CSIRO PUBLISHING
www.publish.csiro.au/journals/ajb Australian Journal of Botany, 2011, 59, 450–459

Genetic variation and phylogeography of *Psammosilene tunicoides* (Caryophyllaceae), a narrowly distributed and endemic species in south-western China

Qing-Ying Zhang[A,B], *Yu-Juan Zhao*[A] *and Xun Gong*[A,C]

[A]Key Laboratory of Biodiversity and Biogeography, Kunming Institute of Botany, Chinese Academy of Sciences, Kunming 650204, China.
[B]Industrial Crops Research Institute, Yunnan Academy of Agricultural Sciences, Kunming 650205, China.
[C]Corresponding author. Email: gongxun@mail.kib.ac.cn

Abstract. *Psammosilene tunicoides* is a narrowly distributed and endemic species in south-western China. An investigation of sequence variation at two chloroplast DNA (cpDNA) regions (*rp*L16, *trn*Q-5′*rps*16) and one nuclear DNA (nDNA) locus (*GPA*1) were carried out to survey the population structure and population history of the species. Among the 22 populations across its geographical range, nine chloroplast haplotypes and 17 nuclear alleles were identified. Both cpDNA and nDNA consistently revealed high levels of population differentiation ($G_{ST} = 0.604$ and 0.540, respectively), suggesting a distinct phylogeographic structure ($N_{ST} > G_{ST}$, $P < 0.01$). This high genetic differentiation might be a combined effect of breeding system, limited pollen and seed dispersal and geographic isolation of populations. The level of haplotype diversities (cpDNA, $h_T = 0.575$; nDNA, $h_T = 0.724$) were high, but the nucleotide diversities (cpDNA, $\pi = 0.00099$; nDNA, $\pi = 0.00105$) were low. These results together with the star-like phylogenetic pattern and neutrality tests indicate that *P. tunicoides* has experienced a population expansion event in its evolution. Limited genetic exchange after population expansion was supported by the pronounced genetic differentiation among populations as well as evidence for 'isolation-by-distance' revealed by cpDNA. Due to high population subdivision and complex landscape, as many populations as possible should be considered for genetic conservation.

Introduction

The south-east part of the Qinghai-Tibet Plateau and its adjacent areas, namely the mountains of south-western China is one of the world's biodiversity hotspots. It contains the richest temperate flora in the world with a high number of endemic and endangered species (Wilson 1992; Myers *et al*. 2000; http://www.biodiversityhotspots.org/xp/hotspots, accessed 8 July 2011). According to the prevailing hypothesis, many species in this region are ancient and have been preserved in many natural refugia formed by the complicated topography and climate (Wu 1998). On the other hand, following the collision of the Indian subcontinent with the Eurasian plate starting ~40 million years ago, frequent speciation events have occurred due to the emergence of new and diversified habitats created during the lifting of the Qinghai-Tibet Plateau (Wu 1998; Liu *et al*. 2006). It is estimated that over 3500 endemic plants (29.2% of 12 000 vascular plants in this region) emerged as a result of this collision (Myers *et al*. 2000). Endemic elements are crucial for floristic and phytogeographical analyses (Zhou and Momohara 2005). For effective conservation of endangered and endemic species, it is important to understand the genetic variation and phylogeography of these plants. However, there is relatively limited information on the genetic diversity and population structure of endemic plants from this region.

Psammosilene tunicoides W. C. Wu et C. Y. Wu (Caryophyllaceae) is a monotypic herbal plant endemic to south-western China. This species was described ~500 years ago and is valued highly in traditional Chinese medicine for its analgesic, hemostyptic, anti-inflammatory, and immune-adjustment properties. Recent research has shown that cyclic octapeptide-psammosilenin A, one of the natural compounds in *P. tunicoides* root, has excellent pharmacological activity and could be developed as a novel cytotoxic, anthelmintic, and antibacterial drug (Ding *et al*. 2000; Dahiya 2008). However, populations of this species have been declining rapidly due to extensive exploitations in recent years such that it is now classified as a rare and endangered species in the Chinese Plant Red Book (Fu and Jin 1992). This perennial herb is characterised by prostrate and dichotomously branched stems of 20–35 cm long, with a long conical, brown yellow, fleshy root. Its small purple flowers are in compound thyrsiferous inflorescences, and its narrowly obovoid seed is located in the clavate capsule (Lu 2001). It flowers from June to September, and is pollinated by bees and flies. The fruit starts to mature from July until October, and are nearly unsplit at maturity and the seeds germinate without seed dormancy after falling to the ground (Lu 2001), thus have a very limited dispersal distance. It takes 2–3 years for seedlings to become sexually mature.

10.1071/BT11024 0067-1924/11/050450

P. tunicoides preferentially inhabits rocky mountain slopes and calcareous rock crevices, but also occasionally occurs in dry pastures, pine forests, with altitudes ranging from ~2000 to 3500 m. Its distribution in south-western China is not continuous but the patchy populations are centred around the north-western Yunnan Province and south-western Sichuan Province. There are also several subcentres of distribution in central and north-east Yunnan. However, its distribution in south-eastern Tibet and south-eastern Yunnan is extremely sparse. Due to its special distribution pattern, *P. tunicoides* is an ideal model to study how historical events affected population history of this species and others in this biodiversity hotspot. Moreover, reversing the increasingly endangered situation of *P. tunicoides* requires a clear understanding of the genetic variation within and among its populations.

Molecular markers and phylogeographic methods provide powerful tools for studying genetic variation and diversity, population structure, and evolutionary processes of species (Avise 2000, 2004). In plants, the chloroplast genome is a single, haploid, non-recombining unit of inheritance, and chloroplast DNA (cpDNA) is typically transmitted maternally only through seeds in angiosperms and within most species, genetic variations in cpDNA are typically low at the intra-specific level. In contrast, the nuclear genomes typically contain multiple chromosomes, are diploid or higher ploidy and have features such as recombination and heterozygosity, biparental inheritance by seed and pollen, and frequent intra-specific genetic variation in most single-copy or low-copy genes (Schaal *et al*. 1998). Therefore, the analyses of gene loci from both the chloroplast and the nuclear genome would provide a comprehensive view of the genetic structure of plant populations and better reveal their phylogeographic patterns (Schaal *et al*. 1998; Petit *et al*. 2005; Ikeda *et al*. 2008). Here, we selected two cpDNA regions and one single-copy nDNA gene as molecular markers. The main objectives of this study were: (1) to estimate the amount of genetic variation at the cpDNA and nuclear DNA (nDNA) loci within and among populations of *P. tunicoides*; (2) to determine the spatial distribution patterns of the alleles and genotypes and provide information about the historical processes; and (3) to make recommendations for future conservation practices for the species.

Materials and methods

Population sampling

The leaf samples used in this study were collected in the Yunnan, Sichuan, and Guizhou Provinces in south-western China. A total of 117 individuals of *P. tunicoides* were collected from 22 populations, covering almost all areas of its distribution ranges. Of these 22 populations, one was from the north-western Guizhou Province, four populations were from the south-western Sichuan Province that borders south-eastern Tibet, and the remaining 16 were from various areas in the Yunnan Province. In Linzhi and Chayu of south-eastern Tibet, localities where this species is described in the literature and in the KUN herbaria (KUN-0512912, KUN-0512918, KUN-0512949 and so on) were visited, but we failed to obtain any samples despite repeated field trips. The sample size, population code, geographical coordinates and altitude for each population are presented in Table 1 and Fig. 1, for all

Table 1. Populations of *Psammosilene tunicoides* analysed in this study and geographical information

Population	Population code	Sample sizes (cpDNA)	Sample sizes (nDNA)	Latitude (N)	Longitude (E)	Altitude (m)
Sichuan, Jiulong	JL	5	5	29°09′	101°25′	2800
Sichuan, Reda	RD	5	6	29°04′	99°41′	3150
Sichuan, Sangpi	SP	5	5	28°56′	99°48′	3300
Sichuan, Muli	ML	5	5	28°22′	100°37′	2810
Sichuan,Yanyuan	YY	5	5	27°24′	101°31′	2700
Yunnan, Geza	GZ	6	6	28°02′	99°46′	3160
Yunnan, Napahai	NP	5	5	27°55′	99°37′	3200
Yunnan, Wufengshan	WF	5	6	27°49′	99°43′	3300
Yunnan, Luguhu	LG	5	5	27°37′	100°43′	2680
Yunnan, ninglang	NL	5	5	27°22′	100°51′	2550
Yunnan, yushuizhai	YS	5	5	26°59′	100°15′	2570
Yunnan, Lijiang	LJ	5	5	26°43′	100°12′	2400
Yunnan, Maershan	HM	5	5	26°28′	100°07′	2770
Yunnan, Heqing	HQ	5	5	26°15′	100°11′	2250
Yunnan, Baoshan	BS	5	5	25°08′	99°11′	2050
Yunnan, Fumin	FM	5	5	25°20′	102°25′	2240
Yunnan, Kunming	KM	5	6	25°09′	102°43′	2170
Yunnan, Anning	AN	5	5	24°59′	102°27′	2200
Yunnan, Gejiu	GJ	5	5	23°32′	103°11′	2200
Yunnan, Huize	HZ	6	6	26°25′	103°15′	2700
Yunnan, Zhanyi	ZY	6	5	25°54′	104°02′	2160
Guizhou, Weining	WN	6	4	26°53′	104°17′	2300
Total/range	22	114	114	23°32′–29°09′	99°11′–104°17′	2050–3300

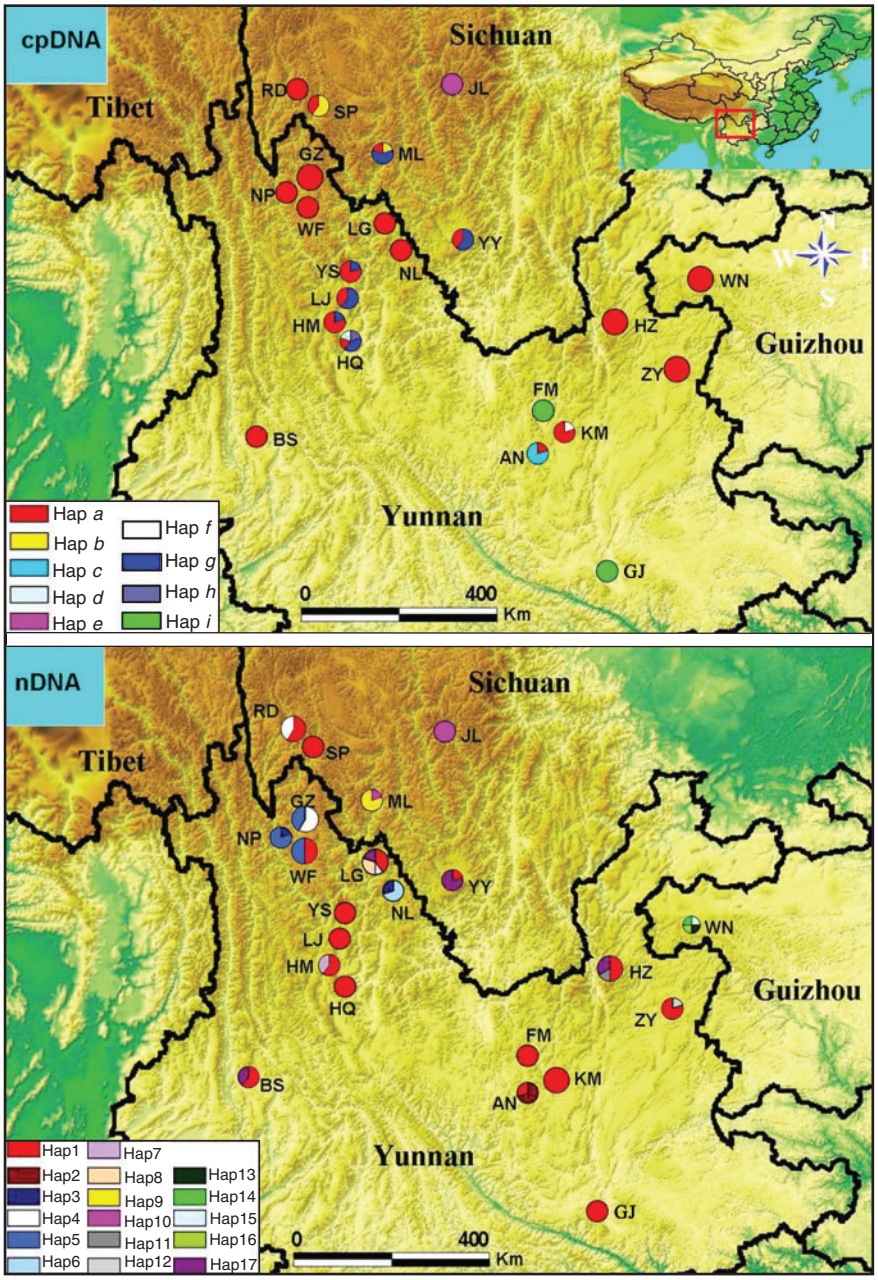

Fig. 1. Geographical distributions of chloroplast DNA and nuclear DNA haplotype frequency among and within populations of *Psammosilene tunicoides* in south-western China. Population codes are the same as Table 1. The pie sizes of sampled populations are proportional to their sample sizes.

22 populations. Our sampled regions spanned an area ~700 km from south to north and 600 km from east to west with an altitude span of almost 1300 m, from 2050 m above sea level in Baoshan (BS) to 3300 m in Wufengshan (WF) and Sanpi (SP). The population range of *P. tunicoides* is generally from tens to several hundred individuals. Five to six samples were taken from the edges, as well as the interior of populations, and individuals at least 10 m apart between two individuals were chosen at random to increase the possibility of detecting variation within each population. Healthy leaves were collected in the field and immediately dried with silica gel until DNA extraction.

DNA extraction, PCR amplification and sequencing

Total genomic DNA was extracted from leaf material of each sample according to the modified CTAB method (Doyle 1991). To find suitable markers containing population-level variation in *P. tunicoides*, several non-coding cpDNA and mtDNA fragments, as well as nDNA fragments, were sequenced from

samples in populations that have relatively larger geographical distances using universal primers described in previous studies (e.g. Olsen and Schaal 1999; Shaw *et al*. 2007). After preliminary screening of nine fragments (cpDNA – *rp*L16, *trn*Q-5′*rps*16, *pet*L-*psb*E; mtDNA – *nad*7; nDNA – ITS, g3pdh, *adh*1, SLXY1, *GPA*1), we chose chloroplast *rp*L16 intron, *trn*Q-5′*rps*16 intergenic spacer and nDNA fragment *GPA*1 for further analysis of all the samples. The primers of *rp*L16 (Jordan *et al*. 1996) and trnQ-5′*rps*16 regions (Shaw *et al*. 2007) were used for PCR amplification and sequencing of chloroplast loci. Nuclear DNA fragments *GPA*1 was amplified with primers *GAP*1FF and *GAP*1–14R, which were originally designed based on sequences of three genera of the grass family, *Oryza*, *Hordeum* and *Zea* and are located on exon 9 and exon 14 of the gene, respectively (Bao and Ge 2004). *GPA*1 encodes a G protein α subunit, and functions in various systems of signal transduction in diverse tissues or cells in flowering plants (Ma 1994; Fujisawa *et al*. 1999). In this study, due to the unsuccessful PCR amplification of the *GPA*1 locus for the WN population, a new reverse primer *GAP*1–14R1 (5′-GCT CCC AAT GCG TGA GCT TTT CCT-3′) was designed based on sequences obtained from other populations. The new primer successfully amplified the *GPA*1 gene for the WN population.

All amplification reactions were carried out in a volume of 20 μL containing 10–20 ng template DNA, 2.0 μL of 10 × buffer, 1.4 μL of $MgCl_2$ (25 mM), 1.1 μL of dNTPs (2.5 mM each), 1 μL of DMSO, 0.35 μL primer (10 μM), 1.5 U of *Taq* DNA polymerase (TaKaRa BIO Inc., Dalian, China) and double-distilled water, respectively. Amplification programs for different fragments were: for the *rp*L16 locus, an initial 4 min at 94°C, followed by 30 cycles of 45 s at 94°C, 30 s at 59°C, 90 s at 72°C, and a final 10 min at 72°C; for the *trn*Q-5′*rps*16 locus, an initial 4 min at 94°C, followed by 30 cycles of 45 s at 94°C, 70 s at 54°C, 90 s at 72°C, and a final 10 min at 72°C; and for the *GPA*1 locus, an initial 4 min at 94°C, followed by 37 cycles of 45 s at 94°C, 70 s at 49°C or 60°C (only for WN population), 90 s at 72°C, and a final 10 min at 72°C.

PCR products were purified from agarose gel followed by the ColumnMate Gel Extraction Kit (Watson, Shanghai, China). Sequencing was performed on an ABI 3730 automated sequencer (Life Technologies, Carlsbad, CA, US). Purified products of chloroplast *rp*L16, *trn*Q-5′*rps*16 and nuclear *GPA*1 were sequenced directly. For the WN population, PCR products of *GPA*1 had to be cloned using pMD18-T Vector (TaKaRa) before sequencing, because we found that eight heterozygote individuals had ‘double peaks’ at polymorphic sites when PCR products were sequenced directly. Four of the eight heterozygotes had only one ‘double peak’. The haplotypes of the remaining four heterozygotes were easily inferred through haplotype subtraction (Clark 1990), a method that deduces haplotypes by comparing the heterozygote sequence to haplotypes commonly observed in the total sample. Both strands of each locus were sequenced. ABI sequence chromatograms were checked and corrected, and the contigs were assembled using the SeqMan II software (DNASTAR Inc., Madison, WI, USA). All sequences of *rp*L16, *trn*Q-5′*rps*16 and *GPA*1 loci have been deposited in GenBank under the accession numbers from HQ532909 through HQ532936.

Data analysis

Sequences were aligned using the CLUSTAL W (Thompson *et al*. 1994) followed by manual adjustment implemented in MEGA 3.1 (Kumar *et al*. 2004). Both insertion–deletion events (indels) and simple sequence repeats were taken into account as single mutations, but they were excluded in neutrality tests, as they have been shown to provide relevant phylogeographical information (Caicedo and Schaal 2004; Ikeda and Setoguchi 2006; Caetano *et al*. 2008). Both cpDNA (*rp*L16, *trn*Q-5′*rps*16 sequences were combined) and nDNA sequences were categorised into haplotypes (alleles), and the respective haplotype (allele) distributions were plotted on maps of south-western China using Arcview 3.3 (ESRI Inc., Redlands, CA, USA).

Haplotype diversity (H_d, also known as gene diversity or heterozygosity; Nei 1978) was calculated for each population using DnaSP 4.10 (Rozas *et al*. 2003). Tajima’s *D* (Tajima 1989) and Fu’s *Fs* (Fu 1997) neutrality tests were also conducted with this program. The genetic variation within and among populations was calculated by the AMOVA framework (Excoffier *et al*. 1992) as implemented in ARLEQUIN 3.0 (Excoffier *et al*. 2005), with statistical significance derived by a non-parametric permutation procedure with 1000 repetitions. Estimates of average haplotype diversity within populations (h_S), total haplotype diversity (h_T), two measures of population differentiation G_{ST} and N_{ST}, and the test *U*-statistic were calculated using the program HAPLONST, which is available at http://www.pierroton.inra.fr/genetics/labo/Software/, accessed 8 July 2011. G_{ST} makes use only of the allelic frequencies while N_{ST} also takes into account the overall similarities between the haplotypes. In a *U*-statistic test that compares the values of N_{ST} and G_{ST}, a value of N_{ST} that is significantly higher than the value of G_{ST} usually indicates the presence of phylogeographical structure, i.e. that on average closely related haplotypes are more often found together within the same populations than more distantly related ones (Pons and Petit 1996). The respective topology networks based on the combined chloroplast haplotypes and nuclear alleles data were constructed with TCS 1.21 (Clement *et al*. 2000), a program that calculates with 95% most parsimonious connection limit (Templeton *et al*. 1992).

The pairwise Nei’s (1978) population genetic distances were calculated on the basis of gene frequency differences between populations and were then compared with pairwise geographical distances or altitudinal differences between populations. All four Mantel tests for both cpDNA and nuclear data were conducted based on pairwise geographical or latitude distances and genetic distances with 9999 permutations using GENALEX software (Peakall and Smouse 2006).

Results

Sequence characteristics and haplotype distributions

The maximum length of aligned sequences of *rp*L16 intron was 872 bp with eight polymorphic sites, including seven

nucleotide substitutions and one 12-bp indel, among the 114 individuals of 22 *P. tunicoides* populations. For the *trn*Q-5′*rps*16 spacer, the lengths of obtained sequences differed from 761 to 805 bp due to indel events at nine sites. Two indels were single nucleotide; the other seven ranged from 5 to 27 bp in length. There were five single nucleotide substitutions. A total of nine haplotypes (*a*–*i*) were identified when *rp*L16 and *trn*Q-5′*rps*16 sequences were combined (Table 2). A network of the combined chloroplast haplotypes showed the relationships among the haplotypes (Fig. 2*a*). Haplotype frequencies in each population are shown in Table 3 with geographical distributions overlaid on Fig. 1. As shown in Fig. 1, the cpDNA haplotypes in *P. tunicoides* were not geographically randomly distributed. The most frequent haplotype was haplotype *a* (observed 75 times; 65.8%), which occurred in 19 populations except for FM, GJ, and JL populations. Haplotype *b* was only found in the northern distribution range, and haplotype *g* was distributed in the centre of its distribution range while haplotype *i* (observed 10 times; 8.8%) was only detected in the FM and GJ populations in the southern distribution range and it differed from other haplotypes by more than 14 mutational steps (Fig. 2*a*). The remaining haplotypes were all private found in only one population.

For the nuclear locus *GPA*1, a total of 228 sequences was obtained and/or inferred from the 114 individuals, representing two alleles per individual. The maximum length of aligned sequences was 1237 bp and there were 25 polymorphic sites (Table 4). Twenty polymorphic sites were base substitutions and five were single nucleotide indels, which lead to a small degree of length polymorphism compared with the two chloroplast loci. A total of 17 alleles (1–17) were identified. The allele frequencies in each population and their geographical distribution are shown in Table 3 and Fig. 1. Similar to chloroplast data, the alleles are not evenly distributed. The most common allele (allele 1) was found in 16 of the 22 sampled populations in 52.6% of the individuals. The allele 17 was observed 18 times (7.9%) and is only one mutational step (polyA/T) away from the allele 1. A network of nuclear alleles is shown in Fig. 2*b*.

Neutrality test

The results of the neutrality tests were shown in Table 5. Population expansion results in star-like haplotype networks with a significantly biased frequency spectrum of polymorphisms, which can be detected by negative values of the Tajima's *D*-statistic (Tajima 1989). For both chloroplast and nuclear loci, the values of Tajima's *D* were not significantly negative (chloroplast, $D = -0.81641$, $P > 0.1$; nuclear, $D = -1.62504$, $P > 0.05$), suggesting that the populations were sufficiently old for the frequency spectrum to have recovered after founder events and population growth. As mentioned by Fu (1997), the *Fs*-statistic is sensitive to population expansion and for nuclear locus, was significantly negative ($Fs = -7.134$, $P = 0.001$).

Genetic diversity and genetic structure

The AMOVA results from chloroplast data indicated that 87.25% of the genetic variation was among populations and only 12.75% within populations. Nuclear data similarly suggested that the variation among populations contributed 65.1% of the total genetic variation, while the remaining 34.9% came from within populations. The total F_{ST} values are relatively high and significant (chloroplast, 0.8725, $P < 0.00001$; nuclear, 0.6510, $P < 0.00001$), which indicates that there is significant genetic differentiation among the analysed populations.

Haplotype diversity throughout the range of the species was high (cpDNA, $h_T = 0.575$; nDNA, $h_T = 0.724$), while haplotype diversity within populations was relatively low (cpDNA, $h_S = 0.227$; nDNA, $h_S = 0.333$), which resulted in high genetic differentiation (cpDNA, $G_{ST} = 0.604$; nDNA, $G_{ST} = 0.540$) (Table 6), consistent with results from the above AMOVA

Table 2. Polymorphic sites of the aligned cpDNA sequences of *rp*L16 intron and *trn*Q-5′*rps*16 spacer in *Psammosilene tunicoides*

# indicates presence of an insertion while – indicates absence. #1: GAAAAAAAGAAA; #2: TAAAGTGTCTAAAGTGTAAGAGTTTAT; #3: TATATGA; #4: TTTTTTTTT; #5: ATTAA; #6: TTTTAATTA; #7: ATAAAAAGTTTATAATTTGT; #8: CATATC

	*rp*L16 intron (872 bp)								*trn*Q-*rps*16 spacer (840 bp)													
Haplotype (cpDNA)	1	2	2	4	5	6	6	6	1	1	3	4	4	4	4	4	5	5	6	6	7	7
	8	4	8	5	7	0	4	5	0	9	6	4	4	7	7	8	0	1	2	7	1	2
	6	0	5	8	6	9	3	8	6	9	2	1	2	0	7	6	0	1	1	3	4	3
		|							|		|				|	|	|		|		|	
		2							1		3				4	4	5		6		7	
		5							3		6				8	9	0		4		1	
		1							2		8				5	0	8		0		9	
a	C	–	C	C	C	G	A	A	–	A	–	C	C	–	–	#5	#6	T	–	T	#8	T
b	A	–	C	C	C	G	A	A	–	A	–	C	C	–	–	#5	#6	T	–	T	#8	T
c	C	–	T	C	C	G	A	A	–	A	–	C	C	–	–	#5	#6	T	–	T	#8	T
d	C	–	C	C	C	G	C	A	–	A	–	C	C	–	–	#5	–	T	–	T	–	C
e	C	–	C	C	C	G	A	A	–	–	–	C	C	C	#4	#5	#6	T	–	G	#8	T
f	C	–	C	C	C	G	A	A	–	A	–	C	C	T	–	#5	#6	T	#7	T	#8	T
g	C	–	C	C	C	G	A	A	–	A	–	C	C	–	–	#5	–	T	–	T	#8	T
h	C	–	C	C	C	G	A	A	–	A	–	C	C	–	–	#5	–	T	–	T	–	C
i	A	#1	C	T	A	C	A	G	#2	–	#3	A	A	C	–	–	#6	A	–	T	#8	T

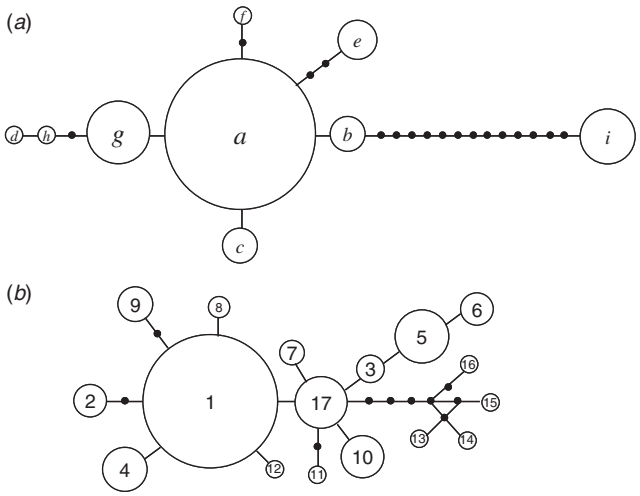

Fig. 2. Haplotype networks derived from: (*a*) the nine haplotypes (*a*–*i*) based on the *rp*L16 intron and *trn*Q-5′*rps*16 spacer of chloroplast DNA genome and (*b*) the 17 nuclear *GPA*1 gene haplotypes (1–17) resolved among the samples of *Psammosilene tunicoides*. Circles represent different haplotypes with size proportional to their relative frequency and dots represent putative interior haplotypes. Each line between haplotypes represents a mutational step.

analysis. The *U*-statistic test for phylogeographic structure showed that N_{ST} was significantly higher than G_{ST} ($N_{ST} > G_{ST}$; chloroplast, $U = 2.60$, $P < 0.01$; nuclear, $U = 4.28$, $P < 0.01$). This indicated that the haplotype distributions were highly structured and that closely related haplotypes were more often found within a population rather than between populations. Both chloroplast and nuclear data consistently indicated that there were low levels of gene flow among populations thus producing significant phylogeographic structure within *P. tunicoides*.

The relationship between genetic and geographical distances

Mantel tests between Nei's pairwise genetic distances (Nei 1978) and the two-dimensional geographical distances (based on longitudinal and latitudinal coordinates), found a significant positive correlation among the sampled populations based on chloroplast data ($r = 0.31$, $P < 0.05$). However, while the nuclear data also showed a positive relationship, the correlation was statistically insignificant ($r = 0.13$, $P = 0.145$). No significant correlation was found between altitudinal differences and population genetic distances (chloroplast, $r = 0.03$, $P = 0.439$; nuclear, $r = 0.04$, $P = 0.450$).

Discussion

Genetic diversity and differentiation

Based on the two cpDNA and one nuclear locus, our investigation of 22 natural populations of *P. tunicoides* revealed a high level of haplotype diversity at the species level. The cpDNA haplotype diversity (h_T = 0.575) is comparable to or lower than those of several other species that occur in the Qinghai-Tibet Plateau and its adjacent areas, such as *Metagentiana striata* (0.558, Chen *et al*. 2008), *Aconitum gymnandrum* (0.739, Wang *et al*. 2009),

Table 3. Haplotype distributions and measures of diversity based on cpDNA and nDNA in *Psammosilene tunicoides*
n, number of sequences of in each population; H_O, observed frequency of heterozygotes; H_d, haplotype diversity

	cpDNA Haplotype												nDNA Haplotype																			
Population	*a*	*b*	*c*	*d*	*e*	*f*	*g*	*h*	*i*	*n*	H_d	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	*n*	H_d	H_o
JL					5					5	0										10								10	0	0
RD	5									5	0	7			5														12	0.530	0.167
SP	2	3								5	0.6	10																	10	0	0
ML	1	1					3			5	0.7									8	2								10	0.356	0
YY	2						3			5	0.6	2																8	10	0.356	0
GZ	6									6	0				7	5													12	0.530	0.167
NP	5									5	0			2		8													10	0.356	0.400
WF	5									5	0	6				6													12	0.545	0
LG	5									5	0	4			1				3									2	10	0.778	0.400
NL	5									5	0			3			7												10	0.467	0.200
YS	4						1			5	0.4	10																	10	0	0
LJ	2						3			5	0.6	10																	10	0	0
HM	4						1			5	0.4	6						4												10	0.533	0
HQ	1			1			2	1		5	0.9	10																	10	0	0
BS	5									5	0	6																4	10	0.533	0
FM									5	5	0	10																	10	0	0
KM	4					1				5	0.4	12																	12	0	0
AN	1		4							5	0.4	3	7																10	0.467	0.200
GJ									5	5	0	10																	10	0	0
HZ	6									6	0	6										2						4	12	0.667	0
ZY	6									6	0	8											2						10	0.356	0
WN	6									6	0													2	2	2	2		8	0.857	0
Total	75	4	4	1	5	1	13	1	10	114	0.55	120	7	5	13	19	7	4	3	8	12	2	2	2	2	2	2	18	228	0.702	

Table 4. Polymorphic sites of the aligned nDNA sequences of *GPA*1 in *Psammosilene tunicoides* (– indicates absence)

	Nuclear *GPA*1 (1237 bp)																								
Haplotype (nDNA)	0 0 2 2	0 0 3 8	0 1 3 1	0 1 4 4	0 1 5 3	0 1 9 5	0 2 0 3	0 3 4 4	0 3 9 4	0 3 9 7	0 4 2 5	0 4 4 4	0 4 6 6	0 4 7 2	0 4 7 3	0 4 7 4	0 5 9 7	0 7 6 8	0 8 6 8	0 8 8 2	0 8 8 3	0 9 9 0	1 0 2 7	1 0 4 7	1 0 5 7
1	T	G	C	C	G	C	G	G	G	G	T	–	T	T	T	–	T	G	A	C	G	A	C	C	A
2	T	G	C	T	G	C	G	G	G	G	T	–	T	T	T	–	T	G	A	C	G	A	T	C	A
3	T	G	C	C	G	C	A	G	G	G	T	–	T	T	–	–	T	G	A	C	G	A	C	C	A
4	T	G	C	C	G	C	G	G	G	G	T	–	T	T	T	–	T	G	A	A	G	A	C	C	A
5	T	G	C	C	G	C	A	G	G	G	T	–	T	T	–	–	T	G	A	C	A	A	C	C	A
6	T	T	C	C	G	C	A	G	G	G	T	–	T	T	–	–	T	G	A	C	A	A	C	C	A
7	T	G	C	C	G	C	G	G	G	T	T	–	T	T	–	–	T	G	A	C	G	A	C	C	A
8	T	G	C	C	G	C	G	G	G	G	T	–	T	T	T	–	T	A	A	C	G	A	C	C	A
9	T	G	C	C	G	C	G	G	A	G	T	–	T	T	T	T	T	G	A	C	G	A	C	C	A
10	T	G	G	C	G	C	G	G	G	G	T	–	T	T	–	–	T	G	A	C	G	A	C	C	A
11	A	G	C	C	G	C	G	G	G	G	T	T	T	T	–	–	T	G	A	C	G	A	C	C	A
12	T	G	C	C	G	C	G	T	G	G	T	–	T	T	T	–	T	G	A	C	G	A	C	C	A
13	T	G	C	C	A	T	G	G	G	G	C	–	–	–	–	–	T	G	A	C	G	A	C	T	A
14	T	G	C	C	A	C	G	G	G	G	C	–	–	–	–	–	C	G	A	C	G	A	C	T	A
15	T	G	C	C	A	C	G	G	G	G	C	–	C	–	–	–	T	G	G	C	G	A	C	T	A
16	T	G	C	C	A	C	G	G	G	G	C	–	T	–	–	–	T	G	A	C	G	G	C	T	T
17	T	G	C	C	G	C	G	G	G	G	T	–	T	T	–	–	T	G	A	C	G	A	C	C	A

Table 5. Haplotype numbers, polymorphic sites, nucleotide diversity (π) and estimates of neutrality for cpDNA and nDNA in *Psammosilene tunicoides*

**, indicates significant values with $P < 0.01$; no *, not significant

	No. of haplotypes	No. of polymorphic sites	π	Tajima's *D*	Fu's *Fs*
cpDNA	9	22	0.00099	–0.81641	0.444
nDNA	17	25	0.00105	–1.62504	–7.134**

Table 6. Gene diversity and differentiation parameters for cpDNA and nDNA in *Psammosilene tunicoides*

h_S = diversity within populations, h_T = the total haplotype diversity, G_{ST}, N_{ST} = two measures of population differentiation. Standard error is shown in parentheses. * indicates that N_{ST} is significantly larger than G_{ST}. ** $P < 0.01$

	h_S	h_T	G_{ST}	N_{ST}
cpDNA	0.227 (0.0639)	0.575 (0.0975)	0.604 (0.1014)	0.905** (0.0555)
nDNA	0.333 (0.0609)	0.724 (0.0829)	0.540 (0.0669)	0.651** (0.0508)

**N_{ST} is significantly different from G_{ST} ($P < 0.01$).

Dipentodon sinicus (0.902, Yuan *et al*. 2008) and *Primula secundiflora* (0.996, Wang *et al*. 2008). For nuclear allele diversity, there are no similar reports available for comparison and our study is the first to use single-copy nuclear sequences to address the genetic diversity of endemic plant species in this biodiversity hotspot. When compared with studies of other species from other geographic regions based on single-copy nDNA sequences, such as *Zizania latifolia* (0.660, Xu *et al*. 2008) from China, *Solanum pimpinellifolium* (0.841, Caicedo and Schaal 2004) from Peru, and *Castanopsis carlesii* (0.689–0.885, using three single-copy genes, Ikeda *et al*. 2008) from Japan, *P. tunicoides* has a similar haplotype diversity (0.724).

Results from nuclear and chloroplast markers revealed similar patterns of genetic diversity in *P. tunicoides*-significant differentiation among populations but low diversity within populations (cpDNA, $G_{ST} = 0.604$; nDNA, $G_{ST} = 0.540$). This result is also consistent with previous studies based on amplified fragment length polymorphisms (AFLP, $G_{ST} = 0.624$, Dai *et al*. 2007) and the direct amplification of length polymorphism (DALP, $G_{ST} = 0.7899$, Qu *et al*. 2010) of this species. In general, maternally inherited cpDNA markers display higher G_{ST} values (mean 0.63) than biparentally inherited nDNA markers (mean 0.18) in angiosperm species (Petit *et al*. 2005). In this study, the G_{ST} value revealed by cpDNA data is slightly higher than that of nDNA, suggesting that pollen flow is almost as restricted within population as that of seed flow. The restricted gene flow contributes to the congruence of cpDNA and nDNA on the genetic structure and history events (to be discussed further in the following section).

The high level among-population differentiation within *P. tunicoides* is likely related to the breeding system, pollen and seed dispersal mechanisms, and genetic drift or geographic isolation of populations. First, according to recent crossing experiments (Professor Z. G. Qian, pers. comm.), *P. tunicoides* has a mixed mating system (outcrossing and autogamy). In our present study, of the total 114 samples, only eight individuals (~7% of the samples) were heterozygotes at the *GPA*1 locus, suggesting that selfing is likely very important in the reproduction of *P. tunicoides* in wild populations. Second, the pollen of *P. tunicoides* is dispersed by insects and the seeds are dispersed mainly by gravity within the vicinity of the mother plants. These mechanisms of short-distance dispersal can lead to low gene flow among populations. Third, its high among-population differentiation is consistent

with the 'patchiness' or 'island' distribution pattern of *P. tunicoides*. Throughout the range of *P. tunicoides*, most of the populations are spatially isolated from each other by uninhabitable areas, which may be related to the complicated topography and strong environmental heterogeneity of this hotspot region.

Historical processes

Earlier studies based on random amplified polymorphic DNA by Yan *et al*. (2003), AFLP by Dai *et al*. (2007), and DALP by Qu *et al*. (2010), failed to detect the presence of a phylogeographic signal among *P. tunicoides* populations. However, in this study both cpDNA and nDNA datasets documented strong phylogeographic signals and suggested the likely historical processes underlying the pattern. The values of Tajima's *D* were not significantly negative (chloroplast, $D = -0.81641$, $P > 0.1$; nuclear, $D = -1.62504$, $P > 0.05$.), indicating that *P. tunicoides* has likely experienced population expansion. Despite this, the more sensitive Fu's *Fs*-statistic showed a significantly negative value for nDNA sequences, providing further support for the hypothesis of population expansion. Additionally, high haplotype diversity (cpDNA, $h_T = 0.575$; nDNA, $h_T = 0.724$), low nucleotide diversity (cpDNA, $\pi = 0.00099$; nDNA, $\pi = 0.00105$), and star-like haplotype networks with the most common haplotype at the cores (Fig. 2) all suggest population expansion from an ancestral population with a small effective population size (Avise 2000, 2004; Novaes *et al*. 2010). It is believed that *P. tunicoides* is a relict of the Tethyan Tertiary Flora (Wu *et al*. 2003), that underwent repeated episodes of contraction and expansion of their geographic ranges following changes in temperature associated with glaciations during the Quaternary (Hewitt 2004). This hypothesis could also explain the population structure observed here that included range expansion from refugia following glaciation. The haplotype networks from cpDNA and nDNA data showed the central position for the most common haplotypes (*a* and *1*, respectively), existing in almost all populations. According to coalescent theory, these haplotypes would have a great probability of producing mutational derivatives and may represent ancestral haplotypes (Crandall and Templeton 1993). In the cpDNA haplotype network, haplotype *g* derived from haplotype *a*, had a high frequency distribution in north-western Yunnan and south-western Sichuan, where populations HQ and ML had the highest haplotype diversities. This might imply that these areas are potential glacial refugia for *P. tunicoides*. A recent study of a rare woody plant, *Nouelia insignis*, in this region also demonstrated that the Jinsha River drainage area (northern Yunnan and southern Sichuan) might be a refugium (Gong *et al*. 2011).

Genetic analyses revealed a significant phylogeographic structure in *P. tunicoides*. Both cpDNA and nDNA dataset detected a phylogeographic signal with the *U*-statistic test ($N_{ST} > G_{ST}$, $P < 0.01$), indicating that haplotype distributions were highly structured and that closely related haplotypes were commonly found within the same populations. A clear geographical pattern of genetic variation was also evident from the haplotype frequency distribution map (Fig. 1). For example, cpDNA haplotype *b* was found only in the north, haplotype *g* only in the centre, and haplotype *i* only in the south. In the cpDNA dataset the significant positive correlation between genetic and geographical distances ($P < 0.05$) further supports the 'isolation-by-distance' pattern and limited genetic exchange between populations. This is consistent with the current isolated and fragmented distribution of *P. tunicoides*. Heterogeneous and isolated ecological environments were formed in this hotspot region due to the uplifting of the Qinghai-Tibet Plateau (Wu *et al*. 2003). High mountains and deep valleys formed during this time has created a fragmented ecological niche for *P. tunicoides*, presenting considerable barriers to gene flow, and thus may dramatically influence the population differentiation. Moreover, human activities and ~500 years of medical usage history have likely contributed to the decline of populations of *P. tunicoides* and promoted further fragmentation.

Chloroplast DNA and nDNA data used in this study demonstrated congruent population differentiation, range expansion events and geographic structure of *P. tunicoides.* The usefulness of *GPA*1 suggests that single-copy nDNA data can be efficient markers in phylogeographic studies, and this is consistent with the result from other studies (e.g. Ikeda *et al*. 2008; on *Cardamine nipponica*; Xu *et al*. 2008; on *Zizania latifolia*). Nevertheless, geographic structure revealed by *GPA*1 in this study was not completely consistent with that revealed by cpDNA. In the WN population, *GPA*1 revealed very high variations compared with those of cpDNA. This is not an unexpected result as cpDNA has a different evolutionary mechanism (maternal inheritance) compared with the nuclear gene. High *GPA*1 variations in WN population probably ascribe to the stochastic variance caused by mutation and recombination, as coding regions of the gene are often under quite strong selective pressures (Hey and Machado 2003).

Implications for conservation

The information of genetic diversity and population structure is important for designing conservation strategies for threatened and endangered species (Frankel *et al*. 1995; Avise and Hamrick 1996). Strong genetic differentiations and low gene flow among populations revealed by our study indicate that protecting one or two populations will not capture the majority of the genetic diversity. The strong evidence for isolation-by-distance and its current isolated and fragmented distribution pattern also suggest that *in situ* conservation should be put into practice broadly as a long-term strategy. Such a strategy will preserve not only the genetic diversity of *P. tunicoides*, but also its habitat diversity. In addition, a transplanting trial conducted in a greenhouse in Kunming city found phenological delay with individuals from the southern populations flowering much earlier than those from the northern populations, suggesting that it is better to protect as many populations as possible in the north and south. However, considering the current distribution pattern of the *P. tunicoides* and the reserves in this region, it is very difficult to protect all populations. North-western Yunnan and south-western Sichuan (the modern distribution centre) should be the primary areas to be protected. In particular, the areas containing unique haplotypes (e.g. AN, JL and GJ for cpDNA haplotype; WN,

ML and HZ for nDNA haplotype), should be given special attention. Further, the persistence of small populations such as AN, WN and GJ (heavily threatened by excavating and loss of habitat) might be facilitated by transferring (or seeds collecting) to nearby reserves.

Acknowledgements

The authors thank Jian-Ping Xu (Centre for Environmental Genomics, Department of Biology, McMaster University) for carefully reading an early draft of the manuscript and for his critical comments that greatly improved the manuscript. Thanks also go to Zai-Wei Ge (Kunming Institute of Botany, the Chinese Academy of Sciences) for his kind help in the sample collection. This work was funded by the National Basic Research Program of China (973 Program: 2007CB411600).

References

Avise JC (2000) 'Phylogeography: the history and formation of species.' (Harvard University Press, Cambridge, Massachusetts, USA)

Avise JC (2004) 'Molecular markers natural history, and evolution.' (Sinauer and Associates Press: Sunderland, MA)

Avise JC, Hamrick JL (1996) 'Conservation genetics, case histories from nature.' (Chapman and Hall Press: New York)

Bao Y, Ge S (2004) Origin and phylogeny of *Oryza* species with the CD genome based on multiple-gene sequence data. *Plant Systematics and Evolution* **249**, 55–66. doi:10.1007/s00606-004-0173-8

Caetano S, Prado D, Pennington RT, Beck S, Oliveira-Filho A, Spichiger R, Naciri Y (2008) The history of seasonally dry tropical forests in eastern South America: inferences from the genetic structure of the tree *Astronium urundeuva* (Anacardiaceae). *Molecular Ecology* **17**, 3147–3159. doi:10.1111/j.1365-294X.2008.03817.x

Caicedo AL, Schaal BA (2004) Population structure and phylogeography of *Solanum pimpinellifolium* inferred from a nuclear gene *Molecular Ecology* **13**, 1871–1882. doi:10.1111/j.1365-294X.2004.02191.x

Chen SY, Wu GL, Zhang DJ, Gao QB, Duan YZ, Zhang FQ, Chen SL (2008) Potential refugium on the Qingha-Tibet Plateau revealed by the chloroplast DNA phylogeography of the alpine species *Metagentiana striata* (Gentianaceae). *Botanical Journal of the Linnean Society* **157**, 125–140. doi:10.1111/j.1095-8339.2008.00785.x

Clark AG (1990) Inference of haplotypes from PCR-amplified samples of diploid populations. *Molecular Biology and Evolution* **7**, 111–122.

Clement M, Posada D, Crandall KA (2000) TCS: a computer program to estimate gene genealogies. *Molecular Ecology* **9**, 1657–1659. doi:10.1046/j.1365-294x.2000.01020.x

Crandall KA, Templeton AR (1993) Empirical tests of some predictions from coalescent theory with applications to intraspecific phylogeny reconstruction. *Genetics* **134**, 959–969.

Dahiya R (2008) Total synthesis and biological potential of *Psammosilenin A. Archiv der Pharmazie* **341**, 502–509. doi:10.1002/ardp.200800006

Dai ZB, Zhu CC, Qian ZG (2007) Genetic diversity analysis of germplasm resources of *Psammosilene tunicoides. Chinese Traditional and Herbal Drugs* **38**, 1070–1074.

Ding ZT, Wang YC, Zhou J, Tan NH, Wu HM (2000) Cyclic peptides from the roots of *Psammosilene tunicoides. Acta Botanica Yunnanica* **22**, 331–336.

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

Excoffier L, Smouse PE, Quattro JM (1992) Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. *Genetics* **131**, 479–491.

Excoffier L, Laval G, Schneider S (2005) Arlequin (version 3.0): an integrated software package for population genetics data analysis. *Evolutionary Bioinformatics Online* **1**, 47–50.

Frankel OH, Brown AHD, Burdon JJ (1995) 'The conservation of plant biodiversity.' (Cambridge University Press: Cambridge)

Fu LG, Jin JM (1992) 'Chinese plant red data book: rare and endangered plants.' (Science Press: Beijing, China)

Fu YX (1997) Statistical tests of neutrality of mutations against population growth, hitchhiking and background selection. *Genetics* **147**, 915–925.

Fujisawa Y, Kato T, Ohki S, Ishikawa A, Kitano H, Sasaki T, Asahi T, Iwasaki Y (1999) Suppression of the heterotrimeric G protein causes abnormal morphology, including dwarfism, in rice. *Proceedings of the National Academy of Sciences of the United States of America* **96**, 7575–7580. doi:10.1073/pnas.96.13.7575

Gong X, Luan SS, Hung KH, Hwang CC, Lin CJ, Chiang YC, Chiang TY (2011) Population structure of *Nouelia insignis* (Asteraceae), an endangered species in southwestern China, based on chloroplast DNA sequences: recent demographic shrinking. *Journal of Plant Research* **124**, 221–230. doi:10.1007/s10265-010-0363-0

Hewitt GM (2004) Genetic consequences of climatic oscillations in the Quaternary. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences* **359**, 183–195. doi:10.1098/rstb.2003.1388

Hey J, Machado CA (2003) The study of structured populations – new hope for a difficult and divided science. *Nature Reviews. Genetics* **4**, 535–543. doi:10.1038/nrg1112

Ikeda H, Setoguchi H (2006) Phylogeography of *Arcterica nana* (Ericaceae) suggests another range expansion history of Japanese alpine plants. *Journal of Plant Research* **119**, 489–495. doi:10.1007/s10265-006-0012-9

Ikeda H, Senni K, Fujii N, Setoguchi H (2008) Consistent geographic structure among multiple nuclear sequences and cpDNA polymorphisms of *Cardamine nipponica* Franch. et Savat. (Brassicaceae). *Molecular Ecology* **17**, 3178–3188. doi:10.1111/j.1365-294X.2008.03821.x

Jordan WC, Courtney MW, Neigel JE (1996) Low levels of intraspecific genetic variation at a rapidly evolving chloroplast DNA locus in North American duckweeds (Lemnaceae). *American Journal of Botany* **83**, 430–439. doi:10.2307/2446212

Kumar S, Tamura K, Nei M (2004) MEGA3: Integrated software for molecular evolutionary genetics analysis and sequence alignment. *Briefings in Bioinformatics* **5**, 150–163. doi:10.1093/bib/5.2.150

Liu JQ, Wang YJ, Wang AL, Hideaki O, Abbott RJ (2006) Radiation and diversification within the *Ligularia-Cremanthodium-Parasenecio* complex (Asteraceae) triggered by uplift of the Qinghai-Tibetan Plateau. *Molecular Phylogenetics and Evolution* **38**, 31–49. doi:10.1016/j.ympev.2005.09.010

Lu DQ (2001) *Psammosilene*. In 'Flora of China'. (Eds CY Wu, H Raven) (Science Press: Beijing).

Ma H (1994) GTP-binding proteins in plants: new members of an old family. *Plant Molecular Biology* **26**, 1611–1636. doi:10.1007/BF00016493

Myers N, Mittermeier RA, Mittermeier CG, Fonseca G, Kent J (2000) Biodiversity hotspots for conservation priorities. *Nature* **403**, 853–858. doi:10.1038/35002501

Nei M (1978) Estimation of average heterozygosity and genetic distance from small number of individuals. *Genetics* **89**, 583–590.

Novaes RML, Filho JPDL, Ribeiro RA, Lovato MB (2010) Phylogeography of *Plathymenia reticulata* (Leguminosae) reveals patterns of recent range expansion towards northeastern Brazil and southern Cerrados in Eastern Tropical South America. *Molecular Ecology* **19**, 985–998. doi:10.1111/j.1365-294X.2010.04530.x

Olsen KM, Schaal BA (1999) Evidence on the origin of cassava: phylogeography of *Manihot esculenta. Proceedings of the National Academy of Sciences of the United States of America* **96**, 5586–5591. doi:10.1073/pnas.96.10.5586

Peakall R, Smouse PE (2006) Genalex 6: genetic analysis in Excel. Population genetic software for teaching and research. *Molecular Ecology Notes* **6**, 288–295. doi:10.1111/j.1471-8286.2005.01155.x

Petit RJ, Duminil J, Fineschi S, Hampe A, Salvini D, Vendramin GG (2005) Comparative organization of chloroplast, mitochondrial and nuclear diversity in plant populations. *Molecular Ecology* **14**, 689–701. doi:10.1111/j.1365-294X.2004.02410.x

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

Qu Y, Yu H, Wu G, Ma RF, Li YY (2010) Genetic diversity and population structure of the endangered species *Psammosilene tunicoides* revealed by DALP analysis. *Biochemical Systematics and Ecology* **38**, 880–887. doi:10.1016/j.bse.2010.09.007

Rozas J, Sanchez-DelBarrio JC, Messeguer X, Rozas R (2003) DnaSP, DNA polymorphism analyses by the coalescent and other methods. *Bioinformatics (Oxford, England)* **19**, 2496–2497. doi:10.1093/bioinformatics/btg359

Schaal BA, Hayworth DA, Olsen KM, Rauscher JT, Smith WA (1998) Phylogeographic studies in plants: problems and prospects. *Molecular Ecology* **7**, 465–474. doi:10.1046/j.1365-294x.1998.00318.x

Shaw J, Lickey EB, Schilling EE, Small RL (2007) Comparison of whole chloroplast genome sequences to choose noncoding regions for phylogenetic studies in angiosperms: the tortoise and the hare III. *American Journal of Botany* **94**, 275–288. doi:10.3732/ajb.94.3.275

Tajima F (1989) Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. *Genetics* **123**, 585–595.

Templeton AR, Crandall KA, Sing CF (1992) A cladistic analysis of phenotypic associations with haplotypes inferred from restriction endonuclease mapping and DNA sequence data. III. Cladogram estimation. *Genetics* **132**, 619–633.

Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research* **22**, 4673–4680. doi:10.1093/nar/22.22.4673

Wang FY, Gong X, Hu CM, Hao G (2008) Phylogeography of an alpine species *Primula secundiflora* inferred from the chloroplast DNA sequence variation. *Journal of Systematics and Evolutiion* **46**, 13–22.

Wang LY, Abbott RJ, Zheng W, Chen P, Wang YJ, Liu JQ (2009) History and evolution of alpine plants endemic to the Qinghai-Tibetan Plateau: *Aconitum gymnandrum* (Ranunculaceae). *Molecular Ecology* **18**, 709–721. doi:10.1111/j.1365-294X.2008.04055.x

Wilson EO (1992) 'The diversity of life.' (Harvard University Press: Cambridge)

Wu ZY (1998) Delineation and unique features of the Sino-Japanese floristic region. In 'Sino-Japanese flora: its characteristics and diversification'. (Eds DE Boufford, H Ohba) pp. 1–35. (Tokyo University Press: Tokyo)

Wu ZY, Lu AM, Tang YC, Chen ZD, Li DZ (2003) 'The families and genera of angiosperms in China: a comprehensive analysis.' (Science Press: Beijing)

Xu XW, Ke WD, Yu XP, Wen J, Ge S (2008) A preliminary study on population genetic structure and phylogeography of the wild and cultivated *Zizania latifolia* (Poaceae) based on Adh1a sequences. *Theoretical and Applied Genetics* **116**, 835–843. doi:10.1007/s00122-008-0717-3

Yan YW, Xie H, Pu CX, Zhang J, Qian ZG (2003) RAPD analysis of three populations of *Psammosilene tunicoides* W. C. Wu et C. Y. Wu. *Journal of Yunnan College of Traditional Chinese Medicine* **26**, 21–23.

Yuan QJ, Zhang ZY, Peng H, Ge S (2008) Chloroplast phylogeography of *Dipentodon* (Dipentodontaceae) in southwest China and northern Vietnam. *Molecular Ecology* **17**, 1054–1065. doi:10.1111/j.1365-294X.2007.03628.x

Zhou ZK, Momohara A (2005) Fossil history of some endemic seed plants of East Asia and its phylogeographical significance. *Acta Botanica Yunnanica* **25**, 449–470.

Manuscript received 24 January 2011, accepted 29 June 2011