Biochem Genet (2008) 46:75–87
DOI 10.1007/s10528-007-9131-9

Strong Genetic Differentiation of *Primula sikkimensis* in the East Himalaya–Hengduan Mountains

Feng-Ying Wang · Xue-Jun Ge · Xun Gong · Chi-Ming Hu · Gang Hao

Received: 9 December 2006 / Accepted: 12 September 2007 / Published online: 23 December 2007

Abstract The East Himalaya-Hengduan Mountains region is the center of diversity of the genus *Primula*, and *P. sikkimensis* is one of the most common members of the genus in the region. In this study, the genetic diversity and structure of *P. sikkimensis* populations in China were assessed using inter-simple sequence repeat (ISSR) and chloroplast microsatellite markers. The 254 individuals analyzed represented 13 populations. High levels of genetic diversity were revealed by ISSR markers. At the species level, the expected heterozygosity and Shannon's index were 0.4032 and 0.5576, respectively. AMOVA analysis showed that 50.3% of the total genetic diversity was partitioned among populations. Three pairs of chloroplast microsatellite primers tested yielded a total of 12 size variants and 15 chloroplast haplotypes. Strong cpDNA genetic differentiation ($G_{ST} = 0.697$) and evidence for phylogeographic structure were detected ($N_{ST} = 0.788$, significantly higher than G_{ST}). Estimated rates of pollen-mediated gene flow are approximately 27% greater than estimated rates of seed-mediated gene flow in *P. sikkimensis*. Both seed and pollen dispersal, however, are limited, and gene flow among populations appears to be hindered by the patchiness of the species' habitats and their geographic isolation.

F.-Y. Wang · C.-M. Hu
South China Botanical Garden, Chinese Academy of Sciences, Guangzhou 510650, P.R. China

F.-Y. Wang
Shanghai Botanical Garden, Shanghai 200231, P.R. China

X.-J. Ge
School of Life Sciences, Sun Yat-Sen University, Guangzhou 510275, P.R. China

X. Gong
Kunming Institute of Botany, Chinese Academy of Sciences, Kunming 650204, P.R. China

G. Hao (✉)
College of Life Sciences, South China Agricultural University, Guangzhou 510642, P.R. China
e-mail: haogang07@gmail.com

These features may have played important roles in shaping the genetic structure of *P. sikkimensis*. A minimum-spanning tree of chloroplast DNA haplotypes was constructed, and possible glacial refugia of *P. sikkimensis* were identified.

Keywords Chloroplast microsatellite · East Himalaya–Hengduan Mountains · ISSR · *Primula sikkimensis*

Introduction

The eastern Himalayan region is one of the 25 hotspots of biodiversity (one of two in the northern hemisphere) (Myers et al. 2000). This region's great biodiversity is due to the relatively stable environment and diverse topography. The Hengduan Mountains, a major part of the region, lie at its eastern end, extending from the western and northern parts of the provinces Sichuan and Yunnan, respectively, to east Tibet. They comprise a series of spectacular north–south trending ridges along four major rivers of Asia: the Brahmaputra, Salween, Mekong, and Yangtze. Although covering only ca. 500,000 km^2, the Hengduan Mountains host about 8,000 angiosperm species (Wu 1988; Li and Li 1993; Wu and Wu 1996), of which about 3,000 (ca. 37%) are endemic or mainly restricted to this area (Li and Li 1993). Studies have shown that this region was a center of active speciation for *Rhododendron*, *Primula*, and *Gentiana* (Wu 1988; Takhtajan 1969; Rao 1994). Of the ca. 430 species of *Primula* (Primulaceae), over 75% are concentrated in the Himalayan mountain chain and western China (Richards 2002). The center of diversity for *Primula* is located in the core area of the Hengduan Mountains (Hu 1994; Hu and Kelso 1996).

The spatial genetic variations of taxa are results of their evolutionary history, so information on genetic structure is important for understanding speciation and adaptation (Syamsuardi and Okada 2002). Thus, the eastern Himalaya–Hengduan Mountains region has great potential for analyses of these processes, given its high species diversity, but to date very few species in the region have been analyzed with regard to genetic structure (Ge et al. 2005; Xia et al. 2005; Peng et al. 2005). Furthermore, although the genetic structure of many primrose species has been assessed (Jacquemyn et al. 2004; Reisch et al. 2005; Van Rossum and Triest 2003), none of the species studied to date is from this region. *Primula sikkimensis* Hook is one of the most common primroses in this region. It is widely distributed from western Sichuan and Yunnan to southeast Tibet, Bhutan, Nepal, Sikkim, Myanmar, and northeast India (Hu and Kelso 1996). Understanding the genetic structure of *P. sikkimensis* and the factors that have shaped it would help attempts to elucidate the diversification of this genus in the East Himalaya–Hengduan Mountains region.

The genetic structure of plant populations is determined in large part by the movement of genes via pollen and seed dispersal. The contributions of these two forms of gene dispersal on the genetic structure of natural plant populations can be estimated using a combination of nuclear DNA and chloroplast DNA (cpDNA) markers. Such combinations have provided abundant valuable information on plant

colonization and dispersal (Viard et al. 2001; Newton et al. 2002; Fontaine et al. 2004). Among the various types of molecular markers for detecting genetic diversity, nuclear inter-simple sequence repeats (ISSR) are powerful tools for investigating genetic variation within species (Gupta et al. 1994; Zietkiewicz et al. 1994; Wolfe and Liston 1998). These markers, however, have limitations due to their biparental mode of inheritance. In contrast, uniparentally inherited organellar DNA markers, such as chloroplast simple sequence repeats (cpSSRs), offer alternatives that are free of this limitation and thus are powerful complementary tools for analyzing genetic diversity among populations (Powell et al. 1995). Knowledge of the target species' genome sequence is not required for analyses of either of these types of markers. The ready availability of data and their convenience make them suitable for studying population genetics.

In the present study, both ISSR and cpSSR markers were used to (1) assess the genetic structure of *P. sikkimensis*, (2) estimate gene flow in populations of the species via seed and pollen, and (3) identify possible glacial refugia of this species.

Materials and Methods

Plant Materials and DNA Extraction

Primula sikkimensis is a diploid ($2n = 18$ or 22) perennial herb (2–7 cm high) and an obligate outcrosser with a distylous self-incompatibility system (Hu and Kelso 1996). This species grows in wet meadows, at margins of bogs and wet forests, and beside streams at altitudes of 3,200–4,400 m. The plants analyzed in this study (254 individuals) were sampled from 13 natural populations of *P. sikkimensis* from Sichuan, Tibet, and Yunnan in China (Table 1, Fig. 1). Leaves were collected in the field and were dried directly with silica gel. Total DNA was extracted following the CTAB procedure (Doyle 1991).

ISSR Analysis

In a preliminary study, 100 ISSR primers (Biotechnology Laboratory, University of British Columbia, primer set 9) were screened for PCR amplification. Ten of these primers (UBC nos. 808, 811, 814, 827, 834, 835, 840, 857, 888, 889) that gave clear, reproducible banding patterns were chosen for final analysis. Polymerase chain reactions (PCR) were carried out in 20 µl reaction mixture consisting of 20 ng template DNA, 2.5 mM $MgCl_2$, 0.1 mM dNTPs, 2% formamide, 0.2 µM primer, 1 U *Taq* polymerase, and double-distilled water in an MJ Research 96-well thermal cycler. The amplification products were separated electrophoretically in 1.5% agarose gels with 0.5× TBE buffer and visualized using ethidium bromide staining. The amplified DNA fragments were documented using LabWorks Version 3.0 image analysis software (UVP, Upland, CA).

Table 1 Genetic variation within *Primula sikkimensis* populations from 13 locations in China

Pop. No.	Location	Voucher (IBSC)	*N*	Long. E	Lat. N	H_o	PLP (%)	H_j
1	Luding, Sichuan	Hao 456	16	102°17′	29°51′	0.2700	84.6	0.250
2	Kangding, Sichuan	Hao 462	19	101°58′	30°02′	0.3564	81.3	0.306
3	Yajiang, Sichuan	Hao 464	19	101°02′	30°02′	0.3507	83.5	0.301
4	Wolong, Sichuan	Hao 481	20	103°09′	31°03′	0.3317	75.8	0.272
5	Zhongdian, Yunnan	Hao 497	20	100°03′	27°54′	0.3635	73.6	0.283
6	Zhongdian, Yunnan	Hao 499	20	99°47′	28°30′	0.2898	74.7	0.245
7	Xiangcheng, Sichuan	Hao 504	20	99°50′	28°57′	0.3281	76.9	0.274
8	Daocheng, Sichuan	Hao 508	20	100°22′	28°35′	0.3043	76.9	0.256
9	Deqin, Yunnan	Hao 509	20	98°57′	28°26′	0.3474	78.0	0.286
10	Linzhi, Tibet	Ge & Yuan 2003T-10	20	94°43′	29°55′	0.3399	72.5	0.281
11	Zuogong, Tibet	Ge & Yuan 2003T-6	20	97°53′	29°41′	0.4016	72.5	0.306
12	Mangkang, Tibet	Ge & Yuan 2003T-4	20	98°38′	29°41′	0.2615	72.5	0.219
13	Mangkang, Tibet	Ge & Yuan 2003T-4a	20	98°32′	29°28′	0.2293	38.5	0.199
Pop. level						0.3211	73.9	0.268
Species level						0.5576		0.4032

Note: *N*, Number of samples. H_o, Shannon's information index. PLP, Percentage of polymorphic loci. H_j: Expected heterozygosity

cpSSR Analysis

Chloroplast microsatellite primer sequences were obtained from Weising and Gardner (1999) and Chung et al. (2003). After screening 20 pairs of universal cpSSR primers, three pairs of primers yielding polymorphic products (ccmp4, ccmp5, ccSSR8) were tested across all the samples. PCR was performed in reaction mixtures with 1× PCR buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTP, 0.2 μM each primer, 1 U *Taq* polymerase, and 20 ng of template DNA. Optimal amplification conditions were: initial denaturation for 4 min at 94°C, followed by 35 cycles of 45 s at 94°C, 45 s at 50–62°C, and 45 s at 72°C, with a final extension for 7 min at 72°C. The PCR products were separated in 6% denaturing polyacrylamide gels, detected by silver staining and sized by comparison to a 50 base pair (bp) DNA ladder standard (Invitrogen).

Data Analysis

Only bands that could be unambiguously scored were used in subsequent analyses. ISSR profiles were determined for each individual based on the presence (1) or absence (0) of specific bands. The percentage of polymorphic loci and the expected heterozygosity (H_j) were calculated using PopGene 1.31 (Yeh et al. 1999) and AFLPSurv Version 1.0 (Vekemans et al. 2002), respectively. The detected genetic diversity was compared with that of other species by calculating Shannon's index,

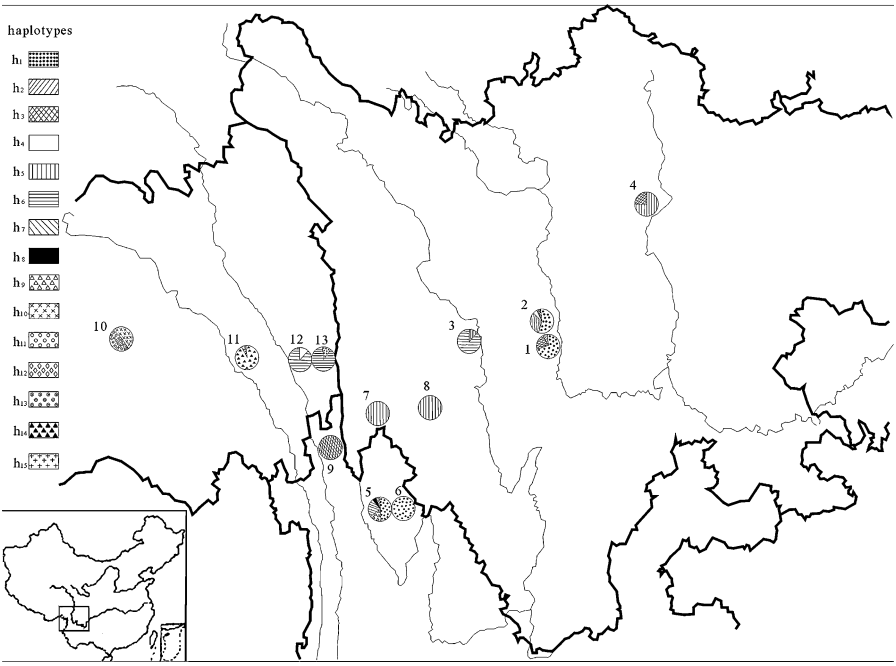

Fig. 1 Sampling locations and distribution of 15 chloroplast haplotypes detected in *P. sikkimensis*. Population numbers correspond to those designated in Table 1. Haplotypes 1 to 15 are described in Table 3

$H_o = -\sum p_i \log_2 p_i$ (Lewontin 1972), in which p_i is the frequency of a given ISSR fragment. Genetic diversity was calculated at two levels, the average diversity within populations and the total diversity.

To describe population structure and variability among populations, analysis of molecular variance (AMOVA) was performed using the Arlequin program (Schneider et al. 2000), where the variation was partitioned among populations and among individuals. To infer phylogenetic relationships among the different populations, a neighbor-joining tree was constructed using MEGA version 3.0 (Kumar et al. 2004) based on average pairwise genetic differences between the populations obtained from AMOVA. Relative branch support was evaluated by bootstrap analysis (Felsenstein 1985), using Phylip (Felsenstein 1993) with 1,000 replicates of searches. To test for a correlation between genetic distance and geographic distance among populations, a Mantel test was performed using Tools for Population Genetic Analysis (TFPGA; Miller 1997).

Three diversity parameters (average intrapopulation diversity, h_S; total diversity, h_T; and differentiation, G_{STcp} and N_{ST}) of the chloroplast genome were estimated by the methods of Pons and Petit using the program Permut (Pons and Petit 1996). N_{ST} was compared with G_{STcp} using U-statistics, which are approximated by Gaussian variables, taking into account the covariance between N_{ST} and G_{STcp}, and a one-sided test (Pons and Petit 1996). G_{STcp} is based solely on allelic frequencies; N_{ST}

also takes into account the differences among haplotypes. An N_{ST} higher than the G_{STcp} usually indicates the presence of phylogeographic structure (Pons and Petit 1996); that is, closely related haplotypes are more often found in the same area than less closely related haplotypes. AMOVA was applied to estimate variance components within and among populations.

To unravel the genealogies of the haplotypes in *P. sikkimensis*, a minimum-spanning tree of the cpSSR haplotypes was constructed with the aid of Arlequin (Schneider et al. 2000).

Results

Genetic Diversity Revealed by ISSR Markers

Of the 100 ISSR primers screened, 10 were selected for further analysis. A total of 91 unambiguous and reproducible electrophoretic bands were scored, all of which were polymorphic. The percentage of polymorphic bands ranged from 38.5% to 84.6%, with an average of 73.9%. The expected heterozygosity was 0.268 and 0.4032 at the population and species levels, respectively, and the corresponding Shannon's index was 0.3211 and 0.5576, respectively (Table 1).

Significant genetic differences among populations were detected by the AMOVA (Table 2). Of the total genetic diversity, 50.3% was attributable to between-population differentiation and the rest (49.7%) resided within populations. The neighbor-joining tree (Fig. 2) demonstrated that the 13 populations could be classified into two groups, one from two populations from Tibet, and the other from 11 populations from west Sichuan, northwest Yunnan, and east Tibet. The Mantel test with 1,000 permutations revealed that the genetic divergence of populations was significantly correlated with geographic distance ($r = 0.5906$, $P = 0.0109$); that is, an "isolation by distance" model was supported.

Genetic Diversity Revealed by Chloroplast Microsatellite Markers

Three pairs of conserved chloroplast microsatellite primers tested (ccmp4, ccmp5, ccSSR8) yielded a total of 12 size variants in 15 different haplotype combinations (Table 3). High levels of cpDNA genetic diversity were detected in *P. sikkimensis*,

Table 2 Analysis of molecular variance of ISSR and chloroplast microsatellite variation in *Primula sikkimensis* populations

	Source of variation	d.f.	Sum of squares	Variance component	% Variation	*P*-value
ISSR	Among population	12	2127.942	8.641	50.3	<0.0010
	Within population	241	2059.125	8.544	49.7	<0.0010
cpSSR	Among population	12	305.576	1.2863	79.16	<0.0010
	Within population	241	81.613	0.3386	20.84	<0.0010

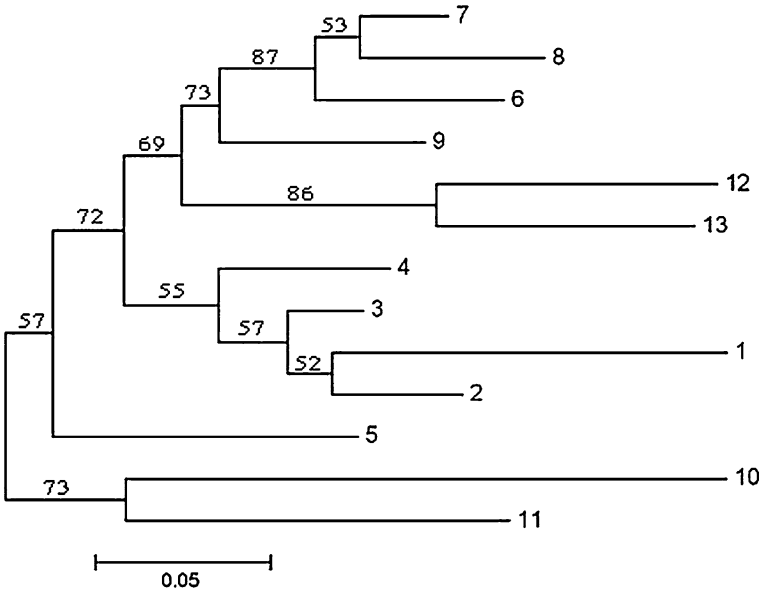

Fig. 2 Unrooted neighbor-joining tree of 13 populations of *P. sikkimensis* based on ISSR markers. Numbers above the branches are bootstrap values supporting the corresponding branch (>50%). Population numbers as in Table 1

Table 3 Haplotype frequency and composition of 13 populations of *P. sikkimensis*

Haplotype	Population													Frequency
	1	2	3	4	5	6	7	8	9	10	11	12	13	
h1	12	11			10	20							2	0.22530
h2	1				1									0.00865
h3	3	1		5					20					0.11463
h4		1										2	18	0.08097
h5		6	3	15			20	20						0.24798
h6			16									18		0.13401
h7					5									0.01923
h8					1									0.00385
h9					3									0.01154
h10										11				0.04231
h11										3				0.01154
h12										1				0.00385
h13										5				0.01923
h14											19			0.07308
h15											1			0.00385
Total	16	19	19	20	20	20	20	20	20	20	20	20	20	1.00000

with $h_T = 0.891$. Most of the genetic diversity was partitioned between populations. The G_{STcp} (0.697) and N_{ST} (0.788) values, based on unordered and ordered alleles, respectively, indicate significant geographic structuring of cpDNA markers. The finding that N_{ST} was greater than G_{ST} ($U = 3.77$, $P < 0.05$) provides a further indication that the cpDNA data contain phylogeographic information.

The distribution of the 15 haplotypes across the sampled range show a complex pattern (Fig. 1). Overall, the frequency of haplotypes 1 and 5 was high (22.5 and 24.8%, respectively), and the frequency of all other haplotypes was less than 15%. Four populations were composed of single haplotypes, five populations had two haplotypes, and four populations possessed multiple polymorphic haplotypes. The genetic compositions of populations 7 and 8 were identical. In the minimum-spanning tree (Fig. 3), haplotype 1 occupies a central position, and all the other haplotypes are separated from it by two mutational differences, except haplotype 8, which is separated by four mutational differences.

Discussion

Genetic Diversity in *P. sikkimensis*

High levels of genetic variation have been found in several primrose species, e.g., *P. ovalifolia* (Shannon's index, H_{SP}: 0.313) (Nan et al. 2003a), *P. obconica* (H_{SP}: 0.435) (Nan et al. 2003b), *P. elatior* (H_T: 0.2936) (Jacquemyn et al. 2004), *P. interjacens* (H_{SP}: 0.4618) (Xue et al. 2004), and *P. farinosa* (H_{SP}: 0.33) (Reisch et al. 2005), but the level of genetic variation we detected in *P. sikkimensis* at the species level was still higher (percentage of polymorphic loci: 100%; Shannon's index H_{SP}: 0.5576; expected heterozygosity H_j: 0.4032).

A wide range of life history and demographic factors, including the geographic distribution, breeding system, and size of population, are important determinants of

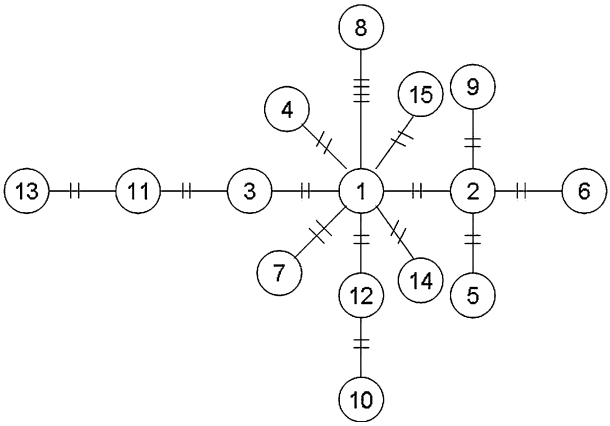

Fig. 3 Minimum-spanning tree among the 15 haplotypes. Connection lengths between haplotypes are represented by the number of marks

the genetic diversity within and among populations of plant species (Hamrick and Godt 1989). Plant species with large geographic ranges tend to have higher genetic diversity than congenerics with more limited distributions (Hamrick and Godt 1989; Hamrick et al. 1992). Among primrose species, the genetic variation of the widespread *P. veris* has been found to be significantly higher than that of its rare relative *P. vulgaris* (Van Rossum et al. 2004). As one of the most common primrose species, the regional range of *P. sikkimensis* in the East Himalaya–Hengduan Mountains may contribute substantially to the high levels of genetic diversity detected in its populations.

The breeding system is another major influence on genetic diversity (Hamrick and Godt 1996). It has been reported that 91% of all *Primula* species are heteromorphic (Richards 2002). The distyly observed in *P. sikkimensis* is a visible manifestation of a self-incompatible reproductive system. Outcrossing species commonly have higher levels of genetic diversity than selfing plants. For example, high genetic diversity has been found in *P. interjacens*, a species with distylous flowers with a narrow distribution in Yunnan (Xue et al. 2004). In contrast, low genetic diversity has been found in *P. scotica*, which lacks heterostylous flowers and has an inbreeding system (Glover and Abbott 1995). Genetic differentiation is expected to be particularly weak in populations of long-lived, perennial, outcrossing species (Loveless and Hamrick 1984), and accordingly reported levels of differentiation are very low for the congenerics *P. elatior* (G_{ST}: 0.04) (Jacquemyn et al. 2004), *P. veris* (G_{ST}: 0.039) (Antrobus and Lack 1993), *P. vulgaris* (F_{ST}: 0.165) (Van Rossum and Triest 2003), and *P. farinosa* (Φ_{ST}: 0.2059) (Reisch et al. 2005). However, in contrast with the expectation of weak genetic differentiation, significantly higher population differentiation was found in *P. sikkimensis*. Of the total molecular variance, 50.3% was attributable to among-population diversity (Table 2), much more than the mean value for outcrossing species reported by Nybom (2004) (Φ_{ST}: 0.27). Strong genetic differentiation has also been found in some other primrose species, such as *P. ovalifolia* (G_{ST}: 0.574) (Nan et al. 2003a), *P. obconica* (G_{ST}: 0.519) (Nan et al. 2003b), and *P. interjacens* (G_{ST}: 0.261) (Xue et al. 2004).

In addition to the breeding system, other factors, such as isolation by gene flow among populations, drift, and inbreeding, may increase genetic differentiation and shape genetic structure (in a local study of *P. elatior*; Jacquemyn et al. 2004). In alpine landscapes, most plant populations are spatially isolated because of the extreme patchiness of their habitats and strong natural fragmentation. Obvious features of the East Himalaya–Hengduan Mountains region are its complex topography and strong environmental heterogeneity. Most parts of the region have series of parallel mountain ranges dissected by deep river valleys that run from north to south and present physical barriers to gene flow. *P. sikkimensis* generally grows at the edge of bogs or forests at altitudes of 3,200–4,400 m. The patchy habitats where *P. sikkimensis* grows on different mountains are isolated by the deep valleys between them or alpine tundra as "terrestrial habitat islands." The varied topography and scattered distribution of the species might strengthen population differentiation by limiting pollen and seed dispersal and, at least in part, biparental inbreeding, thus tending to promote population differentiation. High genetic

differentiation has often been found in other alpine plant species from this region e.g., *Megacodon stylophorus* (G_{ST}: 0.807) (Ge et al. 2005), *Rhodiola alsis* (G_{ST}: 0.703) (Xia et al. 2005), and *Populus cathayanan* (G_{ST}: 0.477) (Lu et al. 2006). The similarity of the differentiation patterns detected in *P. sikkimensis* and other alpine species studied in the East Himalaya–Hengduan Mountains region may be related to the patchy habitats and strong isolation associated with its general geographic features. The weak positive correlation ($r = 0.5906$, $P = 0.0109$) found between genetic distance and geographic distance is consistent with an "isolation by distance" model and suggests that the genetic exchangeability between geographically distant populations is constrained to some extent.

The chloroplast genome is maternally inherited and dispersed solely by seeds in angiosperms (Birky et al. 1983). It has been shown both theoretically and empirically that the level of genetic differentiation among populations is likely to be higher for maternally inherited cpDNA markers than for biparentally inherited nuclear genes because of differences in seed and pollen migration parameters (Ennos 1994; Raspé et al. 2000). In the present study, most chloroplast DNA diversity in *P. sikkimensis* was found to reside among populations. Its substantial differentiation, with a G_{ST} value of 0.697, is close to the mean value (G_{ST}: 0.73) reported for maternally inherited genomes in angiosperm tree species (Petit 1999). Nevertheless, the expected large difference between ISSR and cpSSR markers was not detected; both types of markers revealed high levels of genetic differentiation (0.5028 vs. 0.697). The ratio of seed to pollen flow rates can be estimated using a modified form of an equation published by Ennos (1994): pollen flow/seed flow $= [(1/\text{Gstb} - 1) - 2(1/\text{Gstc} - 1)]/(1/\text{Gstc} - 1)$, where Gstb is the level of population subdivision based on biparentally (nuclear) inherited genomes, and Gstc is the value of subdivision for cytoplastic markers. The estimates here suggest that the rate of pollen-mediated gene flow is 27% greater than the rate of seed-mediated gene flow. According to our field observations, *P. sikkimensis* was visited by insects, which fly limited distances, implying that the range of its pollen dispersal by insects is almost as restricted as that of its seed dispersal.

Although seed dispersal in *P. sikkimensis* has not been explicitly studied, the morphology of its seeds suggests that they are scattered from ripened capsules within the vicinity of the parent plants, rather than being dispersed over longer distances by a more complex mechanism. Pollen and seed dispersal have both been shown to be spatially limited in the congenerics *P. vulgaris* (Cahalan and Gliddon 1985) and *P. sieboldii*, in which the pollen is primarily dispersed by bumblebees over mean distances of 5.4–7.2 m (Ishihama et al. 2006). The observed diversity pattern suggests that the limited pollen- and seed-mediated gene flows among the populations contribute to the high level of population differentiation detected in *P. sikkimensis*.

Possible Glacial Refugia

In the present study, mutational differences between the haplotypes were used to generate a minimum-spanning tree, showing the relationships between them. This

tree (Fig. 3) was not fully resolved, partly because insufficient variation was detected by the cpSSR analyses. They did, however, detect sufficient variation for a broad-scale phylogeographic study of *P. sikkimensis*, and the haplotype network was informative for interpreting the geographic distribution of the haplotypes. According to the minimum-spanning tree, there were two mutation steps between most haplotypes, and a maximum of four between haplotypes 8 and 1. Haplotype 1 is located at the interior position with high frequency of the network, implying that it is an ancestral haplotype.

During the long glacial episodes of the Quaternary, many taxa were restricted to one of the few regional refugia (Hewitt 1996). Then, as the climate warmed and the ice receded, these plants expanded their ranges northward or retreated upslope. It is generally considered in the biogeographic research that the Hengduan Mountains are an important refugium for species surviving the Pleistocene glaciations due to its north-south trending ridges and rivers, and complex topography (Zhang et al. 1997). Chloroplast DNA analysis is extensively used to infer postglacial migration routes of plants in Europe (Taberlet et al. 1998). In this study, the geographic distribution of the cpSSR haplotypes may have arisen through a series of mutations during the course of the quick expansion after the last glaciation. In the Himalaya–Hengduan Mountains region, four glacial and interglacial episodes occurred during the Quaternary period (Sun 2002). The haplotype richness was relatively high in Kangding, Luding (west Sichuan), and Zhongdian (northwest Yunnan), suggesting that these areas may have provided refugia for the species during the Pleistocene, since populations in such areas generally harbor higher haplotype diversity than populations in areas that were subsequently colonized and the interior positions of their haplotypes in the network. These areas are adjacent to low-altitude valleys, which may have provided refugia during the Pleistocene. We cannot exclude the possibility, however, that additional refugia existed elsewhere in other regions, since the sampling was not exhaustive and did not cover the extremes of the species' range.

Acknowledgments This project was financially supported by the National Natural Science Foundation of China (Nos. 30470125, 40671066) and the Natural Science Foundation of Guangdong Province (No. 31255).

References

Antrobus S, Lack AJ (1993) Genetics of colonizing and established populations of *Primula veris*. Heredity 71:252–258

Birky CW Jr, Maruyama T, Fuerst P (1983) An approach to population and evolutionary genetic theory for genes in mitochondria and chloroplasts, and some results. Genetics 103:513–527

Cahalan CM, Gliddon C (1985) Genetic neighbourhood sizes in *Primula vulgaris*. Heredity 54:65–70

Chung SM, Decker-Walters DS, Staub JE (2003) Genetic relationships within the Cucurbitaceae as assessed by consensus chloroplast simple sequence repeats (ccSSR) marker and sequence analyses. Can J Bot 81:814–832

Doyle J (1991) DNA protocols for plants-CTAB total DNA isolation. In: Hewitt GM, Johnston A (eds) Molecular techniques in taxonomy. Springer, Berlin, pp 283–293

Ennos RA (1994) Estimating the relative rates of pollen and seed migration among plants populations. Heredity 72:250–259

Felsenstein J (1985) Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39:783–791
Felsenstein J (1993) Phylip, Phylogenetic inference package, version 3.5.7. A computer program distributed by the author, http://www.evolution.genetics.washington.edu/phylip.html. Department of genetics, University of Washington, Seattle
Fontaine C, Lovett PN, Sanou H, Maley J (2004) Genetic diversity of the shea tree (*Vitellaria paradoxa* C.F. Gaertn), detected by RAPD and chloroplast microsatellite markers. Heredity 93:639–648
Ge XJ, Zhang LB, Yuan YM, Hao G, Chiang TY (2005) Strong genetic differentiation of the East-Himalayan Megacodon stylophorus (Gentianaceae) detected by Inter-Simple Sequence Repeats (ISSR). Biodivers Conserv 14:849–861
Glover BJ, Abbott RJ (1995) Low genetic diversity in the Scotish endemic Primula scotica Hook. New Phytol 129:147–153
Gupta M, Chyi YS, Romero-Severson J, Owen JL (1994) Amplification of DNA markers from evolutionarily diverse genomes using single primers of simple-sequence repeats. Theor Appl Genet 89:998–1006
Hamrick JL, Godt MJW (1989) Allozyme diversity in plant species. In: Brown AHD, Clegg MT, Kahler AL, Weir BS (eds) Plant population genetics, breeding and genetic resources. Sinauer Associates, Sunderland, pp 43–63
Hamrick JL, Godt MJW (1996) Effects of life history traits on genetic diversity in plant species. Philos Trans R Soc Lond B 351:1291–1298
Hamrick JL, Godt MJW, Sherman-Broyles SL (1992) Factors influencing levels of genetic diversity in woody plant species. New For 6:95–124
Hewitt GM (1996) Some genetic consequences of ice ages, and their role in divergence and speciation. Biol J Linn Soc 58:247–276
Hu CM (1994) On the geographical distribution of the Primulaceae. J Trop Subtrop Bot 2:1–14
Hu CM, Kelso S (1996) Primulaceae. In: Wu CY, Raven PH (eds) *Flora of China*, vol 15. Science Press, Beijing, China, and Missouri Botanical Garden, St. Louis, USA, pp 39–189
Ishihama F, Ueno S, Tsumura Y, Washitani I (2006) Effects of density and floral morph on pollen flow and seed reproduction of an endangered heterostylous herb, Primula sieboldii. J Ecol 94:846–855
Jacquemyn H, Honnay O, Galbusera P, Roldán-Ruiz I (2004) Genetic structure of the forest herb Primula elatior in a changing landscape. Mol Ecol 13:211–219
Kumar S, Tamura K, Nei M (2004) MEGA 3: Integrated software for molecular evolutionary genetics analysis and sequence alignment. Brief Bioinform 5:150–163
Lewontin RC (1972) The apportionment of human diversity. Evol Biol 6:381–398
Li XW, Li J (1993) A preliminary floristic study on the seed plants from the region of Hengduan Mountain. Acta Bot Yunnanica 15:217–231
Loveless MD, Hamrick JL (1984) Ecological determinants of genetic structure in plant populations. Ann Rev Ecol Syst 15:65–95
Lu Z, Wang YH, Peng YH (2006) Genetic diversity of *Populus cathayana* populations in southwestern China revealed by ISSR markers. Plant Sci 170:407–412
Miller MP (1997) Tools for population genetic analysis, ver. 1.3. Department of Biological Sciences, Northern Arizona University, Flagstaff, Arizona
Myers N, Mittermeier RA, Mittermeier CG, da Fonseca GAB, Kent J (2000) Biodiversity hotspots for conservation priorities. Nature 403:853–858
Nan P, Peng SL, Shi SH, Ren H, Yang J, Zhong Y (2003a) Interpopulation congruence in Chinese *Primula ovalifolia* revealed by chemical and molecular markers using essential oils and ISSRs. Z Naturforsch 58:57–61
Nan P, Shi SH, Peng SL, Tian CJ, Zhong Y (2003b) Genetic diversity of Primula obconica from central and southwest China as revealed by ISSR markers. Ann Bot 91:329–333
Newton AC, Allnutt TR, Dvorak WS, Del Castillo RF, Ennos RA (2002) Patterns of genetic variation in *Pinus chiapensis*, a threatened Mexican pine, detected by RAPD and mitochondrial DNA RFLP markers. Heredity 89:191–198
Nybom H (2004) Comparison of different nuclear DNA markers for estimating intraspecific genetic diversity in plants. Mol Ecol 13:1143–1155
Peng YH, Lu ZX, Chen K, Luukkanen O, Korpelainen H, Li CY (2005) Population genetic survey of *Populus cathayana* originating from Southeastern Qinghai-Tibetan plateau of China based on SSR markers. Silvae Genet 54:116–122

Petit RJ (1999) Diversité Génétique et Histoire des Populations d'Arbres Forestiers. Dossier D'habilitation à Diriger Des Recherches. Université de Paris-Sud, Université Formation de Recherche Scientifique d'Orsay, Paris

Pons O, Petit RJ (1996) Measuring and testing genetic differentiation with ordered versus unordered alleles. Genetics 144:1237–1245

Powell W, Morgante M, McDevitt R, Vendramin GG, Rafalski JA (1995) Polymorphic simple sequence repeat regions in chloroplast genomes: applications to the population genetics of pines. Proc Natl Acad Sci USA 92:7759–7763

Rao RR (1994) Biodiversity in India: Floristic Aspects. Bisen Singh Mahendra Pal Singh, Dehra Dun

Raspé O, Saumitou-Laprade P, Cuguen J, Jacquemart A-L (2000) Chloroplast DNA haplotype variation and population differentiation in *Sorbus aucuparia* L. (Rosaceae: Maloideae). Mol Ecol 9:1113–1122

Reisch C, Anke A, Röhl M (2005) Molecular variation within and between ten populations of *Primula farinosa* (Primulaceae) along an altitudinal gradient in the northern Alps. Basic Appl Ecol 6:35–45

Richards J (2002). *Primula*, New edition. BT Batford, London

Schneider S, Roessli D, Excoffier L (2000) Arlequin, Version 2.000: a software for population genetics data analysis. Genetics and Biometry Laboratory, University of Geneva, Switzerland

Sun H (2002) Evolution of Arctic-tertiary flora in Himalayan–Hengduan Mountains. Acta Bot Yunnanica 24:671–688

Syamsuardi, Okada H (2002) Genetic diversity and genetic structure of populations of *Ranunculus japonicus* Thunb. (Ranunculaceae). Plant Species Biol 17:59–69

Taberlet P, Fumagalli L, Wust-Saucy AG, Cosson JF (1998) Comparative phylogeography and postglacial colonization routes in Europe. Mol Ecol 7:453–464

Takhtajan A (1969) Flowering plants, origin and dispersal. Oliver and Boyd, Edinburgh

Van Rossum F, De Sousa SC, Triest L (2004) Genetic consequences of habitat fragmentation in an agricultural landscape on the common *Primula veris*, and comparison with its rare congener, *P. vulgaris*. Conserv Genet 5:231–245

Van Rossum F, Triest L (2003) Spatial genetic structure and reproductive success in fragmented and continuous populations of *Primula vulgaris*. Folia Geobot 38:239–254

Vekemans X, Beauwens T, Lemaire M, Roldan-Ruiz I (2002). Data from amplified fragment length polymorphism (AFLP) markers show indication of size homoplasy and of a relationship between degree of homoplasy and fragment size. Mol Ecol 11:139–151

Viard F, El-Kassably YA, Ritland K (2001) Diversity and genetic structure in populations of *Pseudotsuga menziesii* (Pinaceae) at chloroplast microsatellite loci. Genome 44:336–344

Weising K, Gardner RC (1999) A set of conserved PCR primers for the analysis of simple sequence repeat polymorphisms in chloroplast genomes of dicotyledonous angiosperms. Genome 42:9–19

Wolfe AD, Liston A (1998) Contributions of PCR-based methods to plant systematics and evolutionary biology. In: Soltis DE, Soltis PS, Doyle JJ (eds) Plant molecular systematics II. Kluwer, Boston, pp 43–86

Wu ZY (1988) Hengduan mountain flora and her significance. J Jpn Bot 63:297–311

Wu ZY, Wu SG (1996) A proposal for new floristic kingdom (realm). In: Zhang AL, Wu SG (eds) Floristic characteristics and diversity of east Asian plants. Springer-Verlag, Hongkong, China Higher Education Press, Beijing, pp 3–42

Xia T, Chen SL, Chen SY, Ge XJ (2005) Genetic variation within and among populations of *Rhodiola alsia* (Crassulaceae) native to the Tibetan plateau as detected by ISSR makers. Biochem Genet 43:87–101

Xue DW, Ge XJ, Hao G, Zhang CQ (2004) High genetic diversity in a rare, narrowly endemic primrose species: *Primula interjacens* (Primulaceae) by ISSR analysis. Acta Bot Sin 46:1163–1169

Yeh FC, Yang R, Boyle T (1999) PopGene. Microsoft Windows-based freeware for population genetic analysis. Release 1.31. University of Alberta, Edmonton

Zhang RZ, Zheng D, Yang QY, Liu YH (1997) Physical geography of Hengduan mountains. Science Press, Beijing

Zietkiewicz E, Rafalski A, Labuda D (1994) Genome fingerprinting by simple sequence repeat (SSR)-anchored polymerase chain reaction amplification. Genomics 20:176–183