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Evolutionary history of an alpine shrub *Hippophae tibetana* (Elaeagnaceae): allopatric divergence and regional expansion

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Increasing evidence suggests that geological or climatic events in the past promoted allopatric speciation of alpine plants in the Qinghai-Tibetan Plateau and adjacent region. However, few studies have been undertaken to examine whether such allopatric divergences also occurred within a morphologically uniform species. In the present study, we report the evolutionary history of an alpine shrub species, *Hippophae tibetana*, based on examining chloroplast DNA (cpDNA) and nuclear ribosomal internal transcribed spacer (ITS) DNA variations. We sequenced two cpDNA fragments (*trn*L-F and *trn*S-G) and the nuclear ITS region in 183 individuals collected from 21 natural populations. Ten chlorotypes and 17 ITS types were identified. Phylogenetic analyses of both chlorotypes and ITS sequence variations suggested two distinct lineages distributed in the eastern and western region, respectively. On the basis of the fast and low plant substitution rates, these two lineages were estimated to have diverged from each other between 1 and 4 million years ago, during the period of the major glaciations and orogenic processes. In addition, ITS has undergone the accelerated evolution in two populations in the southern Himalaya isolated by the high mountains with a surprising accumulation of the private variations. The east–west split was also supported by an analysis of molecular variance, which partitioned around 91% of the total cpDNA variance between these two groups of populations. A single chlorotype was found for most populations in eastern or western region, suggesting a recent postglacial expansion within each region. Star-phylogeny and mismatch analyses of all chlorotypes within the eastern group of populations suggested an earlier regional expansion before the Last Glacial Maximum (LGM). The local fixture of the different chlorotypes in multiple populations suggested more than one refugia remained for eastern or western region. Coalescent tests rejected the hypothesis that all current populations originated from a single refugium during the LGM. Instead, they supported hypothesis that two lineages diverged before the late Pleistocene. These findings, when taken together, suggested that this species had experienced long allopatric divergence and recent regional range expansions in response to orogenic processes and the climate changes. The evolutionary history of this shrub species highlights importance of geographical isolations to the intraspecific divergence of alpine plants occurring in the world's ruff. © 2010 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2011, **102**, 37–50.

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INTRODUCTION

The genetic structure of the current species reflects the interacting consequences of two fundamental processes: population dynamics in response to the past geological or climatic changes and species' evolutionary ability under the diverse selection (Avise, 2004). Range fragmentation, contraction, and expansion therefore could have distinct genetic signature within the current populations (Hewitt, 2000), which can be recovered by the phylogeographic analyses. The inferred population dynamics and distributional range based on the genetic variations between and within the current populations is largely congruent with those inferred from the fossil evidence, especially in both Europe and North America (Petit & Grivet, 2002; Godbout *et al*., 2005). However, our phylogeographic understandings of both plants and animals are still incomplete because species occurring in the other regions of the world have received little attention.

The Qinghai-Tibetan Plateau (QTP; the largest and highest plateau in the world with mean altitude > 4000 m a.s.l.) is such a region. The QTP had experienced considerable and rapid geological uplifts and glaciations of large areas since the Pliocene. For example, the QTP mountains had been extensively uplifted several times between 1.2 and 3.6 Mya (Li, Shi & Li, 1995). The largest glaciation in the QTP (Naynayxungla Glaciation) started around 1.2 Mya and reached its maximum between 0.6 and 0.8 Mya (Shi & Ren, 1990; Zhou & Li, 1998; Zheng, Xu & Shen, 2002). After this largest glaciation, several cycles of climatic oscillations as a result of mountainous glaciations might continue to the Holocene (Shi, Li & Li, 1998). This region, especially its southeastern part, represents one of the world's biodiversity hotspots (Mittermeier *et al*., 2005), and contains more than 20 000 species of vascular plants with numerous endemic species (Wu, 1988; Li & Li, 1993). Phylogenetic analyses of the species-rich genera suggested that most endemic species occurring there originated *in situ* or adjacently and the species diversifications were calibrated to be highly consistent with environmental and/or climatic changes (Yang, Wang & Hong, 2003; Liu *et al*., 2006; Ran, Wei & Wang, 2006; Zhang *et al*., 2009; Wang *et al*., 2009a). It is feasible that these changes also resulted in the intraspecific divergences (Avise, 2004) because numerous species survived in the high altitude regions for a long time (Wang *et al*., 2009a; Tang *et al*., 2010), although the others colonized the plateau platform postglacially or interglacially after the largest glaciation occurred there (Zhang *et al*., 2005; Meng *et al*., 2007; Chen *et al*., 2008; Yang *et al*., 2008; Wu *et al*., 2010).

In the present study, we report the evolutionary history of *Hippophae tibetana*, an alpine shrub occurring in the QTP and adjacent Himalayan region. *Hippophae* is a small genus of the Elaeagnaceae, comprising between five and seven species (Rousi, 1971; Bartish *et al*., 2002). All species of this genus have received a great deal of attention because of their nitrogen-fixing functions (Lian, Chen & Lian, 1998). These species are dioecious and wind pollinated, with gender being determined genetically (Rousi, 1971; Lian *et al*., 1998; Bartish *et al*., 2000). The juicy fruits are dispersed by birds (Rousi, 1971). *Hippophae tibetana* diverged early from the other species of the genus (Bartish *et al*., 2000, 2002; Sun *et al*., 2002). This species was shown to be morphologically uniform without much of intraspecific variation (Rousi, 1971). It occupies a wide range of habitats from 2800–5000 m (Lian *et al*., 1998). In the present study, we used chloroplast DNA (cpDNA) and nuclear ribosomal internal transcribed spacer (ITS) sequence data to examine intraspecific diversification of *H. tibetana*. The cpDNA is maternally inherited in *Hippophae* (Bartish *et al*., 2002) and the nuclear ITS variation exhibits biparental inheritance (Sun *et al*., 2002). The genetic variations inferred from these two sets of molecular markers can be highly effective to trace intraspecific differentiation of plants and their range responses to the Quaternary changes (Wang *et al*., 2009a). We aimed to address the following questions based on range-wide population sampling: (1) are the intraspecific divergences related to geographical distribution and did past geological changes and climatic glaciations promote the allopatric divergence of this species and (2) did this species show range shifts in response to the Quaternary climatic oscillations?

MATERIAL AND METHODS

POPULATION SAMPLING

We collected 183 individuals from 21 natural populations across the entire distributional range of this species in the QTP (Fig. 1A, Table 1) from 2002–2005. The sampled individuals within each population were located more than 100 m apart. However, in a few small populations with fewer than 100 individuals, only one to five individuals were sampled. We failed to collect sparse distributions spanning the geographical gap between populations 7 and 8 (Fig. 1A) because they were inaccessible during the 4 years of field as a result of floods and road constructions. According to the specimens collected almost 50 years ago, this species was recorded to occur in four sites of this region (Dingqing, Ranwu, Baqing, and Chayu). We used silica gels to dry the collected fresh leaves immediately in the field. We further collected voucher specimens at the same time, which are now deposited in the

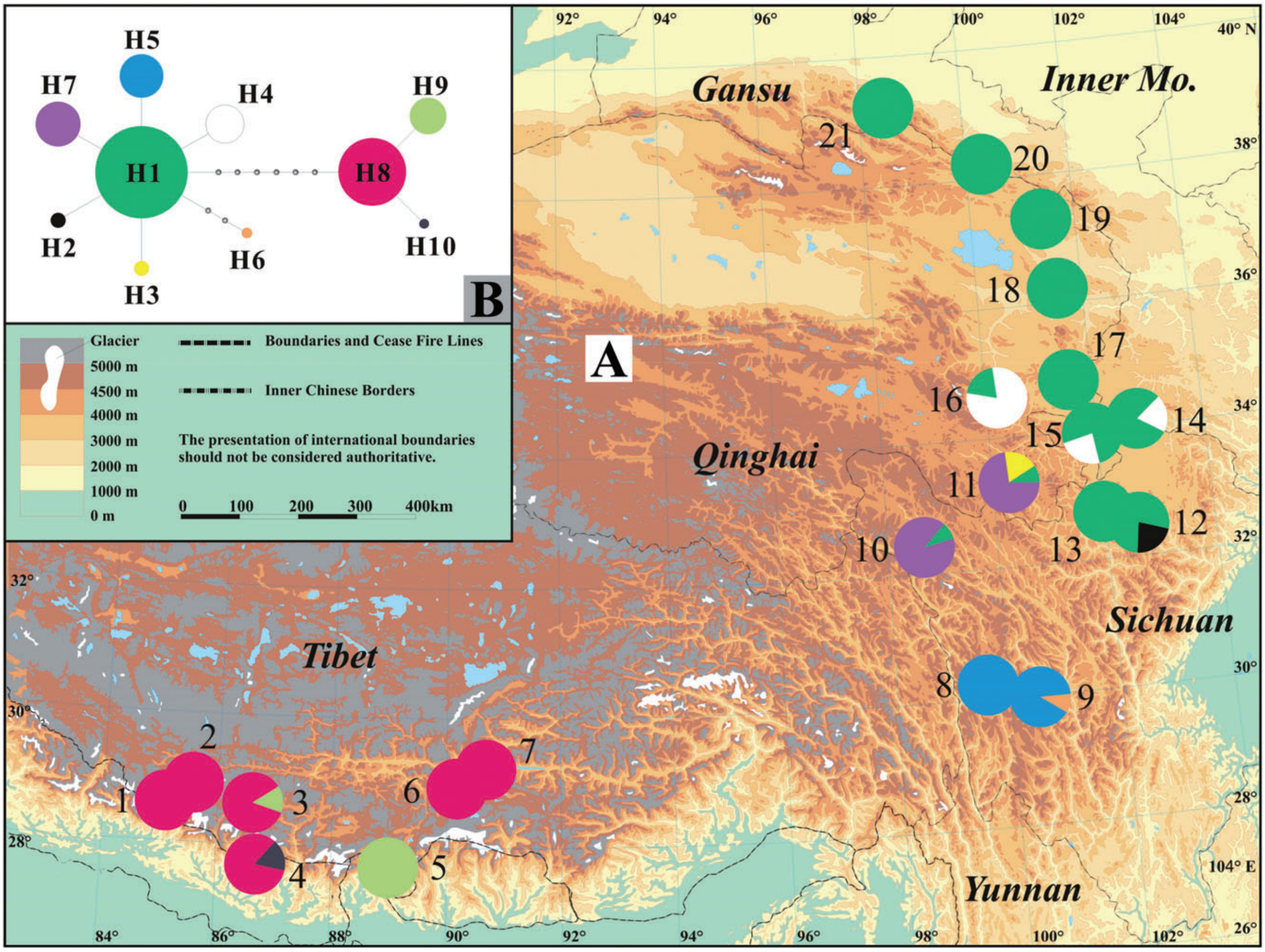

Figure 1. A, map of the sampling sites (Table 1) and the geographic distribution of *Hippophae tibetana* chloroplast DNA (cpDNA) haplotypes. Pie charts show the proportions of chlorotypes within each population. B, the network of chlorotypes as inferred from TCS. Circle sizes are proportional to chlorotype frequencies within the total sample and small dots indicate unidentified chlorotypes.

Herbarium of Lanzhou University. We included the other six congeners (*H. neurocarpa* ssp. *stellatopilosa*, *H. neurocarpa* ssp. *neurocarpa*, *H. salicifolia*, *H. gyantsensis*, *H. rhamnoides* ssp. *yunnanensis*, and *H. rhamnoides* ssp. *sinensis*) and one outgroup (*Elaeagnus umbellata*) for phylogenetic analyses.

DNA EXTRACTION, AMPLIFICATION AND SEQUENCING

We used DNeasy™ Tissue Kit (Qiagen) to isolate total genomic DNA in accordance with the manufacturer's instructions. We amplified two cpDNA fragments (*trn*L-F and *trn*S-G) and the ITS region using the primers: *trn*-L: 5′-CgA AAT Cgg TAg ACg CTA Cg-3′ and *trn*-F: 5′-ATT TgA ACT ggT gAC ACg Ag-3′ (Taberlet *et al*., 1991); *trn*-S: 5′-gCC gCT TTA gTC CAC TCA gC-3′ and *trn*-G: 5′-gAA CgA ATC ACA CTT TTA CCA C-3′ (Hamilton, 1999); and ITS1: 5′-AgA AgT CgT AAC AAg gTT TCC gTA gg-3′ and ITS4: 5′-TCC TCC gCT TAT TgA TAT gC-3′ (White *et al*., 1990). We performed PCR on a GeneAmp® PCR System 9700 (Applied Biosystems) in a 25 µL reaction volume, which contained 0.02 mmol L^{-1} dNTPs, 0.05 mmol L^{-1} of each primer, 1.0 units of TAKARA r*Taq* polymerase (Takara), Takara 10 × reaction buffer (100 mM Tris-HCl, 500 mM KCl, 15 mM $MgCl_2$), and 10–40 ng of genomic DNA. The thermal cycler profile comprised 36 cycles of 94 °C denaturing for 45 s, 60 °C (*trn*L-F, ITS) or 59 °C (*trn*S-G) annealing for 40 s, and 72 °C extending for 60 s preceded by 5 min at 94 °C and terminated with 7 min at 72 °C. PCR products were further purified then with a TIANquick Midi Purification Kit (Tiangen) in accordance with the manufacturer's instructions. Sequencing reactions

Table 1. Locations of populations of *Hippophae tibetana* sampled, sample sizes (*N*), chlorotypes in each population, and estimates of gene diversity (H_E) and nucleotide diversity averaged across loci (π) within populations and groups

						Chlorotypes											
Code	Location	Latitude	Longitude	Alt. (m)	*N*	H1	H2	H3	H4	H5	H6	H7	H8	H9	H10	H_E (SD)	π (SD) (×100)
Western group					**54**								**41**	**12**	**1**	**0.39 (0.07)**	**0.03 (0.03)**
1	Jilong, XZ	28°34.891′	085°15.495′	3800	11								11			0	0
2	Jilong, XZ	28°45.322′	085°34.698′	4609	12								12			0	0
3	Dingri, XZ	28°35.060′	086°37.799′	4340	7								6	1		0.29 (0.20)	0.02 (0.03)
4	Nepal	27°48.694′	086°43.181′	4300	6								5		1	0.33 (0.22)	0.02 (0.03)
5	Yadong, XZ	27°43.283′	088°58.092′	3780	11									11		0	0
6	Jiangzi, XZ	28°53.713′	090°16.345′	4711	6								6			0	0
7	Langkazi, XZ	29°11.142′	090°35.014′	4461	1								1			0	0
Eastern group					**129**	**76**	**2**	**2**	**13**	**17**	**1**	**18**				**0.61 (0.04)**	**0.05 (0.04)**
8	Batang, SC	30°13.991′	099°45.849′	4200	8					8						0	0
9	Litang, SC	30°01.815′	100°22.073′	4015	10					9	1					0.20 (0.15)	0.06 (0.05)
10	Shiqu, SC	32°21.626′	098°39.935′	3990	11	1						10				0.18 (0.14)	0.01 (0.02)
11	Dari, QH	33°18.004′	100°25.886′	4020	11	1		2				8				0.47 (0.16)	0.05 (0.05)
12	Hongyuan, SC	32°30.817′	102°21.718′	3541	9	7	2									0.39 (0.16)	0.03 (0.03)
13	Rruoergai, SC	32°44.257′	102°05.868′	3784	7	7										0	0
14	Langmusi, SC	34°05.239′	102°38.318′	3600	5	4			1							0.40 (0.24)	0.03 (0.04)
15	Maqu, GS	33°59.939′	102°04.508′	3479	17	13			4							0.38 (0.11)	0.03 (0.03)
16	Maqin, QH	34°38.821′	100°14.879′	3580	10	2			8							0.36 (0.16)	0.02 (0.03)
17	Henan, QH	34°39.482′	101°33.480′	3527	12	12										0	0
18	Guide, QH	36°13.676′	101°33.607′	3820	13	13										0	0
19	Datong, QH	37°15.088′	101°24.454′	2980	1	1										0	0
20	Qilian, QH	38°10.350′	100°18.886′	3130	10	10										0	0
21	Qilian, QH	39°12.823′	098°30.310′	3841	5	5										0	0

Alt., altitude; XZ, Tibet; SC, Sichuan; QH, Qinghai; GS, Gansu.

were carried out with the same PCR primers described above and ABI Prism Bigdye™ Terminator, version 3.1, Cycle Sequencing Kit. Purified DNA fragments were separated and called on an ABI 3730XL DNA Analyzer. All sequences were initially aligned with CLUSTAL X, version 1.81 (Thompson *et al*., 1997) and then refined manually. The newly recovered sequences of *H. tibetana* have been deposited in GeneBank under the accession numbers HM769671–HM769721.

PHYLOGENETIC ANALYSIS

Phylogenetic analyses of both cpDNA and the nuclear ITS sequences were carried out by maximum-parsimony (MP), maximum-likelihood (ML), and Bayesian analyses using PAUP*, version 4.0b10 (Swofford, 2002); GARLI, version 1.0 (Zwickl, 2006); and MrBayes, version 3.0 (Huelsenbeck & Ronquist, 2001; Ronquist & Huelsenbeck, 2003). We constructed MP trees using a heuristic search, 100 random additions of sequences, equally weighted characters and nucleotide transformations, with the tree-bisection–reconnection branch swapping, MULTREES, COLLAPSE, and STEEPEST DESCENT options switched on. All indels detected were coded using the simple code method applied by the software GAPCODER (Young & Healy, 2003), and included in the MP analysis. The robustness of MP trees was estimated by 1000 bootstrap replicates. ML analysis in GARLI was performed using random starting trees and 5 000 000 generations per search; 30 searches were performed and the best tree saved. ML bootstrap analysis was carried out with the same settings, using 100 replicates and with five searches per replicate. Bayesian posterior probability (PP) values were calculated by the Markov chain Monte Carlo (MCMC) searches, which were performed, with four chains of 3 000 000 generations and trees sampled every 100th generation when a common model GTR+I+G was adopted. The first 10 000 trees sampled were discarded as 'burn-in', whereas a consensus topology and PP-values were calculated with the remaining trees with PAUP*. For comparison, unrooted statistical parsimony networks for all the recovered chlorotypes and ITS types were also built using TCS, version 1.21 (Clement, Posada & Crandall, 2000). TCS was run with default parsimony connection limit of 95%, all indels in the sequence alignment coded as additional single characters.

DIVERGENCE TIMES

A likelihood-ratio test (Huelsenbeck & Rannala, 1997) was conducted to test the molecular clock hypothesis for the cpDNA and ITS sequences of *H. tibetana* and the other congeners using PAUP*, version 4.0b10 (Swofford, 2002). This test evaluated the hypothesis of rate constancy by calculating the log likelihood of the ML trees with and without the molecular clock enforced. Because the hypothesis of a molecular clock could not be rejected because constrained and unconstrained analyses were not significantly different (HKY+G, $2\ln LR = 16.437$, d.f. = 15, $P = 0.354$) for cpDNA sequences, we then used the Bayesian analyses to estimate the divergence times between the western and eastern lineages with the software BEAST (Drummond *et al*., 2002; Drummond & Rambaut, 2007) under the hypothesis of the molecular clock based on the substitution rates of the chloroplast sequence recovered for most angiosperm species ($\mu = 1.0\text{–}3.0 \times 10^{-9}$ s s^{-1} y^{-1}; Wolfe, Li & Sharp, 1987). Following a burn-in of 500 000 steps, all parameters were sampled once every 100 steps from 5 000 000 MCMC steps. We checked convergence of the stationary distribution by visual inspection of plotted posterior estimates using the software Tracer (Rambaut & Drummond, 2007), and the effective sample size for each parameter sampled was found to exceed 200. However, for ITS sequence data, our tests rejected the molecular clock hypothesis ($P < 0.05$). We therefore used the nonparametric rate smoothing technique of Sanderson (1997) to estimate intraspecific divergences based on the ITS substitution rates ranging from 3.46×10^{-9} to 8.69×10^{-9} s s^{-1} y^{-1} (Richardson *et al*., 2001) recorded for shrubs and herbal plants.

POPULATION AND PHYLOGEOGRAPHIC ANALYSIS

Average gene diversity within populations (H_S), total gene diversity (H_T), and two parameters of differentiation, G_{ST} (coefficient of genetic variation over all populations) and N_{ST} (equivalent coefficient taking into account sequence similarities between haplotypes) were estimated for overall populations and for groups (identified by phylogenetic analyses) *sensu* Pons & Petit (1996), using the software PERMUT (http://www.pierroton.inra.fr/genetics/labo/Software/PermutCpSSR). G_{ST} and N_{ST} were compared using a permutation test with 1000 permutations. The occurrence of significant phylogeographic structure was indicated by N_{ST} being significantly higher than G_{ST} (Pons & Petit, 1996). Hierarchical analysis of molecular variance (AMOVA; Excoffier, Smouse & Quattro, 1992) was performed to characterize the population structure and genetic variation using ARLEQUIN, version 3.0 (Excoffier, Laval & Schneider, 2005). Haplotype diversity (H_E), nucleotide diversity (π) were also calculated using this software.

The signature of demographic expansion was tested by the *D*-test of Tajima (1989) and F_S test of

Fu (1997). Significant *D*-values and large negative F_S-values generally suggest rapid demographic expansions (Hudson, 1990). A mismatch distribution analysis (Schneider & Excoffier, 1999) was also conducted to infer the demographic expansions of *H. tibetana*. One thousand parametric bootstrap replicates were used to generate an expected distribution using a model of sudden demographic expansion (Excoffier *et al*., 2005). *P*-values were then calculated as the proportion of simulations producing a larger sum-of-squared deviation than the observed. The raggedness index of Harpending (Harpending, 1994) of the observed mismatch distribution and its significance were also computed to qualify the smoothness of the observed mismatch distribution. All these demographic tests were performed using ARLEQUIN, version 3.0 (Excoffier *et al*., 2005). When the hypothesis of sudden expansion can not be rejected, the corresponding τ value was used to infer a time scale for the demographic expansion. To estimate *t*, we used the relationships $\tau = 2ut$ (Rogers & Harpending, 1992; Rogers, 1995), where *t* is the expansion time in number of generations and *u* is the mutation rate per generation for the whole analyzed sequence. Values for *u* were calculated as $u = 2\,\mu kg$, where μ is the substitution rate per nucleotide site per year, *k* is the average sequence length of the DNA region, and *g* is the generation time in years.

Coalescent analysis and simulation

We used coalescent simulations of genealogies constrained within models of population divergence to assess the fit of observed gene tree to different phylogeographic hypotheses (Knowles, 2001; Knowles & Maddison, 2002; Carstens *et al*., 2005; DeChaine, 2008). Hypothesis testing was performed using the software MESQUITE, version 2.5 (Maddison & Maddison, 2008). Absolute time (years) was converted to coalescent time (generations) assuming a generation time of 5 years for a congeneric species *H. rhamnoides* (Bartish, Kadereit & Comes, 2006). We used the *S* statistic of Slatkin & Maddison (1989) to evaluate whether the observed genealogies were consistent with the given models by comparing the *S* value of empirical ML genealogy and those of the simulated genealogies.

For all coalescent simulations, effective population size (N_e) was estimated using the θ-values calculated using the software MIGRATE, version 2.4.3 (Beerli, 2002). The search parameters were: ten short chains of 1 000 000 steps, followed by two long chains of 10 000 000 steps; chains were sampled every 100 steps following a burn-in of 10 000 steps, and default settings used for the initial estimate of theta value. The theta value was converted to N_e using the formula $\theta = 2N_e\mu$, with the low substitute rate of chloroplast sequence for the woody plants ($\mu = 1.0 \times 10^{-9}$ s s^{-1} y^{-1}; Wolfe *et al*., 1987) adopted. During coalescent simulations, we set the overall N_e to equal the empirically estimated values, and constrained the N_e of the refugial population to a size proportional to the relative N_e of the population sampled from the site of the putative refugia.

RESULTS

Chloroplast DNA

The total alignment length of the two cpDNA sequences examined was 1482 bp. Nucleotide substitution occurred at 16 sites, of which seven were parsimony informative, and one indel with a length of 49 bp was present in the *trn*S-G region. Combining all base substitutions and length variation character states, a total of ten chlorotypes (H1–H10; see Supporting information, Table S1) were identified from the 183 plants analyzed. Twelve populations (57%) were fixed for a single chlorotype, whereas the remaining nine (43%) were polymorphic (Fig. 1A, Table 1). Seven chlorotypes (H1–H7) were found among populations (8–21) located in the eastern QTP, whereas the other three (H8, H9, and H10) were present only among western populations (1–7) (Fig. 1A). The most dominant chlorotype (H1) among eastern populations was fixed or present in twelve populations (10–21). Chlorotypes H4, H5, and H7 occurred in more than two populations, whereas the remaining three (H2, H3, and H6) were private to populations 9, 11, and 12, respectively. In the western distribution of the species, H8 was found to be the most common chlorotype and very widely distributed in six out of the total seven populations. Chlorotype H9 was shared between populations 3 and 5, whereas H10 occurred only in population 4.

Three phylogenetic analyses by MP, ML, and Bayesian methods produced consistent topology (Fig. 2A). Monophyly of the ten chlorotypes of *H. tibetana* was strongly supported. Within the species, two highly supported clades were recognized: one consisting of H1–H7 and the other H8–H10. Chlorotypes comprising these two clades were geographically distributed in the eastern and western areas of the QTP, respectively (Fig. 1A). The cpDNA haplotype network was congruent with the results from the MP, ML, and Bayesian analyses, in which the two clades were separated by six unidentified chlorotypes (Fig. 1B). The two intraspecific lineages of *H. tibetana* were estimated to have diverged between 1.08 (95% HPD: 0.60–1.60) and 3.25 (95% HPD: 1.77–4.82) Mya based on the fast and slow substitution rates.

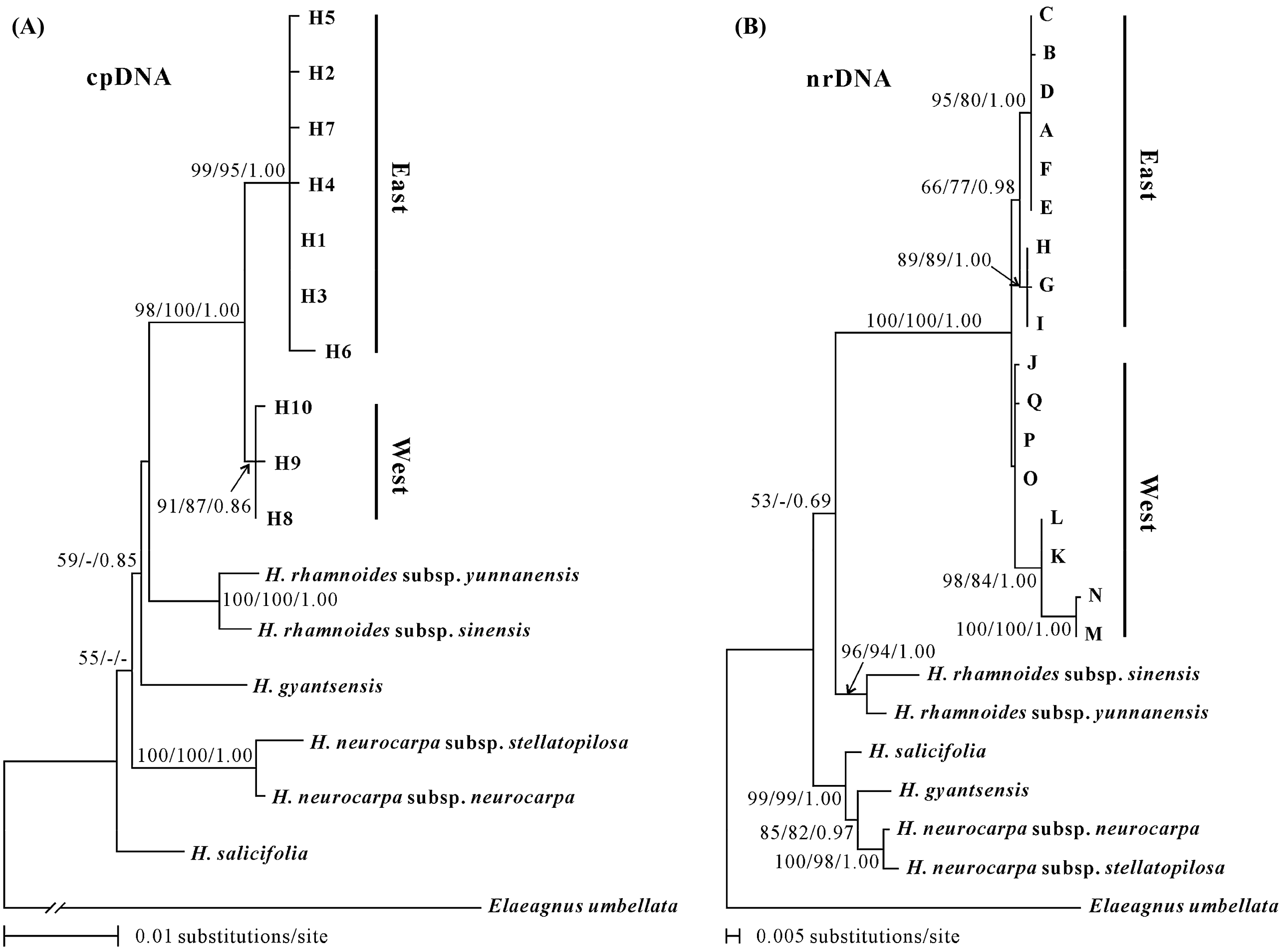

Figure 2. Two maximum-likelihood trees based on phylogenetic analyses of (A) the chloroplast DNA (cpDNA) data set ($-\ln L = 3185.2048$) and (B) the nuclear ribosomal DNA (nrDNA) data set ($-\ln L = 2097.6013$). Support values (maximum parsimony bootstrap/maximum likelihood bootstrap/Bayesian posterior probability) greater than 50% are shown at nodes.

NUCLEAR RIBOSOMAL DNA

We detected 17 different ITS types (A–Q; see Supporting information, Table S2). The length of the alignment of all ITS types was 702 bp. A total of 25 base substitutions and six different length variants (1–5 bp) were found. Seven out of the 17 ITS types were unique to single populations (see Supporting information, Fig. S1A, Table S3). Eight ITS types (J–Q) were found in western populations, whereas the remaining nine types (A–I) were present only in eastern populations. For eastern populations, ITS type A was found to be the most common ITS sequence that was fixed or present in all populations, except for populations 8 and 9. For western populations, almost each population was fixed for a different ITS genotype. Especially, M and N were only found in populations 4 and 5 collected from the southern Himalaya. Seven mutations and five indels were only found for these two ITS genotypes (see Supporting information, Table S2). The phylogenetic topology based on the ITS sequences produced by MP, ML, and Bayesian analyses was broadly congruent with each other, which was also consistent with that based on cpDNA sequences, although with low support: these ITS types were grouped into two major lineages that were geographically distributed in the eastern and western populations (Fig. 2B; see Supporting information, Fig. S1A). Multiple variations private to M and N clustered these genotypes as a sublineage with an extremely long branch within the western lineage. Within the eastern lineage, two sublineages were also identified: one consisting of types A–F that were present in populations 1–7 and the other types G–I that occurred only in populations 8 and 9. The divergence of these two intraspecific lineages was estimated to range from 1.26 (95% HPD: 0.78–1.79) to 3.17 (95% HPD: 1.96–4.48) Mya based on the fast and slow ITS substitution rates.

Population structure and phylogeographic analysis

Chlorotype diversity (H_E) within the 21 populations was in the range 0–0.47, and nucleotide diversity (π) in percent was in the range 0–0.06 (Table 1). The higher mean haplotype diversity was exhibited in the eastern group (0.61). Total genetic diversity H_T (0.782; Table 2) across all populations was much higher than average within-population diversity H_S (0.158) (i.e. the majority of cpDNA diversity is distributed among populations). Consequently, total N_{ST} (0.947) was significantly larger than G_{ST} (0.798; $P < 0.001$), indicating that cpDNA variation of this species on the whole distribution was well geographically structured (Table 2). The hierarchical AMOVA based on chlorotypes revealed that nearly 95% of the total variation was partitioned among populations, whereas just 5% was within populations (Table 3). The east–west split divergence of the species was also supported by AMOVA, with approximately 91% of the variation attributed to this pattern of differentiation (Table 3). The results of demographic expansion histories tested by different analyses are shown in the Supporting information (Table S4). The significant D-value, large negative F_S-value and unimodal mismatch distributions (see Supporting information, Fig. S2) of the eastern lineage suggest a historical demographic expansion. The time of the expansion event was estimated to be 26.4–79.1 Kya.

Coalescent analysis and simulations

We conducted coalescent simulations to test hypotheses concerning the putative glacial refugia of *H. tibetana*. First, all current populations of the species were derived from a single refugium that was located either at the edge or in the interior of the plateau and present towards the end of the Last Glacial Maximum (LGM) (approximately 12 000 years ago; Fig. 3A). Second, the species may have survived the LGM in two isolated refugia that gave rise to the western and eastern lineage, respectively. We assumed that the split between the two lineages occurred at the beginning of the LGM (T2 = 20 000 years ago) and the current populations were derived, respectively, from the two refugia at the end of LGM (T1 = 12 000 years ago; Fig. 3B). Third, the east–west split may have occurred early in the Pleistocene, as estimated by genetic divergences of the ITS and cpDNA sequences, and that all current populations are derived from three independent refugia that existed during the LGM (T1 = 20 000 years ago; Fig. 3C). In this case, the initial divergence of the east and west clade was assigned to be T3 = 3.0 Mya, as estimated based on the low mutation rate ($\mu = 1.0 \times 10^{-9}\ \mathrm{s\ s^{-1}\ y^{-1}}$). We assumed that T2 ranged from the last interglacial period (0.12 Mya; Sangamon interglacial period) to 0.17 Mya (the beginning of Penultimate Glaciation; Shi, 2002; Zheng *et al.*, 2002).

We estimated θ-value based on cpDNA sequences with MIGRATE, version 2.4.3: $\theta_{total} = 1.76 \times 10^{-3}$ [consistency index (CI): 9.40×10^{-4}–2.79×10^{-3}], $\theta_{west} = 1.51 \times 10^{-4}$ (CI: 1.89×10^{-5}–5.74×10^{-4}), $\theta_{east} = 1.24 \times 10^{-3}$ (CI: 4.95×10^{-4}–2.00×10^{-3}). On the basis of these θ-values and assuming $\mu = 1.0 \times 10^{-9}$, each N_e was obtained: $N_{e\ total} = 879\,000$, $N_{e\ west} = 75\,500$, and $N_{e\ east} = 620\,500$. The model of evolution for ingroup sequences used in the coalescent simulations was: TIM, $\pi A = 0.3344$, $\pi C = 0.1681$, $\pi G = 0.1400$, $\pi T = 0.3575$; rA-C = 1.0000, rA-G = 0.0000, rA-T = 0.4116, rC-G = 0.4166, rC-T = 0.3921, rG-T = 1.0000; *pinv* = 0. The fit of each of the three models to the geographical genetic structures was then tested using MESQUITE, version 2.5. The coalescent simulations rejected the single-refugium hypothesis ($P < 0.01$) and the hypothesis of two isolated refugia for the species originating at the start of the LGM ($P < 0.05$) and support that two lineages diverged before the late Pleistocene (hypothesis C; $P = 0.19$).

DISCUSSION

Allopatric divergence

Phylogenetic analyses of ITS and cpDNA sequences from all individuals showed that *H. tibetana* comprised two lineages, respectively, distributed in the eastern and western QTP and adjacent southern Himalaya (Figs 1, 2, Table 1; see also Supporting

Table 2. Estimates of average gene diversity within populations (H_S), total gene diversity (H_T), interpopulation differentiation (G_{ST}), and number of substitution types (N_{ST}) for chlorotypes (mean ± SE)

Regions	H_S	H_T	G_{ST}	N_{ST}
Western group (1–7)	0.103 (0.0655)	0.405 (0.1881)	0.745 (0.2260)	0.753 (0.2160)
Eastern group (8–21)	0.183 (0.0534)	0.637 (0.1136)	0.712 (0.0783)	0.682 (0.0729)
All populations	0.158 (0.0419)	0.782 (0.0574)	0.798 (0.0524)	0.947 (0.0181)

Population numbers of the two groups are shown in parentheses under ‘Regions’. A significantly larger N_{ST} than G_{ST} was detected only in regard to ‘All populations’ ($P < 0.001$).

Table 3. Analysis of molecular variance of chlorotypes and internal transcribed spacer (ITS) types for populations of *Hippophae tibetana*

Source of variation	d.f.	SS	VC	PV (%)	Fixation indices
Chloroplast DNA					
Western group					
Among populations	6	8.624	0.189	83.98	$F_{ST} = 0.840$*
Within populations	47	1.691	0.036	16.02	
Total	53	10.315	0.225		
Eastern group					
Among populations	13	33.511	0.269	66.83	$F_{ST} = 0.668$*
Within populations	115	15.342	0.133	33.17	
Total	128	48.853	0.402		
All populations					
Among populations	20	312.301	1.797	94.47	$F_{ST} = 0.945$*
Within populations	162	17.032	0.105	5.53	
Total	182	329.333	1.902		
Western group versus eastern group					
Among groups	1	270.166	3.516	90.91	$F_{CT} = 0.909$*
Among populations within groups	19	42.135	0.247	6.37	$F_{SC} = 0.701$*
Within populations	162	17.032	0.105	2.72	$F_{ST} = 0.973$*
Total	182	329.333	3.867		
ITS					
Western group					
Among populations	6	220.971	4.921	95.14	$F_{ST} = 0.951$*
Within populations	47	11.826	0.252	4.86	
Total	53	232.796	5.173		
Eastern group					
Among populations	13	80.004	0.673	95.78	$F_{ST} = 0.958$*
Within populations	115	3.415	0.030	4.22	
Total	128	83.419	0.703		
All populations					
Among populations	20	638.536	3.688	97.51	$F_{ST} = 0.975$*
Within populations	162	15.24	0.094	2.49	
Total	182	653.776	3.782		
Western group versus eastern group					
Among groups	1	337.561	4.196	68.48	$F_{CT} = 0.685$*
Among populations within groups	19	300.975	1.837	29.99	$F_{SC} = 0.951$*
Within populations	162	15.24	0.094	1.54	$F_{ST} = 0.985$*
Total	182	653.776	6.128		

*$P < 0.001$ (1000 permutations).

SS, sum of squares; VC, variance components; PV, percentage of variation; F_{CT}, correlation of haplotypes within groups relative to total; F_{SC}, correlation within populations relative to groups; F_{ST}, correlation within populations relative to total.

information Fig. S1, Table S3). Our estimations further suggested that the genetic divergence between the two intraspecific lineages of this species occurred between 1 and 4 Mya based on the fast and slow substitution rates of nuclear and chloroplast DNA recorded for the other angiosperms, corresponding to the middle-late Pleistocene (Zheng *et al*., 2002). Hierarchical AMOVA analyses also revealed that approximately 91% of cpDNA variation was distributed between these two lineages. Coalescent simulations of chlorotype variation agreed with the phylogenetic analyses in indicating subdivision of the species into two major lineages during the early Pleistocene rather than during the LGM.

This allopatric divergence was also recovered for another alpine plant species (Wang *et al*., 2009a) and another animal species (Tang *et al*., 2010). These divergences reflected a strong signature of highly

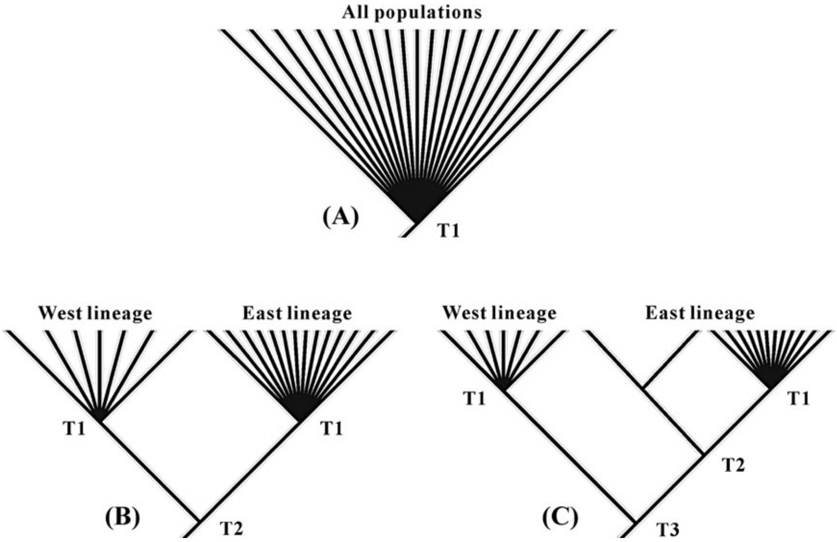

Figure 3. Three models used to test glacial refugia hypothesis. A, a single refuge hypothesis: all current populations were derived from a single refugium located either at the edge or in the interior of the Qinghai-Tibetan Plateau at the end of the Last Glacial Maximum (LGM) (T1 = 12 000 years BP). B, two refugia hypothesis: the two lineages split at the beginning of the LGM (T2 = 20 000 years BP), and all current populations were derived, respectively, from two refugia at the end of the LGM (T1 = 12 000 years BP). C, three refugia hypothesis: the west-east split occurred in early Pleistocene (T3 = 3.0 Mya) and the two sublineages of the east lineage diverged before the start of the LGM, thus three refugia were retained during the last glacial period (T2 ranged from 120 000 to 170 000 years BP; T1 = 20 000 years BP).

restricted gene flow probably posed by the geographical or climatic isolations (Avise, 2004). The estimated timescales for the deep divergences within these three species are highly congruent, between 1 and 1.5 Mya if the fast evolutionary rate were, respectively, adopted. This fits well with the orogenic processes and glaciation events recorded for the QTP during the middle–late Pleistocene (Zheng *et al.*, 2002), although caution is necessary because of the poor palaeogeographic data and fossil records. The latest extensive uplift of the QTP started from the Late Pliocene (3.4 Mya; Li *et al.*, 1995; Searle, 1995; Zhong & Ding, 1996; Shi *et al.*, 1998), and continued to approximately 1 Mya (Harrison *et al.*, 1992; Shi *et al.*, 1998). The largest glacier on the plateau reached the maximum 0.8–0.6 Mya and continued its range until 0.17 Mya after the penultimate glaciations (0.3–0.13 Mya) (Zheng & Rutter, 1998; Zhang, Li & Bian, 2000; Shi, 2002), when an ice sheet is thought to cover an area five to seven times larger than it does today, and glaciers may have been retained in the high mountains of the central QTP regions even during the interglacial warm stages (Shi & Ren, 1990; Wu *et al.*, 2001; Zheng *et al.*, 2002; Owen *et al.*, 2005). The rising of mountains, development of glaciers and/or extremely low temperature in the high mountains during these stages may have blocked gene flow between geographically isolated populations of *H. tibetana* and other species and therefore promoted their deep intraspecific divergence.

Two populations (4 and 5) collected from the southern Himalaya were geographically isolated from those occurring in the western QTP. It is interesting that two ITS genotypes (M and N) of these two populations together have seven mutations and five indels private to them, which is higher than the remaining intraspecific variations recorded for the other 19 populations (see Supporting information, Table S2). When these private variations were included, the two genotypes were still nested within the western ITS lineage but with an extremely long branch (Fig. 2B). Because these two populations shared the same chlorotypes with those occurring in the western QTP (Fig. 1A), it is likely that warm climates and the isolation by the high mountains accelerated the ITS evolution of these two populations since their long-distance dispersals to the southern Himalaya.

The factors underlying high intraspecific differentiation may also have contributed rapid species diversification in the QTP because the species occurring in this region should have experienced similar evolutionary history. Most species-rich genera of the QTP were found to have shown radiative species diversification because of the fast isolations in the multiple ‘islands’ within a short time (Liu *et al.*, 2006; Ran *et al.*, 2006; Wang *et al.*, 2009c; Zhang *et al.*, 2009). For example, in the genus *Gentiana*, the divergences among a few species were estimated to fall within the late Pliocene (Zhang *et al.*, 2009). The present study, as a case study of the intraspecific diversification provides further evidence that geographical isolations caused by both orogenic processes and climatic oscillations greatly promoted species diversification of alpine plants in the QTP and adjacent regions.

REGIONAL EXPANSION

Two deep lineages, respectively, in the eastern and western regions in *H. tibetana* suggest that the species was subdivided into at least two separate glacial refugia through the Pleistocene. Because all populations of the 'western' lineage are completely restricted to the high altitude (Fig. 1A), this suggests that this lineage should have survived glaciations at a high altitude through the Quaternary. However, except for allopatric divergences because of the long isolations, past climatic fluctuations may have promoted range contraction and expansion within a single region without continuous isolations (Avise, 2004). Such large-scale range expansions usually left two distinct genetic signatures: wide distribution of a single genotype and a dominant haplotype with multiple rare alleles (a star-phylogeny pattern) (Avise, 1987; Hewitt, 1996, 1999, 2000; Comes & Kadereit, 1998). In both eastern and western populations, we found wide fixtures of a singe chlorotype. For example, H1 occurs in 12 out of 14 sampled populations in the eastern region, and six populations were fixed for only this chlorotype (Fig. 1A). Similarly, H8 was fixed in seven out of the eight populations in the western region. This distribution of genetic diversity is in agreement with the leading-edge model: the recolonization resulted in loss of diversity in recolonized regions (Comes & Kadereit, 1998). Therefore, we suggest that this species underwent a recent range expansion, probably after the LGM, which resulted in the wide fixture of the two chlorotypes, respectively, in the western and eastern region.

In the network of chlorotypes, a star-phylogeny pattern was detected for the eastern lineage of *H. tibetana* (Fig. 1B), indicating that their origins also resulted from a rapid range expansion (Hudson, 1990). This expansion was also supported by strongly negative Fu's F_S-values, Tajima's D-values and unimodal mismatch distributions (see Supporting information, Fig. S2, Table S4). These findings suggested a possible earlier expansion of *H. tibetana* in the eastern region in addition to the recent expansions. On the basis of a mutation rate of 1.0–3.0×10^{-9} s s^{-1} y^{-1} and a generation time of 5 years for a congeneric species *H. rhamnoides* (Bartish *et al.*, 2006), the estimated times of range expansion are 26.4–79.1 Kya, before the LGM (12–20 Kya). The period of the earlier range expansion for *H. tibetana* is comparable to those inferred for the QTP endemic *Pedicularis longiflora* (Yang *et al.*, 2008) and snow finch (Qu *et al.*, 2005). This early expansion produced all recovered chlorotypes in the eastern range where populations 8 and 9 were mainly fixed for chlorotype H5, whereas populations 10 and 11 were fixed for H7. However, chlorotype H2 was only fixed in the population 12 and H4 was widely fixed for populations 14, 15 and 16. Because these chlorotypes probably originated earlier than the LGM, it is likely that at least four LGM refugia may have retained this species in the eastern region. The widespread distribution of H1 in the northeastern region probably originated from the recent expansion after the LGM as we noted earlier. In the western region, populations 3 and 5 were fixed for a different chlorotype (H9) from the remaining populations therefore two LGM refugia may have been retained. These analyses collectively suggested that multiple microrefugia may have been maintained for this alpine shrub. Coalescent simulations suggested that at least three independent refugia (one for western lineage and two for sublineages in the eastern region) (Fig. 3) had existed. The possible existence of multiple refugia for this species during the LGM is also consistent with phylogeographic studies of a few other alpine plant or animal species occurring in the QTP (Yang *et al.*, 2008; Wang *et al.*, 2009a, b; Opgenoorth *et al.*, 2010; Tang *et al.*, 2010).

CONCLUSIONS

Our phylogeographic study of *H. tibetana* suggested strong allopatric divergence between western and eastern populations in this species, which might be promoted by the orogenic processes and climate oscillations during the Quaternary. This deep intraspecific divergence provides further evidence for the hypothesis that these historical events had promoted species diversification in this region. In response to climate fluctuations, *H. tibetana* may have experienced at least two cycles of demographic expansions during the Last Interglacial Period and at the end of the LGM. The latest expansion probably resulted in genetic homogeneity in a few populations in the western or northeastern QTP. However, the chlorotypes originated from the early expansion distributed in the different populations suggested multiple refugia for this species during the LGM. In addition, ITS fragment has undergone the accelerated evolution in two populations of the southern Himalaya isolated by the high mountains. The peculiar evolutionary history of this shrub species occurring in the world's ruff highlights importance of the Quaternary oscillations and orogenic processes to the intraspecific divergence.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. (A) The geographic distribution of *Hippophae tibetana* internal transcribed spacer (ITS) types. Unique ITS types particular to each population are presented in yellow, with their corresponding codes given. (B) The network of ITS types as inferred from TCS.

Figure S2. Mismatch distribution analysis showing histogram of observed mismatch frequencies and best-fit curve of the sudden expansion model. A, west lineage. B, east lineage, C, total data set.

Table S1. Variable sites of aligned sequences of two cpDNA fragments (*trn*L-F and *trn*S-G) from which ten chlorotypes of *Hippophae tibetana* were identified. Sequences are numbered from the 5′- to the 3′- end in each region.

Table S2. Variable sites of aligned sequences of the internal transcribed spacer from which 17 internal transcribed spacer (ITS) types of *Hippophae tibetana* were identified. Sequences are numbered from the 5′- to the 3′- end.

Table S3. Frequencies of internal transcribed spacer (ITS) types, sample sizes (N) and estimates of gene diversity (H_E) and nucleotide diversity averaged across loci (π) within populations and groups. Unique ITS types particular to each population were given under u.

Table S4. Results of Tajima's D and Fu's F_S tests, and mismatch analyses for *Hippophae tibetana* lineages based on chlorotypes.