Plant Syst Evol (2014) 300:51–61
DOI 10.1007/s00606-013-0859-x

ORIGINAL ARTICLE

Phylogeographic patterns of the *Aconitum nemorum* species group (Ranunculaceae) shaped by geological and climatic events in the Tianshan Mountains and their surroundings

Xiao-Long Jiang · Ming-Li Zhang · Hong-Xiang Zhang · Stewart C. Sanderson

Received: 9 October 2012 / Accepted: 27 May 2013 / Published online: 16 June 2013
© Springer-Verlag Wien 2013

Abstract To investigate the impacts of ancient geological and climatic events on the evolutionary history of the *Aconitum nemorum* species group, including *A. nemorum* s. str., *A. karakolicum*, and *A. soongoricum*; a total of 18 natural populations with 146 individuals were sampled, mainly from grassy slopes or the coniferous forest understory of the Tianshan Mountain Range and its surroundings. Two cpDNA intergenic spacer regions (*trn*S-*trn*G and *psb*A-*trn*H) were sequenced and 16 haplotypes were identified. These were clustered into three divergent lineages which almost entirely corresponded to the three species. Analysis of molecular variance indicated restricted gene flow, mainly among species. High levels of genetic distance were detected among eastern populations in *A. nemorum* s. str. and *A. karakolicum* from spatial genetic landscape analysis. Neutral tests and mismatch distribution analysis suggest that *A. nemorum* s. str. experienced demographic expansions during interglacial periods. Based on haplotype distribution and the median-joining network, it was inferred that this species underwent two periods of eastward expansion. Our molecular dating indicates that the lineages of the complex separated during the period of the late Tertiary to late Pleistocene (11.74–0.064 million years ago), which was most likely triggered by recent rapid uplift of the Tianshan Mountains, while genetic variation at the intra-specific level might be attributed to climatic cycles in the late Quaternary.

Keywords *Aconitum* · Phylogeography · Tianshan Mountains · Speciation · Glacial refugia · Restricted gene flow

Electronic supplementary material The online version of this article (doi:10.1007/s00606-013-0859-x) contains supplementary material, which is available to authorized users.

X.-L. Jiang · M.-L. Zhang (✉) · H.-X. Zhang
Key Laboratory of Biogeography and Bioresource in Arid Land, Xinjiang Institute of Ecology and Geography, Chinese Academy of Sciences, Urumqi 830011, China
e-mail: zhangml@ibcas.ac.cn

X.-L. Jiang · H.-X. Zhang
University of Chinese Academy of Sciences, Beijing 100049, China

M.-L. Zhang
State Key Laboratory of Systematic and Evolutionary Botany, Institute of Botany, Chinese Academy of Sciences, Beijing 100093, China

S. C. Sanderson
Shrub Sciences Laboratory, Intermountain Research Station, Forest Service, US Department of Agriculture, Utah 84601, USA

Introduction

Phylogeography, a new discipline which began at 1980s, plays an important role in connecting population genetics and phylogenetic systematics (Avise 2000). Since the recent rapid development of this discipline, it has been recognized as an appropriate method to detect the present spatial genetic structures of species and infer the antecedent historical events (Liu et al. 2009; Hardy et al. 2002; Avise 2000). For example, in Europe and North America, phylogeographic studies have shown many temperate plant species to have experienced multiple processes of south retreat–north colonization in accordance with glacial–interglacial cycles (Hewitt 2000). Due to complex topography and high levels of species diversity, the consideration of hypotheses on how plant species in mountainous regions have responded to paleoclimatic changes and geological events has also attracted numerous botanists and ecologists

(Zhou et al. 2012; Liu et al. 2009; Hewitt 2004; Wang et al. 2009; Muellner et al. 2005; Stewart et al. 2010). Many mountain plants are deemed to have retreated to low altitudes to avoid alpine glaciations (Wulff 1943), although some cold-tolerant species might have been able to survive on mountain platforms during glacial periods (Wang et al. 2009). For example, the European distributed, cold-tolerant species *Trollius europaeus* experienced migrations to northern areas even during glacial periods (Espíndola et al. 2012).

Among regions harboring multiple genetic haplotypes in China (Liu et al. 2012), the Qinghai-Tibetan Plateau (QTP) and adjacent southwestern China, due to an exceptionally high biodiversity, have been regarded as a study hotspot (Liu et al. 2009, 2012; Qiu et al. 2011a, 2011b; Zhou et al. 2012; Sun et al. 2010). High species richness and genetic diversity in this region are associated with rapid uplifting of the southeastern QTP during the Miocene (Clark et al. 2005) and the Quaternary glacial–interglacial cycles (Cun and Wang 2010; Jia et al. 2012). For example, Jia et al. (2012) recently investigated the phylogeographic structure of *Hippophae rhamnoides*, showing this species to have originated in the QTP and dispersed across Eurasia during the Pliocene and Quaternary.

Attention has now also been directed to the phylogeography of the region of arid Northwestern China, where the spatial genetic structure and demographical history of a number of eremophytes have been recently assessed (Meng and Zhang 2011; Li et al. 2012; Su et al. 2012). Investigating the phylogeographical patterns of these plants can allow for an understanding of the effects of geological and/or climatic events on plant species in the area. However, the forest and grassland species widespread in relatively humid, high altitude zones of mountain ranges have as yet been given little focus.

The Tianshan Mountains, located between the Tarim and Dzungarian basins in Northwestern China, were mainly formed as a result of the intensive Cenozoic India–Asia collision (Sun et al. 2004). Although the chronological process of this uplift is still in debate, it is believed that the most recent uplift of the Tianshan Mountains occurred in the late Tertiary (Sun et al. 2004; Wang 2010). Also, according to evidence from analysis of palynology and ancient alpine glaciers (Zhao et al. 2009; Yan et al. 1998), climatic cycles of cold-dry to warm-humid conditions were experienced during the late Quaternary in the Tianshan Mountains. These geological events and climatic changes are believed to have profoundly affected the genetic structure and distribution patterns of species. Zhang and Zhang (2012) showed that cold-dry climates during glacial periods in these mountains may have triggered specific and intra-specific divergence within the *Delphinium naviculare* species group.

The Quaternary climatic oscillations were generally more recent than the divergence between species. For example, uplifts in the QTP during the early Miocene to Pliocene promoted speciation in *Cupressus* (Xu et al. 2010a). Considering the timescale of the uplift of the Tianshan Mountains, we study the phylogeography of the *Aconitum nemorum* species group, containing *A. nemorum* s. str., *A. soongoricum*, and *A. karakolicum*, mainly occurring in the forest understory, on grassy slopes, and along mountain streams, to provide new perspectives on the evolutionary history of this species group in relation to events in this area. These taxa belong to Series *Grandituberosa* Steinb. of *Aconitum*, which have been shown to be monophyletic and have obvious morphological discontinuities (Luo et al. 2005) based on characters of the follicles and seeds. The seeds are released when the ventral suture dehisces, and their distance of dispersal is small (Guan et al. 1979). This limited dispersal ability would lead to lower levels of gene flow among and within the species. Therefore, we attempt to study phylogeography of this multi-species group employing two cpDNA noncoding regions, which are maternally inherited and non-recombining in most flowering plants (Schaal et al. 1998). We address the following two issues: (1) inference of the spatial genetic structure of the *A. nemorum* species group and (2) effects that the Tianshan Mountains uplift and Quaternary glaciations in Northwestern China may have had on shaping this structure.

Materials and methods

Taxon and population sampling

A total of 146 individuals from 18 natural populations, including 6 populations (48 samples) of *A. nemorum* s. str., 9 populations (72 samples) of *A. soongoricum*, and 3 populations (26 samples) of *A. karakolicum*, were collected in this study (Table 1). Our sampling locations covered most of the range of the *A. nemorum* species group in China. The latitude, longitude, and altitude of each locality were recorded using a global positioning system (GPS). To avoid the collection of clones, we sampled individuals separated by at least 50 m. Leaves were sampled randomly and quickly dried with silica gel and stored frozen until extraction. Voucher specimens of each individual were deposited in the Herbarium of Xinjiang Institute of Ecology and Geography, Chinese Academy of Science (XJBI).

Laboratory procedures

Total genomic DNA was extracted from dried leaf tissue following a modified cetyltrimethyl ammonium bromide

Table 1 Details of information on sample localities for the 18 *Aconitum nemorum* species group populations

Population	Latitude	Longitude	*N*	Haplotype	*h*	π
A. nemorum s. str.			48			
QTB	43.581	89.529	7	H12, H14, H15	0.822	0.857
QTJ	43.582	89.829	8	H12	0	0
FK	43.879	88.122	8	H11, H16	0.559	0.429
WLMQ	43.246	87.181	10	H11, H13, H16	1.242	0.711
MNS	43.876	86.102	8	H13, H16	0.559	0.429
GNSL	43.245	84.642	7	H13, H16, H15	0.822	1.048
A. soongoricum			72			
YW	43.503	93.984	8	H2	0	0
HM	43.287	93.81	8	H2	0	0
BNK	43.542	92.97	8	H2	0	0
JMN	47.166	86.152	8	H2, H3	0.559	0.429
HF	46.989	85.962	8	H1	0	0
EM	46.943	84.549	8	H2	0	0
TC	45.795	82.798	8	H1	0	0
TL1	45.926	83.310	8	H1	0	0
TL2	45.743	83.055	8	H1	0	0
A. karakolicum			26			
WQ	44.935	80.091	9	H8, H9	0.474	0.778
ZS	42.684	80.770	9	H4, H5, H7, H10	1.051	5.5
GNSD	43.145	84.542	8	H6, H7	0.248	0.5
Total			146			

(CTAB) protocol (Doyle and Doyle 1987). To get a preliminary screen of chloroplast variation, 8 universal primers were used initially for 18 samples from 18 natural localities. Two chloroplast regions, *psb*A-*trn*H (Sang et al. 1997) and *trn*S-*trn*G (Hamilton 1999), were found to display more variations than the other six markers examined (*trn*L–*trn*F, *atp*B–*abc*L, *rps*16–*trn*K, *psb*K–*psb*A, *ycf*6–*psb*M, and rps12–rpl20). We then performed amplifications on all individuals using these two pairs of primers. Polymerase chain reaction (PCR) amplifications were carried out in 30 μl reaction volumes consisting of 1.5 μl of 10× PCR buffer (Takara, Japan), 2 μl of 25 mM $MgCl_2$, 1.8 μl of each primer (Sangon, Shanghai, China) at 50 ng/μl, 3 μl of 2.5 mM dNTP solution in an equimolar ratio, 0.46 μl of Taq DNA polymerase (5 U/μl, Takara, Japan), and 0.5 μl of genomic DNA (10–100 μg). Amplification using these primers began with an initial hotstart at 95 °C for 4 min, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 52 °C for 30 s, extension at 72 °C for 90 s, and a final extension at 72 °C for 10 min. Amplification products were purified, and sequenced by a commercial laboratory (Shanghai Sangon Biological Engineering Technology & Service, Shanghai, China).

Nucleotide sequences were edited in Seqman (Lasergene, DNASTAR Inc., Madison, Wisconsin, USA), and aligned using ClustalX version 1.81 (Thompson et al. 1997) with default parameters, and then refined manually. The cpDNA haplotypes were identified based on nucleotide variations in the species examined (see below). They are deposited in GenBank database (GenBank accession no.: KC837082–KC837094 and KC837096 for *psb*A-*trn*H, and accession no.: KC837076–KC837080 for *trn*S-*trn*G).

Genetic diversity and phylogeographic structure analysis

The plastid (cpDNA) haplotypes and polymorphic sites were assessed for the concatenated alignment sequences of 146 individuals of the *A. nemorum* species group using DnaSP 5.0 (Librado and Rozas 2009). Estimations of population genetic indexes [haplotypes diversities (*h*), nucleotide diversity (π) and analysis of molecular variance (AMOVA)] were computed in Arlequin 3.1 (Excoffier et al. 2005). Three groups based on these taxa were prespecified in the AMOVA analysis. A median-joining network (Bandelt et al. 1999) was constructed using the program Network 4.6.1.0 (available at http://www.fluxus-engineering.com/sharenet_rn.htm) to evaluate phylogenetic relationships among haplotypes. Possible historical demographic expansions of the three species were examined using the Tajima *D* test (Tajima 1989) and *F*s test of Fu (1997). *D* values significantly different from 0 are usually correlated with selection, bottlenecks, or population expansion, and a significantly negative *F*'s value indicates

a recent demographic expansion. Mismatch distribution analysis (Schneider and Excoffier 1999) was also used to infer the demographic histories of the species. Unimodal pairwise mismatch distributions indicate that populations have experienced recent demographic expansion, while multimodal distributions are related to demographic equilibrium or decline (Slatkin and Hudson 1991; Rogers and Harpending 1992). Raggedness index (r) and P values were computed to test the significance of the population expansion model. All these analyses were conducted in Arlequin 3.1 (Excoffier et al. 2005). We estimated the expansion time for lineages using the equation $\tau = 2ut$ (where τ is the time in number of generations elapsed since the sudden expansion episode, u the mutation rate per generation for the total length of analyzed sequence, and t is the time measured in generations). Values of u were calculated in equation $u = 2\mu kg$, where μ is the mutation rate per nucleotide site per year (s/s/y), k the length of the cpDNA fragment, and g is the generation time in years. According to the average substitution rates of cpDNA genes in Angiosperms, we used a range of the mutation rate from 1.0×10^{-9} to 3.0×10^{-9} s/s/y (Wolfe et al. 1987) to estimate the expansion time. We used 2 years as the generation time in accordance with previous studies (Li 1995).

To determine possible genetic discontinuities among populations within species, a genetic landscape shape analysis was carried out using Alleles in Space (AIS) (Miller 2005) for each species. First, a connectivity network was generated on the basis of the geographic coordinates of sampling locations using the Delaunay triangulation rule (Brouns et al. 2003; Watson 1992). Then, the connectivity network was combined with the genetic distance matrix to form a landscape shape interpolation. The result of this procedure is a three-dimensional surface plot where x- and y-axes are equivalent to population geographical coordinates and the z-axis is genetic distance.

Phylogenetic analysis and estimation of divergence times

We reconstructed phylogenetic relationships of the haplotypes in the *A. nemorum* species group using two methods, maximum likelihood (ML) and Bayesian inference (BI). We chose *Aconitum gymnandrum*, *Consolida ajacis,* and *Delphinium elatum* as outgroups for the study. The cpDNA sequences of *C. ajacis* (GenBank accession no.: AF216578 for *psb*A-*trn*H and accession no.: JF331819 for *trn*S-*trn*G) and *A. gymnandrum* (GenBank accession no.: FJ418150.1 for *psb*A-*trn*H and accession no.: JF331856.1 for *trn*S-*trn*G) were downloaded from the GenBank database, and those of *D. elatum* (GenBank accession no.: KC837095 for *psb*A-*trn*H and accession no.: KC837081 for *trn*S-*trn*G) were obtained in this study.

PhyML 3.0 (Guindon et al. 2010) was used to perform the ML analysis. A HKY + I model of substitution was selected by Modeltest 3.7 (Posada and Crandall 1998) using the Akaike information criterion (AIC) (Kelchner and Thomas 2007). This model was also used in BI and BEAST analysis (see below). To evaluate clade support values, 1,000 replicates of bootstrap analysis were performed. BI analyses were carried out in MrBayes 3.2 (Ronquist et al. 2012). A Markov chain Monte Carlo (MCMC) was run for 20 million generations with two parallel searches using four chains, each starting with a random tree. Trees were sampled every 1,000 generations and the first 10 % was discarded as burn-in. Tracer 1.5 (Rambaut and Drummond 2007) was used to check whether the log likelihood (lnL) of sampled trees reached a stationary distribution.

As fossil record and specific substitution rates in *Aconitum* were lacking, we used the range of the synonymous substitution rates of cpDNA genes ($1.0–3.0 \times 10^{-9}$ s/s/y) (Wolfe et al. 1987) to estimate divergence time.

BEAST version 1.6.1 (Drummond and Rambaut 2007) was used to estimate dates of lineage divergence. To choose optimal parameters for the BEAST analysis, an initial MCMC was run for 20 million generations using an uncorrelated lognormal clock model and a constant population size. TRACER version 1.5 (Rambaut and Drummond 2007) was used to examine the parameter ucld.stdev and whether it was greater than 1 or close to 0. A parameter value greater than 1 would suggest that our data was appropriate for a relaxed molecular clock model. The final MCMC was run for 20 million generations with sampling every 1,000 generations. Two independent runs achieved the same results. The effective sample sizes (ESS) of each parameter from TRACER version 1.5 were required to have values greater than 200. The maximum clade credibility (MCC) tree was generated using TreeAnnotator version 1.6.1 (Drummond and Rambaut 2007).

Results

Sequence diversity and haplotype distribution

The lengths of the *psb*A-*trn*H and *trn*S-*trn*G sequences were 268 and 739 bp, respectively. A total of 13 nucleotide substitutions and 14 indels/inserts were found in the concatenated *psb*A-*trn*H and *trn*S-*trn*G sequences (Table S1). According to the presence of nucleotide substitutions and indels, a total of 16 haplotypes were identified in the 18 populations, including 6 haplotypes (H11, H12, H13, H14 H15, H16) in *A. nemorum* s. str., 3 (H1, H2, H3) in *A. soongoricum*, and 7 (H4, H5, H6, H7, H8, H9, H10) in *A. karakolicum* (Table S1; Fig. 1). No haplotypes were shared among any of the three species. The nucleotide

composition of the two cpDNA spacers consisted of A (32.67 %), T (36.67 %), G (14.39 %), and C (16.27 %). The intra-population haplotype diversity (h) and nucleotide diversity (π) of *A. soongoricum* were 0, expectation for population JMN was 0.559 and 0.429, respectively; *A. nemorum* s. str. and *A. karakolicum* had high intra-population haplotype diversity (h) and nucleotide diversity (π), except for the QTJ and GNSD populations (Table 1).

Nine of 18 populations were fixed for private haplotypes, including 8 [YW (H2), HM (H2), BNK (H2), EM (H2), HF (H1), TC (H1), TL1 (H1), TL2 (H1)] belonging to *A. soongoricum*, whereas the remainder were polymorphic (Fig. 1). In *A. karakolicum*, each of the populations had two or more haplotypes, but only haplotype H7 was shared, between populations ZS and GNSD; in *A. nemorum* s. str., haplotype H14 was private in population QTB, but all of the other haplotypes were shared among two or more populations (Fig. 1).

Phylogeographic analysis

Neutral tests were carried out in Arlequin 3.1, the results of which revealed dissimilar patterns for the three species. For the Tajima's D test, only *A. karakolicum* showed negative values, while values for the other two species were larger than zero (Table 2). The value of Fs was negative in *A. nemorum* s. str., but greater than zero in *A. soongoricum* and *A. karakolicum* (Table 2). AMOVA analysis showed that a large proportion of the variation occurred among species (82.9 %), accompanied by 15.4 % of variation among populations within species and 1.7 % of variation within populations (Table 3). Populations of *A. nemorum* s. str. displayed unimodal distributions, while populations of *A. soongoricum* and *A. karakolicum* had multimodal distributions in mismatch distribution analysis (Fig. 2). The expansion time for *A. nemorum* s. str. was estimated at 190–63.35 thousand years ago.

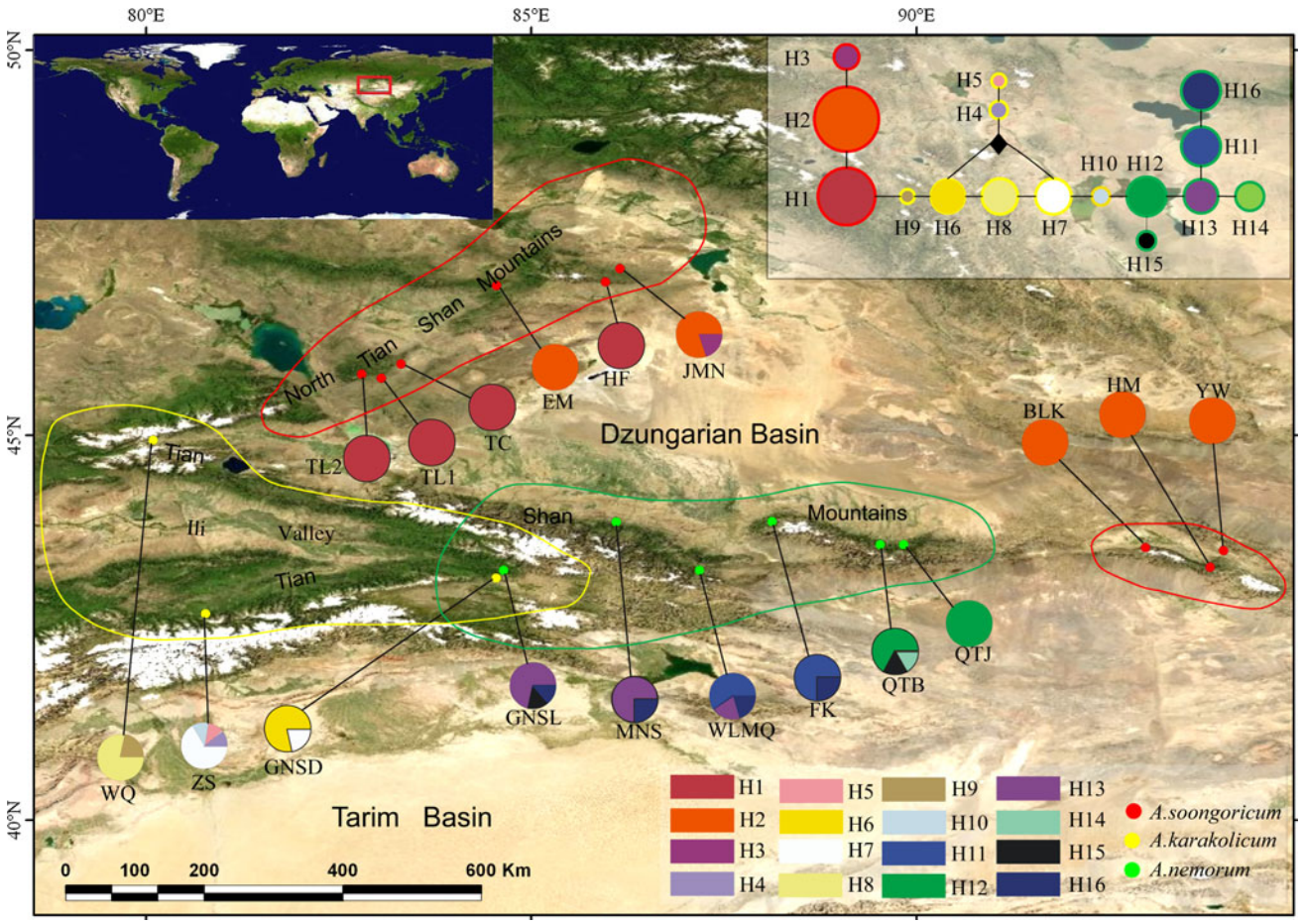

Fig. 1 Geographical distribution and genealogical relationships of the 16 haplotypes in the *Aconitum nemorum* species group from the Tianshan Mountains. The *red*, *yellow*, and *green closed curves* represent the main distribution ranges of *A. soongoricum*, *A. karakolicum* and *A. nemorum* s. str., respectively. The *pie charts* reflect the frequency of occurrence of each haplotype in each population. *Haplotype colors* correspond to those shown in the *lower right corner panel*. The median-joining network for the 16 haplotypes is shown in the *upper right corner* and the *sizes* of the *circles* in the network are proportional to haplotype frequencies. An intermediate haplotype not found among the analyzed individuals is indicated by a *black rhombus*

Table 2 Results of neutral tests and mismatch analysis

	Tajima's *D* test		Fu's *F* test		Mismatch distribution		
	D	*P*	*Fs*	*P*	τ	Θ_0	Θ_1
A. nemorum s. str.	0.336	0.642	−2.215*	0.011	1.721	0	–
A. soongoricum	6.039	0.989	21.83	0.992	–	–	–
A. karakolicum	−0.954	0.05	0.542	0.308	–	–	–

* Values are significant

Table 3 AMOVA analysis results of chlorotype frequencies for population groups of *Aconitum nemorum* species group, partitioned by species

Source of variation	*df*	SS	VC	PV	Fixation index
Among groups	1,417.68	15.21	15.21 Va	82.87	$F_{SC} = 0.90$
Among populations	347.53	2.83	2.83 Vb	15.42	$F_{ST} = 0.98$
Within populations	40.28	0.31	0.31 Vc	1.71	$F_{CT} = 0.83$
Total	1,805.49	18.35	18.35		

Genetic landscape shape analysis showed different spatial genetic patterns for *A. soongoricum* than for the other species. For *A. nemorum* s. str. and *A. karakolicum*, higher peaks occurred for the eastern populations, and genetic distances decreased progressively from east to west. *A. soongoricum* had higher peaks in the widely separated southeastern and northwestern populations (Fig. 3).

Phylogenetic analysis and divergence dating estimation

The phylogenetic trees of haplotypes from the BI and ML methods had similar topologies (Figs. S1, S2). This phylogeny contained three clades showing high bootstrap values (Figs. S1, S2) and placed *A. soongoricum* and *A. karakolicum* in Clade 1 and Clade 2, respectively, except that one transitional haplotype of *A. karakolicum* was placed in the *A. nemorum* s. str. clade (Clade 3).

Similar to BI and ML analysis, we obtained the same three clades from BEAST divergence time estimations. The divergence time between Clade 1 and the other two clades was estimated at 11.74–3.88 million years ago, and that of Clade 2 and Clade 3 at 5.45–1.79 million years ago (Fig. 4). Intra-specific divergence time was indicated as Pliocene to late Pleistocene (Fig. 4).

Discussion

Phylogenetic relationships and restriction of gene flow

Significant phylogenetic and phylogeographical structure for the *A. nemorum* species group was shown by this study (Table 3; Fig. 4; Figs. S1, S2), and the identified haplotypes were clustered into three major clades. Although haplotype H10 from *A. karakolicum* was placed in the *A. nemorum* s. str. clade (Clade 3), the haplotype network shows that each species has its own specific lineage (Figs. 1, 4) and that each species has clear boundaries. Gene flow is restricted among species, as evidenced by high levels of genetic differentiation (Table 3). Gene flow in maternally inherited organellar DNA usually occurs by exchange of seeds, and long-distance dispersal of these usually relies on water, animals, and wind (Manzano and Malo 2006; Nathan and Muller-Landau 2000). In the *A. nemorum* species group, the distribution range of *A. soongoricum* is different from that of *A. karakolicum* and *A. nemorum* s. str., and so little gene flow between this species and the other two would be expected. In addition, since fruits and seeds of the species group are not specialized for dispersal, this would tend to limit dispersal distance. The distribution ranges of *A. karakolicum* and *A. nemorum* s. str. overlap only at Gongnaisi Valley (populations GNSL and GNSD for *A. nemorum* s. str. and *A. karakolicum*, respectively). Evidenced from our field observations, *A. karakolicum* fruition is in mid-August when *A. nemorum* s. str. is only flowering. The difference in blooming phases of the two species likewise restricts the potential for mating between them and enhances the overall restriction of gene flow within the species group.

Potential refugia and demographic dynamics of the *A. nemorum* species group

Each species sampled showed a distinct expansion/contraction pattern. Neutral tests ($D = 0.336$, $P = 0.642$; $Fs^* = -2.215$, $P = 0.011$) and mismatch distribution analysis (Fig. 2) revealed that *A. nemorum* s. str. likely experienced demographic expansions. The dominant haplotypes of the species in the eastern and western parts of the distribution were H12 and H13, respectively. Phylogenetic network analysis showed that these two haplotypes are closely related to those of *A. karakolicum* (Fig. 1). Thus, they apparently originated from the Ili Valley, where *A.*

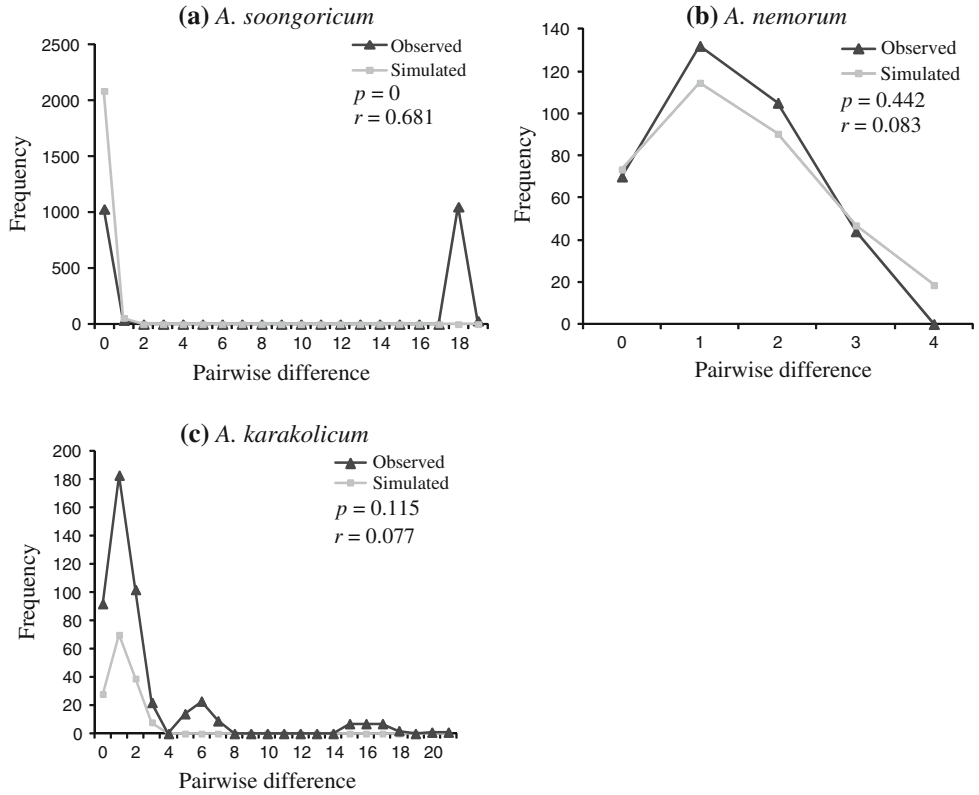

Fig. 2 The mismatch distributions of cpDNA for the three species; *r* represents the raggedness index and *p* represents the significance of a simulation value greater than the observed value

karakolicum is present. Meanwhile, haplotype H12 only observed in populations QTB and QTJ is nearer to the haplotypes of *A. soongoricum* in the phylogenetic tree (Fig. 4), and is ancestral to H13. A decreasing trend of genetic variation from one area to another is usually associated with species range expansion (Hewitt 2000). Thus, based on the phylogenetic network and haplotype spatial distributions, we infer that *A. nemorum* s. str. experienced an expansion from west (Ili Valley) eastwardly along the Tianshan Mountains. The high level of genetic distance between populations QTB and QTJ (Fig. 3) indicates that divergence between these and the remainder of the species has continued for a lengthy period. Considering the multiple glaciations in the Tianshan Mountains during the late Quaternary (Xu et al. 2010b), an appropriate explanation for the spatial distribution patterns of haplotypes might be that haplotype H12 expanded from the Ili Valley to the location of population QTB and surrounding regions during an interglacial period. However, the western populations (FK, WLMQ and MNS) probably contracted to the Ili Valley during the next glacial. The present distribution of haplotypes has likely resulted from expansion from the Ili Valley and from population QTB to the east during interglacials. This status is similar to findings for the *D. naviculare* species group (Zhang and Zhang 2012). The expansion time of that species was estimated as 190–63.35 thousand years ago, which includes the last interglacial period (130–74 thousand years ago) (Nie et al. 1996). Palynological evidence from the Kansu Loess Plateau and Caiwopu Lake, showing dominant components of woody plants (41 %), indicates a warm-humid climate during the last interglacial in the Tianshan Mountains (Group of Comprehensive Survey for Xinjiang Resources Development, Chinese Academy of Sciences 1989). This favorable climate provided suitable habitats for *A. nemorum* s. str. and promoted demographic expansion from west to east during the last interglacial period.

Ili Valley, a biodiversity hotspot of Northwestern China (Tang et al. 2006), located near the juncture between the northern and southern branches of the Tianshan Mountains, is shown to be a biotic glacial refuge where plants persist during glacial periods (Zhang et al. 2008; Zhang and Zhang 2012). In terms of our genetic structure analysis, we can infer that the Ili Valley and the area of the QTB population were potential glacial refugia for the *A. nemorum* species group.

Expansions for the *A. karakolicum* and *A. soongoricum* populations were not supported by neutral tests and mismatch distribution analysis ($Fs > 0$ and multimodal distributions). Although only three populations of *A.*

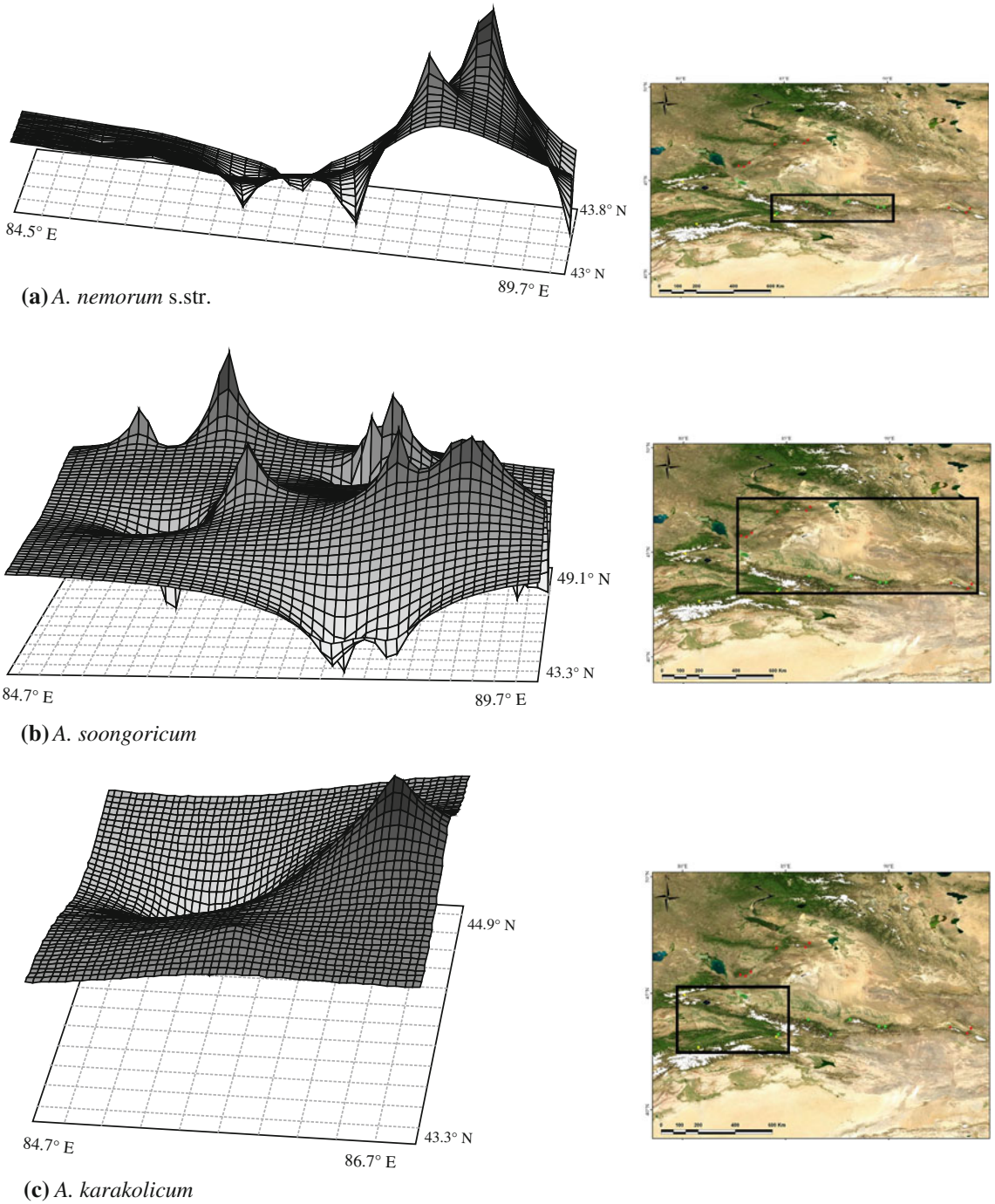

Fig. 3 Results of genetic landscape shape interpolation analysis (*left*) for each of the three species. The *x*- and *y*-axes correspond to geographic locations within the populations analyzed in the study, and the *z*-axis shows genetic distances. The *black rectangle* in the *right map* shows the locations of each of the study species

karakolicum were sampled in this study, the high intra-populational genetic diversity of this species indicated that it contracted to several refugia of the Ili Valley during the glacial period and divergence in situ, and did not experience interglacial expansion, as evidenced by the high level of genetic distance in the genetic landscape shape interpolation (Fig. 3).

Genetic divergence at intra- and inter-specific levels in the *A. nemorum* species group

The estimated divergence time of *A. soongoricum* (Clade 1) from the other two clades ranges from 11.74 to 3.88 million years ago (Fig. 4), while that of Clade 2 and Clade 3 was from 5.45 to 1.79 million years ago.

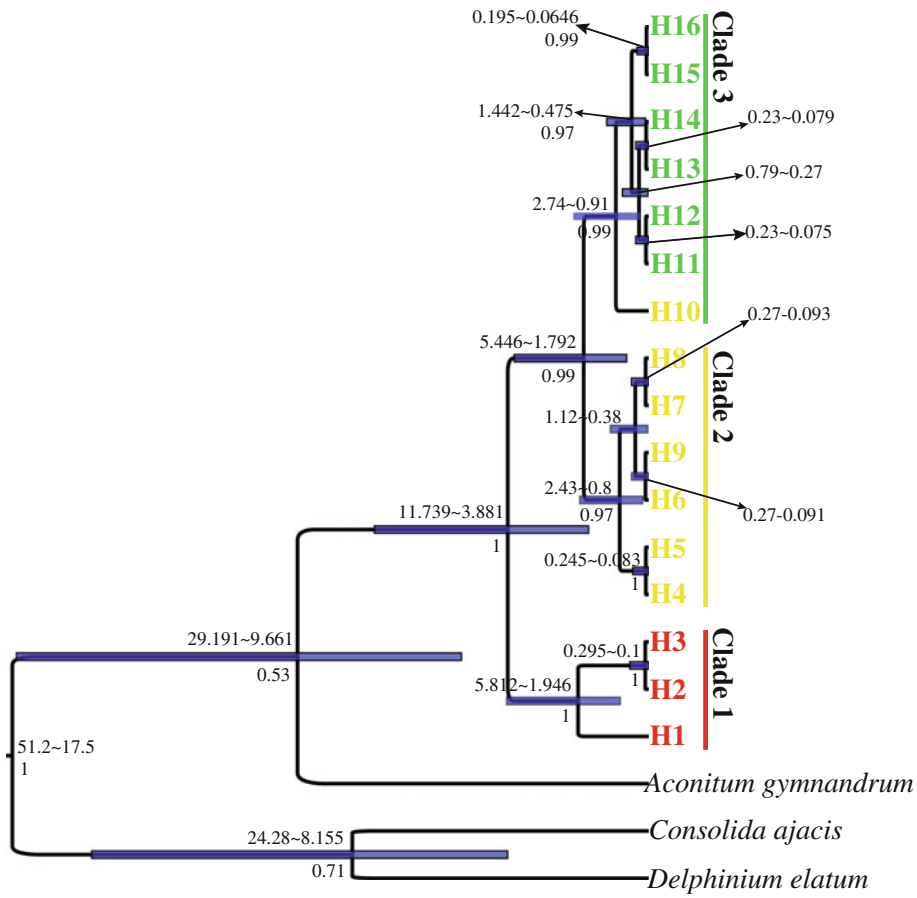

Fig. 4 BEAST Bayesian divergence time estimates of the *Aconitum nemorum* species group based on combined *trn*S-*trn*G and *psb*A-*trn*H cpDNA sequence data. The *values above the branching points* represent the range of divergence time (in million years ago); the *values below* represent posterior probabilities, and those larger than 0.5 are shown. The *red*, *yellow* and *green* haplotype names represent haplotypes belonging to *A. soongoricum*, *A. karakolicum* and *A. nemorum* s. str., respectively

Haplotype divergences within the species began in the Pliocene and mainly occurred at 1–0.064 million years ago. The timescales of interspecies and intraspecies divergence should at least be generally associated with geological and climatic events in Northwestern China. During the period of late Tertiary to early Pleistocene, the Tianshan Mountains were intensively raised at the same time as uplift of some parts of the northern QTP (Sun et al. 2004; Wang 2010). The rapid uplift of the Tianshan Mountains not only changed the topography but also resulted in enhancement of aridity in the surrounding region. Later, accelerated drying occurred in this area at 0.6–0.2 million years ago, as shown by evidence from loess sediments at Dongwanzhen in Shawan County on the northern slope of the Tianshan Mountains (Shi et al. 2006). Glacial advances resulted in a dry and cold climate in the Tianshan Mountains during the last glacial period (Xu et al. 2010b), and increased dryness may have been the reason for the lack of expansion among the study species subsequent to the Pleistocene.

The consistent timing between the molecular dating and geological events and Quaternary climatic fluctuations suggests that complex historical events occurring in montane habitats have promoted genetic divergence, local adaptation, speciation and dispersal of the *A. nemorum* species group. *A. soongoricum* is mainly distributed in the eastern and northern Tianshan Mountains (also called the Western Dzungaria Mountains), while *A. nemorum* s. str. and *A. karakolicum* are distributed in the central part. *A. soongoricum* does not overlap in distribution with the other two species. Rapid uplift of the Tianshan Range during the late Tertiary would likely have been the cause of the separation of the three species. Our phylogeographical history of the *A. nemorum* species group is somewhat similar to those of other taxa occurring along the Tianshan Mountains, for instance, the *Bufo viridis* subgroup (Zhang et al. 2008) and the *D. naviculare* species group (Zhang and Zhang 2012), which have relationships to mountain uplift and Quaternary glaciation.

In conclusion, we report phylogeographic structure and divergence times for the *A. nemorum* species group, and show that geographical isolation and the limited dispersal ability of seeds have resulted in restricted gene flow at inter-specific and intra-specific levels. In the intervals between glaciations in the Tianshan Mountains during the late Quaternary, *A. nemorum* s. str. underwent at least two periods of eastward expansion from glacial refugia. Ancient geological and climatic events thus likely affected

the evolution and current distribution of the *A. nemorum* species group. Genetic divergence among the three species from late Tertiary to early Quaternary appears to have been driven principally by rapid uplift of the Tianshan Mountains. The cold-dry to warm-humid climatic cycles during the late Quaternary are inferred to have promoted genetic divergence within the species.

Acknowledgments We are grateful to Kai-Qing Xie and Jian Zhang in Shihezi University for help in the material collection. Two anonymous reviewers and Dr. Isabel Sanmartin are deeply grateful for their helpful comment and suggestion on the manuscript. Funding was provided by CAS Important Direction for Knowledge Innovation Project (No. KZCX2-EW-305), and Xinjiang Institute of Ecology and Geography, Chinese Academy of Sciences.

References

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

Bandelt HJ, Forster P, Rohl A (1999) Median-joining networks for inferring intraspecific phylogenies. Mol Biol Evol 16:37–48

Brouns G, De Wulf A, Constales D (2003) Delaunay triangulation algorithms useful for multibeam echosounding. J Surv Eng-Asce 129:79–84

Clark MK, House MA, Royden LH, Whipple KX, Burchfiel BC, Zhang X, Tang W (2005) Late Cenozoic uplift of southeastern Tibet. Geol 33:525–528

Cun YZ, Wang XQ (2010) Plant recolonization in the Himalaya from the southeastern Qinghai-Tibetan Plateau: geographical isolation contributed to high population differentiation. Mol Phylogenet Evol 56:972–982

Doyle JJ, Doyle JL (1987) A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochem Bull 19:11–15

Drummond AJ, Rambaut A (2007) BEAST: bayesian evolutionary analysis by sampling trees. BMC Evol Biol 7:214

Espíndola A, Pellissier L, Maiorano L, Hordijk W, Guisan A, Alvarez N (2012) Predicting present and future intra-specific genetic structure through niche hindcasting across 24 millennia. Ecol Lett 15:649–657

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

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

Group of Comprehensive Survey for Xinjiang Resources Development, Chinese Academy of Sciences (1989) Geology and environment in the Quaternary period in Xinxjiang. China Agriculture Press, Beijing

Guan KJ, Xiao PG, Wang WC, Pang KY (1979) Flora of China, vol 27. Science Press, Beijing

Guindon S, Dufayard J-F, Lefort V, Anisimova M, Hordijk W, Gascuel O (2010) New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. Syst Biol 59:307–321

Hamilton MB (1999) Four primer pairs for the amplification of chloroplast intergenic regions with intraspecific variation. Mol Ecol 8:521–523

Hardy ME, Grady JM, Routman EJ (2002) Intraspecific phylogeography of the slender madtom: the complex evolutionary history of the Central Highlands of the United States. Mol Ecol 11:2393–2403

Hewitt G (2000) The genetic legacy of the Quaternary ice ages. Nature 405:907–913

Hewitt G (2004) Genetic consequences of climatic oscillations in the Quaternary. Philos T Roy Soc B 359:183–195

Jia DR, Abbott RJ, Liu TL, Mao KS, Bartish IV, Liu JQ (2012) Out of the Qinghai-Tibet Plateau: evidence for the origin and dispersal of Eurasian temperate plants from a phylogeographic study of *Hippophae rhamnoides* (Elaeagnaceae). New Phytol 194:1123–1133

Kelchner SA, Thomas MA (2007) Model use in phylogenetics: nine key questions. Trends Ecol Evol 22:87–94

Li LQ (1995) The geographical distribution of Subfam. Helleboroideae (Ranunculaceae). Acta Phytotaxon Sin 33:537–555

Li ZH, Chen J, Zhao GF, Guo YP, Kou YX, Ma YZ, Wang G, Ma XF (2012) Response of a desert shrub to past geological and climatic change: a phylogeographic study of *Reaumuria soongarica* (Tamaricaceae) in western China. J Syst Evol 50:351–361

Librado P, Rozas J (2009) DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. Bioinformatics 25:1451–1452

Liu YF, Wang Y, Huang HW (2009) Species-level phylogeographical history of *Myricaria* plants in the mountain ranges of western China and the origin of *M. laxiflora* in the Three Gorges mountain region. Mol Ecol 18:2700–2712

Liu JQ, Sun YS, Ge XJ, Gao LM, Qiu YX (2012) Phylogeographic studies of plants in China: advances in the past and directions in the future. J Syst Evol 50:267–275

Luo Y, Zhang F, Yang QE (2005) Phylogeny of *Aconitum* subgenus *Aconitum* (Ranunculaceae) inferred from ITS sequences. Plant Syst Evol 252(1–2):11–25

Manzano P, Malo JE (2006) Extreme long-distance seed dispersal via sheep. Front Ecol Enviro 4:244–248

Meng HH, Zhang ML (2011) Phylogeography of *Lagochilus ilicifolius* (Lamiaceae) in relation to Quaternary climatic oscillation and aridification in northern China. Biochem Syst Ecol 39:787–796

Miller MP (2005) Alleles In Space (AIS): computer software for the joint analysis of interindividual spatial and genetic information. J Hered 96:722–724

Muellner AN, Tremetsberger K, Stuessy T, Baeza CM (2005) Pleistocene refugia and recolonization routes in the southern Andes: insights from *Hypochaeris palustris* (Asteraceae, Lactuceae). Mol Ecol 14:203–212

Nathan R, Muller-Landau HC (2000) Spatial patterns of seed dispersal, their determinants and consequences for recruitment. Trends Ecol Evol 15:278–285

Nie GZ, Liu JQ, Guo ZT (1996) The major stratigraphic boundaries and climatic events in Weinan loess section since 0.15 Ma BP: based on chronological evidences. Quat Sci 16:221–231

Posada D, Crandall KA (1998) MODELTEST: testing the model of DNA substitution. Bioinformatics 14:817–818

Qiu YX, Li Y, Zhai SN, Guo YP, Ge XJ, Comes HP (2011a) Glacial survival east and west of the 'Mekong-Salween Divide' in the Himalaya-Hengduan Mountains region as revealed by AFLPs and cpDNA sequence variation in *Sinopodophyllum hexandrum* (Berberidaceae). Mol Phylogenet Evol 59:412–424

Qiu YX, Fu CX, Comes HP (2011b) Plant molecular phylogeography in China and adjacent regions: tracing the genetic imprints of Quaternary climate and environmental change in the world's most diverse temperate flora. Mol Phylogenet Evol 59:225–244

Rambaut A, Drummond A (2007) Tracer v1.4. http://beast.bio.ed.ac.uk/Tracer

Rogers AR, Harpending H (1992) Population-growth makes waves in the distribution of pairwise genetic-differences. Mol Biol Evol 9:552–569

Ronquist F, Teslenko M, van der Mark P, Ayres DL, Darling A, Hohna S, Larget B, Liu L, Suchard MA, Huelsenbeck JP (2012) MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. Syst Biol 61:539–542

Sang T, Crawford DJ, Stuessy TF (1997) Chloroplast DNA phylogeny, reticulate evolution, and biogeography of *Paeonia* (Paeoniaceae). Am J Bot 84:1120–1136

Schaal BA, Hayworth DA, Olsen KM, Rauscher JT, Smith WA (1998) Phylogeographic studies in plants: problems and prospects. Mol Ecol 7:465–474

Schneider S, Excoffier L (1999) Estimation of past demographic parameters from the distribution of pairwise differences when the mutation rates very among sites: application to human mitochondrial DNA. Genetics 152:1079–1089

Shi ZT, Fang XM, Song YG, An ZS, Yang SL (2006) Loess sediments in the north slope of Tianshan Mountains and it's indication of desertification since Middle Pleistocene. Mar Geol Quat Geol 36:109–114

Slatkin M, Hudson RR (1991) Pairwise comparisons of mitochondrial-DNA sequences in stable and exponentially growing populations. Genetics 129:555–562

Stewart JR, Lister AM, Barnes I, Dalén L (2010) Refugia revisited: individualistic responses of species in space and time. Proc R Soc B 277:661–671

Su ZH, Zhang ML, Cohen J (2012) Phylogeographic and demographic effects of Quaternary climate oscillations in *Hexinia polydichotoma* (Asteraceae) in Tarim Basin and adjacent areas. Plant Syst Evol

Sun JM, Zhu RX, Bowler J (2004) Timing of the Tianshan Mountains uplift constrained by magnetostratigraphic analysis of molasse deposits. Earth Planet Sci Lett 219:239–253

Sun H, Zhang YH, Volis S (2010) Chloroplast phylogeny and phylogeography of *Stellera chamaejasme* on the Qinghai-Tibet Plateau and in adjacent regions. Mol Phylogenet Evol 57: 1162–1172

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

Tang Z, Wang Z, Zheng C, Fang J (2006) Biodiversity in China's Mountains. Front Ecol Environ 4:347–352

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res 25:4876–4882

Wang LN, Ji JQ, Sun DX (2010) The uplift history of south-western Tianshan: implications from AFT analysis of detrital samples. Chin J of Geophys 53:931–945

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). Mol Ecol 18:709–721

Watson DF (1992) Contouring: a guide to the analysis and display of spatial data (with programs on diskette). Pergamon Press, Oxford

Wolfe KH, Li WH, Sharp PM (1987) Rates of Nucleotide substitution vary greatly among plant mitochondrial, chloroplast, and nuclear Dnas. Proc Natl Acad Sci USA 84:9054–9058

Wulff EV (1943) An introduction to historical plant geography. Chronica Botanica Company, Waltham

Xu T, Abbott RJ, Milne RI, Mao K, Du FK, Wu G, Ciren Z, Miehe G, Liu J (2010a) Phylogeography and allopatric divergence of cypress species (Cupressus L.) in the Qinghai-Tibetan Plateau and adjacent regions. BMC Evol Biol 10:194

Xu XK, Kleidon A, Miller L, Wang SQ, Wang LQ, Dong GC (2010b) Late Quaternary glaciation in the Tianshan and implications for palaeoclimatic change: a review. Boreas 39:215–232

Yan S, Mu G, Xu Y, Zhao Z (1998) Quaternary environmental evolution of the Lop Nur region, China. Acta Geographical Sinica 53:332–340

Zhang HX, Zhang ML (2012) Genetic structure of the *Delphinium naviculare* species group tracks Pleistocene climatic oscillations in the Tianshan Mountains, arid Central Asia. Palaeogeogr Palaeoclimatol Palaeoecol 353–355:93–103

Zhang YJ, Stock M, Zhang P, Wang XL, Zhou H, Qu LH (2008) Phylogeography of a widespread terrestrial vertebrate in a barely-studied Palearctic region: green toads (*Bufo viridis* subgroup) indicate glacial refugia in Eastern Central Asia. Genetica 134:353–365

Zhao JD, Liu SY, He YQ, Song YG (2009) Quaternary glacial chronology of the Ateaoyinake River Valley, Tianshan Mountains, China. Geomorphology 103:276–284

Zhou WW, Wen Y, Fu JZ, Xu YB, Jin JQ, Ding L, Min MS, Che J, Zhang YP (2012) Speciation in the *Rana chensinensis* species complex and its relationship to the uplift of the Qinghai-Tibetan Plateau. Mol Ecol 21:960–973