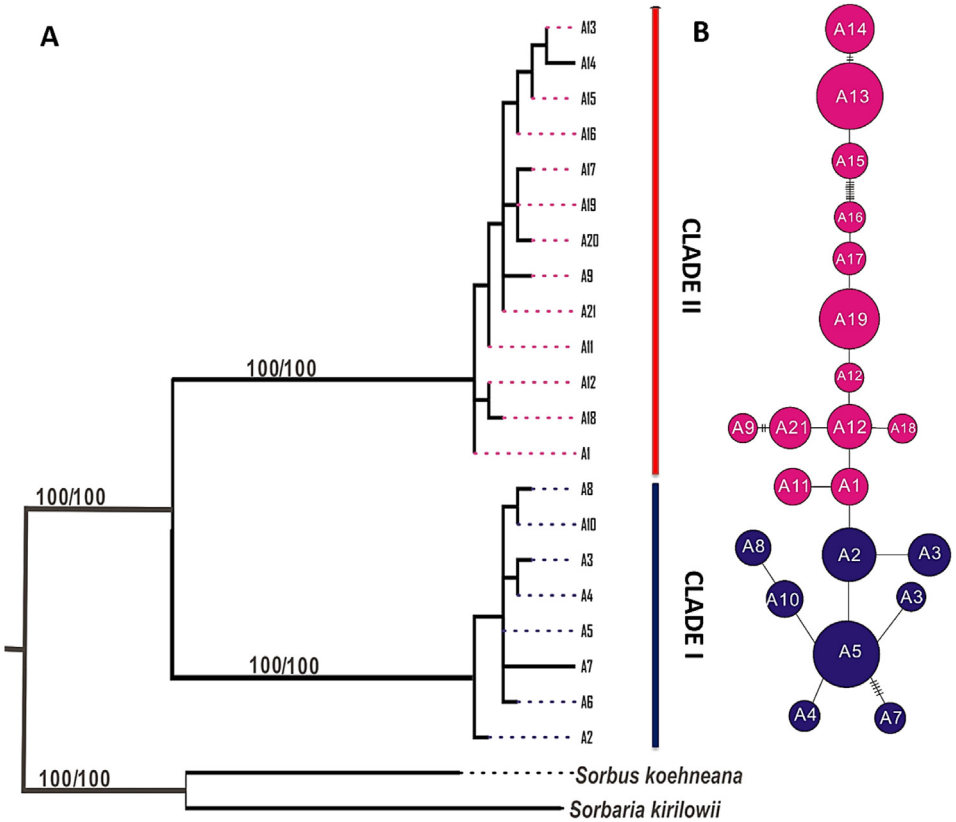

Fig. 2. (A) Maximum likelihood (ML) and maximum parsimony (MP) tree as implemented in PAUP* 4.0 (Swofford, 2003), using *Sorbaria kirilowii* and *Sorbus koehneana as* outgroup taxa. (B) Maximum parsimony median-joining network among all 21 haplotypes of *Spiraea alpina*. NOTE: The number above the branches indicates bootstraps support, the first for ML, the next for MP.

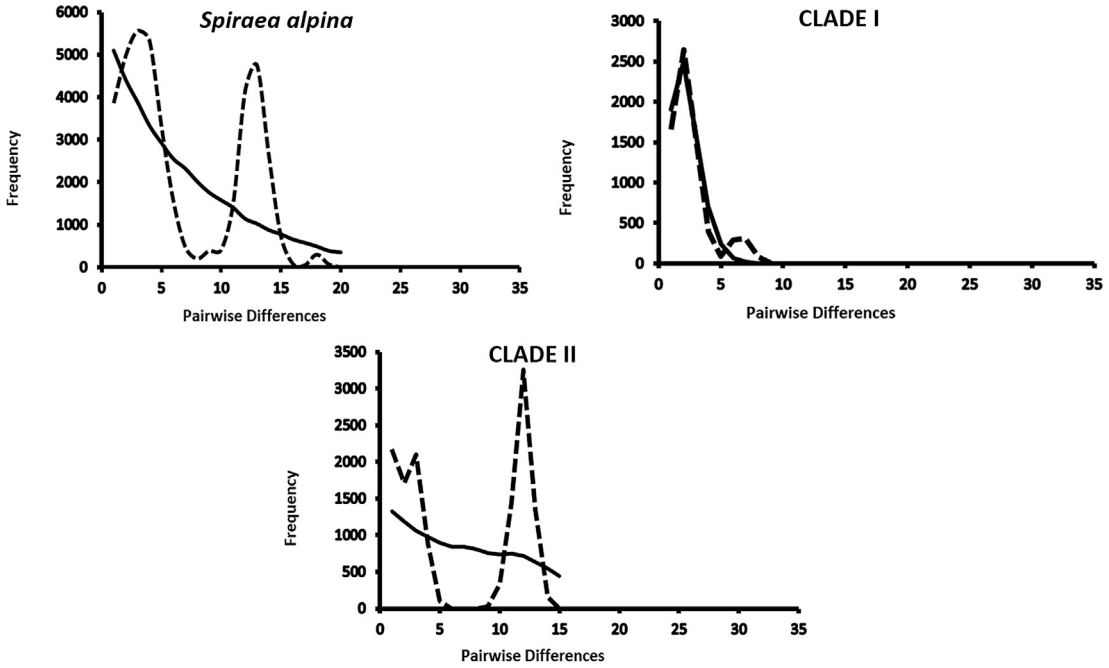

Fig. 3. Mismatch distribution of the number of pairwise nucleotide differences for nrDNA sequence data in *Spiraea alpina*, haplotypes of Clade I, and haplotypes of Clade II. Solid lines are drawn from the observed differences among sequences, whereas dashed lines (squares) represent simulated differences under a model of sudden population expansion (Rogers and Harpending, 1992).

Fig. 4). Clade I diverged at 3.69 MYA, while Haplogroup II diverged at 3.94 MYA. However, most intraspecific divergence occurred during the last glacial maximum 1.52 to 0.60 MYA. The oldest haplotype according to BEAST was A7, while the youngest haplotypes were A13 and A14.

4. Discussion

4.1. Genetic diversity and variation

The nuclear ribosomal (18S–5.8S–26S) internal transcribed spacer is the most popular non-plastid DNA fragment. Based on nrDNA, 21 haplotypes were identified for *S. alpina* in a total of 284 individuals with high haplotype diversity ($H_d = 0.90$). Similar results were obtained for *Rhodiola alsia* (Gao et al., 2012). The same species was studied based on cpDNA, had 10 chlorotypes within 528 individuals (Zhang et al., 2012). Such high genetic diversity reveals that a low level of recombination was responsible for the plastid genome to achieve a high level of genetic diversity. Analysis of molecular variance (AMOVA) revealed that 25.87% of the total genetic diversity was between the populations, while 74.13% was among the populations. Most of the previous studies suggested that high genetic diversity between populations reflects a clear phylogeographic structure (Avise, 2004). Such genetic compositions are resulted due to strong bottlenecks, and further founder effects to favor and fix different alleles in isolated refugia (Birky, 2001). The AMOVA results were further strengthened through high $N_{ST} = 0.693$ result, which was significantly different from $G_{ST} = 0.473$ ($N_{ST} > G_{ST}$; $P < 0.01$). Similar results were reported for other alpine species (Wang et al., 2008; Yang et al., 2008). In *Pedicularis longiflora* this pattern was due to the large-scale range recolonization from edge refugia (Yang et al., 2008). However In this study and in *Potentilla glabra* (Wang et al., 2009b), such results are due to the strong bottlenecks and small-scale range expansion within the local regions. The high nucleotide diversity $Pi = 0.01$ in *S. alpina* suggests that the concert evolution was not fast enough for this species to homogenize the ITS copies (Lorenz-Lemke et al., 2005).

4.2. Glacial refugia

Plant populations show high genetic diversity and fidelity in isolated regions (Petit et al., 2003). The current study revealed three different isolated regions with high genetic diversity and uniqueness for *S. alpina*. These regions could be the potential refugia for this alpine shrub. These refugia constitute the south-eastern rear edge of the QTP, north of the Tanggula mountains and south-west of the Tanggula Mountains. The first refugium with high genetic diversity includes 13 populations (P6–P12, P21, P26–P30) with five endemic haplotypes (A4, A7-A8, A12, and A18). This region has long been considered a potential refugium for QTP flora (Chen et al., 2008; Gao et al., 2012; Zhang et al., 2012). The second potential refugium includes five populations (P1, P22–P25). In this region, P1 has the highest genetic diversity ($h = 1$) and high nucleotide diversity

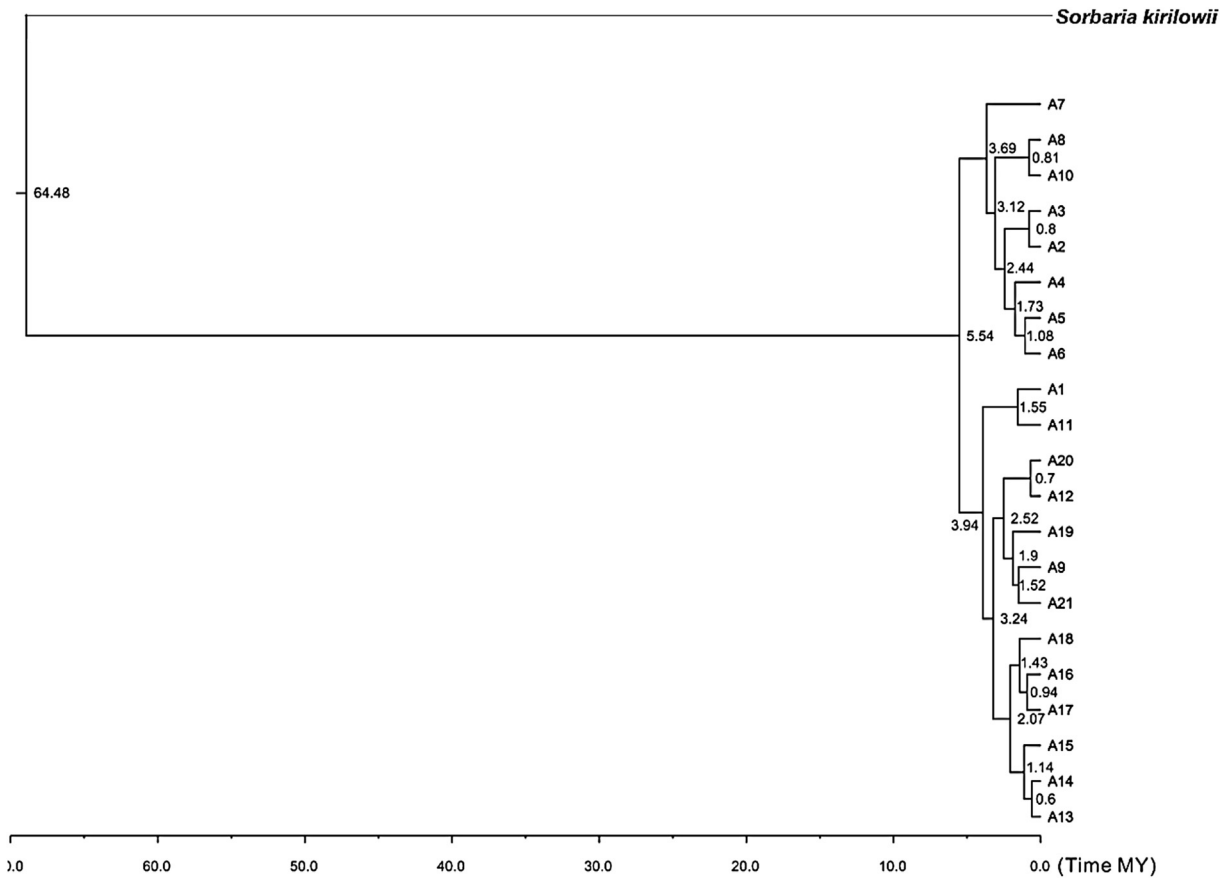

Fig. 4. Bayesian chronogram of *Spiraea alpina* haplotypes. The axis scale represents million years (MY); numbers near the nodes represent divergence time in million years (estimated with the substitution rate of 5.69×10^{-9} s s^{-1} y^{-1}) at 95% highest posterior density.

($\pi = 0.002$), located at north of the Tanggula Mountains; followed by P26 with $h = 0.709$ genetic diversity and $\pi = 0.003$ nucleotide diversity. This region has no endemic haplotype, with high genetic diversity mostly mixes the haplotypes. High genetic diversity may be caused by mixing of organisms from different origins in the region (Petit et al., 2003). The third potential refugium existed in the south-west of the Tanggula Mountains, with 13 populations and four endemic haplotypes (A1, A6, A13, and A21). Among these populations, P5 and P14 include six haplotypes in each, while P13 with seven haplotypes. Populations in this region are isolated from the other populations by the long Yellow River and the Tanggula mountain range. Similar results were found by Gao et al. (2012) and Zhang et al. (2012). The mismatch distribution, neutrality tests, and sudden expansion model (Supplementary Table S1; Fig. 4) rejected the phenomenon of range expansion for *S. alpina*, confirmed the bottleneck and retreat phenomena (Rogers and Harpending, 1992). The results for time in generations elapsed since the sudden expansion ($\tau(t)$) in MY, revealed that *S. alpina* was at the QTP platform 3.27 MYA, before the Quaternary climatic oscillations. Clade I showed total range expansion of 0.02 MY, while Clade II expanded for 0.07 MY.

4.3. Intraspecific divergence in Spiraea alpina

The QTP uplifting process affected organic evolution in the region. Molecular dating, in a phylogenetic context, is required to determine whether diversification is linked to acute uplifting and eco-environmental processes during the last 3.60 MY or not. The more recent acute uplifting of the QTP is divided into two phases. During phase A, the initial major uplift of the QTP occurred in Early Miocene, from 23 to 15 to 8 MYA (Patriat and Achache, 1984; Klootwijk et al., 1985). In phase B, acute uplifting of QTP proceeded, which raised the altitude of QTP from an average of 3000 m to an average of 5000 m during the last 4 MY (Shi et al., 1998; Zheng et al., 2002; Zhisheng et al., 2001). The main lineages based on ITS sequences showed diversification during 1.08–0.60 MY, except for A7, which is the most basal haplotype. The divergence times for main lineages of ITS sequence types lie within the extensive climatic oscillation of the Quaternary (Zheng et al., 2002). This indicates that climatic oscillations caused by glacial and interglacial repeats during the Pleistocene may be an important driving factor for intraspecific divergence in *S. alpina*. Most studies on the alpine flora of the QTP have highlighted the effect of the Pleistocene period on intraspecific diversification (Wang et al., 2009a; Gao et al., 2012). Repeated glacial and interglacial cycles during the Pleistocene likely created allopatric fragmentation and isolation of populations, which accelerated the process of intraspecific

diversification. The existence of three isolated regions harboring relatively high genetic diversity suggests allopatric intraspecific divergence of *S. alpina*.

This study contributes towards the phylogeography of *S. alpina* and elaborates the role of nuclear ribosomal internal transcribed spacers. It is of particular interest that we could use nuclear ITS fragments, instead of the usual plastid chloroplast DNA fragments used in most plant population genetic studies at the QTP, to elucidate a complete picture of population genetics for *S. alpina*. Unlike plastid DNAs, nuclear ITS fragments are much more prone to variation in severe climatic oscillations. The results revealed that *S. alpina* retreated into three different isolated refugia during the Quaternary glaciation. Unlike the results using cpDNA fragments, which suggested multiple refugia for this shrub, our results consist only three refugia, these being the south-east rear edge of the QTP, north of the Tanggula mountains and south-west of the Tanggula Mountains. The results showed that the severe climatic oscillations during Quaternary had a profound effect on intraspecific divergence. Further research about evolution, speciation, and phylogeography at this important bio-diverse hub, is required to explore the evolutionary trends, speciation phenomenon, and phylogeographic structure of more flora and fauna at QTP.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bse.2014.08.013.

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