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Research Article

Chloroplast DNA variation and phylogeography of *Ligularia tongolensis* (Asteraceae), a species endemic to the Hengduan Mountains region of China

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Abstract In this research, we aimed to study the genetic variation and phylogeographic pattern of *Ligularia tongolensis*, a perennial herb endemic to the Hengduan Mountains region of China. We sequenced two chloroplast DNA (cpDNA) intergenic spacers (*trn*Q-5′*rps*16, *trn*L-*rpl*32) in 140 individuals from 14 populations of three groups (Jinshajiang vs. Yalongjiang vs. Wumeng) within this species range. High levels of haplotype diversity (Hd = 0.814) and total genetic diversity (Ht = 0.862) were detected at the species level, based on a total of 12 haplotypes identified. Low levels of intrapopulation diversity (Hs = 0.349), high levels of genetic divergence (Gst = 0.595, Nst = 0.614, Fst = 0.597), and the absence of isolation by distance tests were also found in *L. tongolensis*. Furthermore, H2 and H5, the dominant haplotypes that located at internal nodes and deviated from extinct ancestral haplotype in the network, were found to be shared between Jinshajiang and Yalongjiang groups. These results indicate that past fragmentation may be the important factor responsible for the present phylogeographical pattern of *L. tongolensis*. Meanwhile, the locations occupied by each group might have served as independent refugia for *L. tongolensis* during the Quaternary glaciation. Unimodal mismatch distribution and star-like genealogies indicated this species underwent past demographic expansion events, with expansion ages of 274 ka BP.

Key words genetic variation, *Ligularia tongolensis*, phylogeography, Quaternary glaciation.

The Hengduan Mountains region (HMR), which lies at the southeastern edge of Qinghai-Tibetan Plateau (QTP), is listed as an Indo-Burma and Himalaya biodiversity hot spot, one of 10 biodiversity hot spots in the world (Myers et al., 2000). It contains more than 12 000 species of plant and is especially rich in endemic species and genera (Li, 1994; Hao, 1997; Wang, 2000; Sun, 2002). In contrast to European and North American flora, which have been outlined by many phylogeographic studies (Petit et al., 1997; Abbott et al., 2000; Abbott & Brochmann, 2003; Hewitt, 2004; Petit et al., 2005; Anderson et al., 2006; Soltis et al., 2006; Fér et al., 2007; Grassi et al., 2009), only a few plant species have been phylogeographically studied in the HMR and QTP (Chen et al., 2008; Wang et al., 2008; Yang et al., 2008; Wang et al., 2009; Wu et al., 2010). Although each species had its own history, all results showed that the Quaternary glaciation had an extensive effect on the genetic structure and distribution pattern of the species. Some researchers thought that the low altitude region at the eastern part of QTP (Wu et al., 2010) or the southeastern edge of QTP (HMR) (Chen et al., 2008; Yang et al., 2008) was the refugia of plants during the Quaternary glaciation. However, different lines of evidence showed there were multiple refugia at the interior of QTP platform (Wang et al., 2009). Nevertheless, all species mentioned above are distributed widely not only at the edge but also on the platform of QTP. It will be interesting to research when and how other species distributed only at the edge of QTP, such as the HMR, responded to the glaciation.

Here, we report a phylogeographic study of *Ligularia tongolensis* (Franch.) Hand.-Mazz. (Asteaceae), which is endemic to HMR. It is a diploid perennial herb ($2n = 2x = 58$) (Pan et al., 2004), now sporadically occurring in a narrow and fragmented range (Fig. 1). The genus *Ligularia*, including approximately 140 species, is distributed in Asia and Europe. Among them, 61 species are endemic to the HMR, which was

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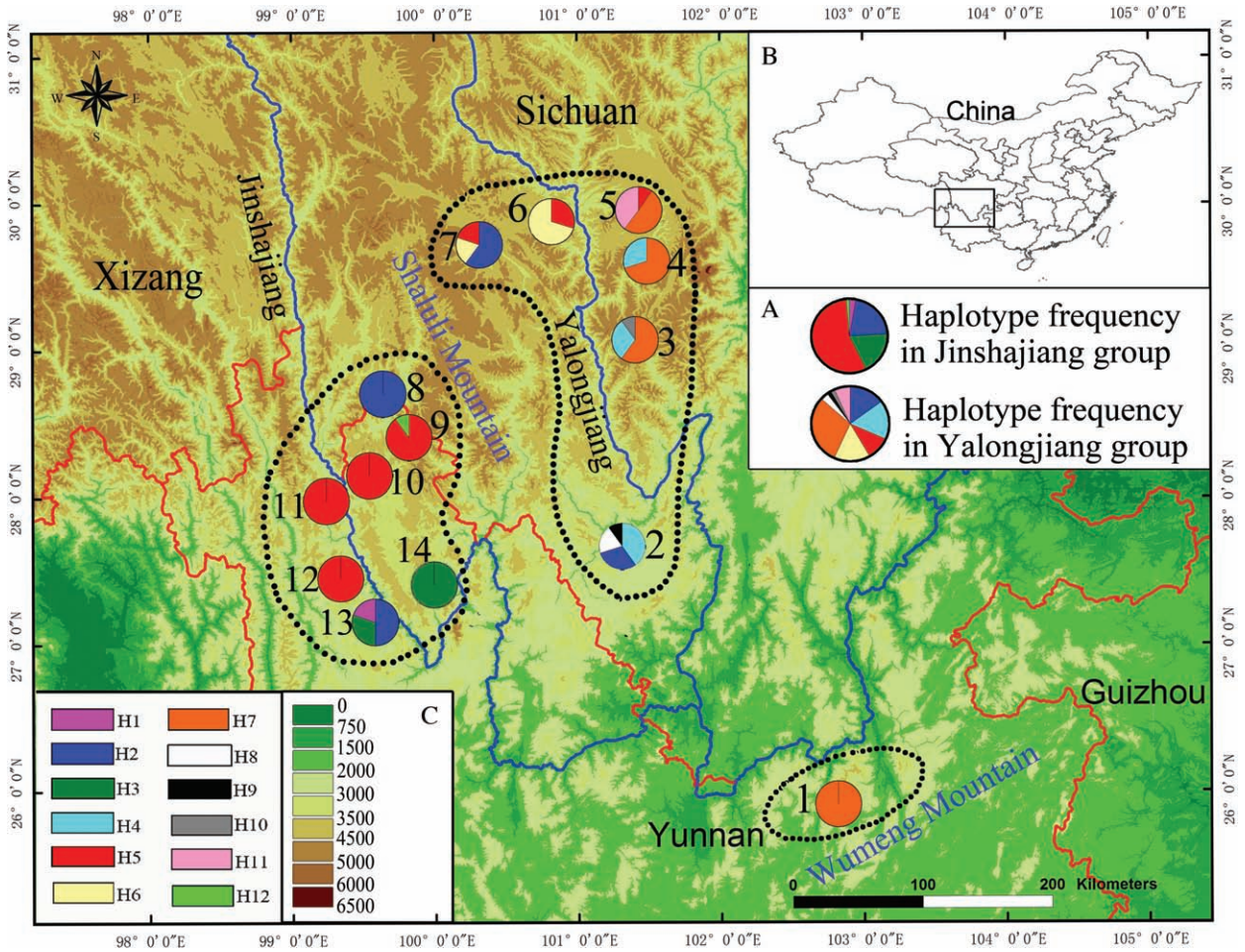

Fig. 1. Sample locations and chloroplast (cp)DNA haplotype distribution of *Ligularia tongolensis* in China. Main panel shows population names. Wumeng Mountain: 1, Wumengshan, Yunnan. Yalongjiang watershed: 2, Linchang, Sichuan; 3, Wuxuhai, Sichuan; 4, Pengbuxi, Sichuan; 5, Xinduqiao, Sichuan; 6, Yajiang, Sichuan; 7, Litang, Sichuan. Jinshajiang watershed: 8, Xiaoxueshan, Yunnan; 9, Xiaohengshan, Yunnan; 10, Gezan, Yunnan; 11, Nixi, Yunnan; 12, Shikashan, Yunnan; 13, Dabaoshan, Yunnan; 14, Qianhushan, Yunnan. Frequency of cpDNA haplotypes in each population is indicated by the pie diagrams. **A,** Frequency of cpDNA haplotypes in Jinshajiang and Yalongjiang groups. **B,** Map of China, indicating the distribution range of *Ligularia tongolensis* (square). **C,** Altitudinal scale (m). The haplotype frequency map was constructed using ArcGIS 9.0.

thought to be the diversity center and distribution center of this genus (Liu, 1989; Liu et al., 1994). *Ligularia tongolensis* is one of the *Ligularia* species endemic to the HMR. However, its distributional range is isolated by high mountains and deep valleys (Fig. 1). *Ligularia tongolensis* is involved in outcrossing, pollinated mainly by *Bombus lucorum* (Wang et al., 2007) and reproduces via achene (Liu, 1989) that is columniform, slippery, and 5 mm in length. The achene has pappi and is dispersed only by wind; there is no special mechanism for its dispersal. *Ligularia tongolensis* has an extremely varied habitat, ranging from forest fringes at a 2100 m altitude to alpine meadows at a 4000 m altitude. It is distributed intermittently throughout two sides of Shaluli Mountain, and restricted to only one place at Wumeng Mountain (Fig. 1). Shaluli Mountain, the broadest mountain range in the HMR, is located between Yalongjiang and Jinshajiang in the southeastern QTP (Fig. 1). Wumeng Mountain is located in the northeast of Yunnan Province and northwest of Guizhou Province, and extends from northeast to southwest with an average altitude of 2000 m, and the highest peak of 4000 m. It is one of the highest mountains among the same latitudinal belt in the northern hemisphere. According to its geographic distribution, there could be differentiation between populations of *L. tongolensis* on Wumeng Mountain and the west and east sides of Shaluli Mountain, which are Jinshajiang and Yalongjiang.

Previously, using inter-simple sequence repeats (ISSRs), Wang et al. (2007) estimated the genetic variation and differentiation levels for *L. tongolensis*. The results revealed a high level of genetic variation and

differentiation. Limited gene flow was detected due to the habitats' discontinuity. However, the ISSR analysis did not reveal any clear phylogeographical structure for this species. Chloroplast DNA (cpDNA) is transmitted by maternal inheritance in most angiosperms, and often shows a more highly geographical structure than the nuclear genome (Schaal et al., 1998; Petit et al., 2003). Furthermore, as it has a low frequency of genetic recombination (Birky et al., 1989; Ennos, 1994; Martinez et al., 1997), the maternal lineage can be traced at the level of whole chloroplast genome. Thus, in this study, we investigated the genetic variation and phylogeographic pattern of *L. tongolensis* using two cpDNA regions, *trn*Q-5′*rps*16 and *trn*L-*rpl*32. We focused on the following issues: (i) the distribution of genetic variation and the genetic structure of *L. tongolensis*, especially its association with geographic areas; (ii) the driving forces shaping the population structure; and (iii) the influence of the Quaternary glaciation on the species.

1 Material and methods

1.1 Population sampling

In this research, we carried out extensive field investigations to cover the whole distributional range of *L. tongolensis* from year 2001 to 2009. A total of 14 populations were collected, covering the entire geographic range for this species (Fig. 1). However, no other populations could be found, neither between populations 1 and 2 nor in other mountain areas. This was possibly due to the low elevation of these areas (Fig. 1). Leaves were collected from the plants at intervals of 5 m then dried directly in silica gel. Ten individuals per population, a total of 140 individuals from 14 populations, were sampled and analyzed in this study. Although *L. tongolensis* is distributed sympatrically with other *Ligularia* species, all sampled population individuals belong to the same species morphologically. In the phylogenetic analysis, the sequences of one individual of *Ligularia nelumbifolia* Hand.-Mazz. and two individuals of *Ligularia hodgsonii* Hook., which are two co-occurring species with *L. tongolensis*, were included to verify if all recovered haplotypes form a monophyletic lineage. Voucher specimens were deposited in the Kunming Institute of Botany, Chinese Academy of Sciences (KUN).

1.2 DNA extraction, PCR amplification, and sequencing

Total genomic DNA was extracted using a modified cetyltrimethyl ammonium bromide protocol (Doyle & Doyle, 1990). Fourteen pairs of cpDNA primers (Shaw et al., 2007) were tested to detect possible intraspecific variations across the 14 individuals from each population surveyed. Finally, we chose the *trn*Q-5′*rps*16 and *trn*L-*rpl*32 intergenic spacers for the full survey, as they contained the most polymorphic sites after a preliminary primer screening. Both of these chloroplast regions were successfully amplified and sequenced from all individuals. Polymerase chain reaction was carried out in a reaction volume of 20 μL, containing 10 ng of template DNA, 2.0 μL of 10 $\times$ PCR buffer with $(NH_4)_2SO_4$, 1.5 μL $MgCl_2$ (25 mmol/L), 1.5 μL dNTP (2.5 mmol/L each), 0.15 mmol/L each primer, and 1.5 units TaKaRa Taq polymerase (Takara, Shiga, Japan). DNA amplification was carried out in a T1 thermocycler (Biometra, Göttingen, German), programmed for an initial 5 min of denaturation at 94 °C, followed by 29 cycles of 1 min at 94 °C, 1 min annealing at 50 °C, 1.5 min extension at 72 °C, and a final extension cycle of 5 min at 72 °C. All PCR products were purified directly by a PCR product purification kit, following the manufacturer's protocol (Sangon Biological Engineering Technology & Services, Shanghai, China). Sequencing reactions were carried out with the same primers used in the amplification reactions in an ABI 3770 automated sequencer at Sangon.

1.3 Data analysis

Sequences were manually assembled and aligned using ClustalX version 1.81 (Thompson et al., 1997) then manually adjusted. Length variations in mononucleotide repeats (poly A or T stretches) were excluded, given that they are prone to homoplasy. A matrix of combined sequences of the two cpDNA regions (*trn*Q-5′*rps*16 and *trn*L-*rpl*32) was constructed for the following analyses.

The program SAMOVA version 1.0 (Dupanloup et al., 2002) was implemented to define groups of populations (K) that are geographically homogeneous and maximally differentiated from each other. In the analysis, K was varied from 2 to 13 with each simulation, starting from 100 random initial conditions. The F_{CT} index (Wright, 1978) of genetic differentiation among initial K groups was recorded to determine the best grouping of populations. However, the SAMOVA tests failed to reveal any meaningful phylogeographic groupings. The F_{CT} fluctuated irregularly when K (the number of groups) was raised from 2 to 13. This result can not be used as the basis of grouping. So we divided the populations based on their geographic distribution, which can help us detect the existence of genetic differentiation among regions. All populations could be divided into three geographic groups. The first group with only one population (1) was located at Wumeng Mountain

Table 1 Details of sample localities in China for the 14 *Ligularia tongolensis* populations studied

Population	Locality, province	Latitude, longitude	Altitude (m)	Haplotypes (no. of individuals)	*H*d	π
Wumeng Mountain						
1	Wumengshan, Yunnan	24.63°N, 103.12°E	3200	*H7(10)*	0.000	0.00000
Yalongjiang watershed				H2(9), H4(10), H5(6), H6(9), *H7(39)*, H8(2), H9(1), H10(1), H11(4)		
2	Linchang, Sichuan	27.76°N, 101.33°E	3700	H2 (3), H4 (4), H8 (2), H9 (1)	0.778	0.00277
3	Wuxuhai, Sichuan	29.09°N, 101.24°E	3700	H4 (3), *H7 (6)*, H10 (1)	0.600	0.00224
4	Pengbuxi, Sichuan	29.42°N, 101.30°E	3300	H4 (3), *H7(7)*	0.467	0.00213
5	Xinduqiao, Sichuan	30.04°N, 101.27°E	3510	H5 (1), *H7 (5)*, H11 (4)	0.644	0.00095
6	Yajiang, Sichuan	29.59°N, 100.54°E	3550	H5 (3), H6 (7)	0.467	0.00027
7	Litang, Sichuan	29.49°N, 100.20°E	3950	H2 (6), H5 (2), H6 (2)	0.622	0.00081
Jinshajiang watershed				H1(2), H2(15), H3(3), *H5(39)*, H12(1)		
8	Xiaoxueshan, Yunnan	28.80°N, 99.59°E	3700	H2 (10)	0.000	0.00000
9	Xiaohengshan, Yunnan	28.49°N, 99.78°E	3600	*H5 (9)*, H12 (1)	0.200	0.00046
10	Gezan, Yunnan	28.26°N, 99.55°E	3500	*H5 (10)*	0.000	0.00000
11	Nixi, Yunnan	28.03°N, 99.25°E	3520	*H5 (10)*	0.000	0.00000
12	Shikashan, Yunnan	27.50°N, 99.41°E	3500	*H5 (10)*	0.000	0.00000
13	Dabaoshan, Yunnan	27.27°N, 99.65°E	3200	H1 (2), H2 (5), H3 (3)	0.689	0.00067
14	Qianhushan, Yunnan	27.41°N, 99.83°E	3400	H3 (10)	0.000	0.00000
Total					0.814	0.00174

Chloroplast DNA haplotype distribution, estimates of haplotype diversity (*H*d), and nucleotide diversity (π) are indicated for each population. Italicized text shows major dominant haplotype in geographic regions.

(Table 1, Fig. 1). The second group of populations (2–7) was located on the Yalongjiang watershed, and the third group of populations (8–14) was distributed on the Jinshajiang watershed (Table 1, Fig. 1). The following analysis with subgroups was done according to this grouping.

Haplotype diversity (*H*d) and nucleotide diversity (π) were determined using DnaSP version 4.0 (Rozas et al., 2003). A haplotype frequency map was constructed using ArcGIS 9.0. Two parameters for population differentiation (*G*st, *N*st) and two for genetic diversity (*H*s, *H*t) were analyzed using the program Permut version 1.0 (Pons & Petit, 1996) (available at http:// www.pierroton.inra.fr/genetics/labo/Software/PermutCpSSR/index.html) with 10 000 permutations. We compared *G*st and *N*st using *U*-statistic. A significantly higher *N*st over *G*st usually indicates the presence of phylogeographic structure and that the populations are strongly differentiated genetically. If *N*st is equal to *G*st, then it is likely the haplotypes are phylogenetically equivalent. Finally, if *N*st is significantly smaller than *G*st, then the relative geographic distribution of haplotypes is likely unrelated to their genetic distances (Pons & Petit, 1996).

Next we sought to infer any phylogenetic relationships among haplotypes of *L. tongolensis*. Maximum parsimony analysis was carried out using the program PAUP* version 4.0b10 (Swofford, 2002). Gaps were treated as missing data. Full heuristic tree searches were carried out with 100 replications of "random" sequence entries, TBRbranch swapping, the "MulTrees" option in effect, and using unweighted characters. Branch supporting values were assessed by bootstrap analysis with 1000 replicates of the full heuristic searches using the above settings.

In addition, a cpDNA haplotype network was estimated by the TCS program version 1.21 (Clement et al., 2000). In this analysis, indels were treated as single mutation events.

To explain regional differences, we quantified the proportion of total genetic variance between groups and those between populations within groups using AMOVA (Excoffier et al., 1992) in the program Arlequin version 3.0 (Excoffier et al., 2005). The *F* statistic (*F*st) was calculated, and the significance was tested using 10 000 permutations.

To test for isolation by distance (IBD) between populations, estimates of F_{ST} were regressed against the natural logarithm of geographic distance for all pairs of populations (Rousset, 1997). Pairwise *F*st values were calculated using Arlequin, and the geographic distance between populations was calculated using the program found at http://www.indo.com/distance/. A Mantel test was used to evaluate the significance with 1000 permutations with IBDWS version 3.16 (Jensen et al., 2005).

We estimated the coalescence times (the most recent common ancestor, TMRCA) of all cpDNA haplotypes for *L. tongolensis* using the program Beast version 1.6.0 (Drummond & Rambaut, 2007). Five independent analyses were run for 3.0×10^7 generations under the GIR + G substitution model, which was chosen by hierarchical likelihood ratio tests implemented in Modeltest version 3.7 (Posada & Crandall, 1998). Convergence of the chains to the stationary distribution was checked using the software TRACER version 1.5 (Rambaut & Drummond, 2009). To estimate TMRCA

in years, an appropriate rate is necessary, but neither a well-documented cpDNA evolutionary rate nor a fossil for calibrating the nucleotide substitution rate has been reported for *Ligularia*. Therefore, we used the rate of 1.52×10^{-9} substitutions per site per year (s/s/y), an evolutionary rate recently proposed by Yamane et al. (2006).

To assess any evidence of a recent population expansion, we compared the observed number of differences between pairs of cpDNA haplotypes to their theoretical distribution using a sudden (stepwise) expansion model (Rogers & Harpending, 1992) with the software DnaSP version 4.0 (Rozas et al., 2003). Mismatch distributions for each sample to distinguish between models invoke past exponential growth and historical population stasis. The parameters of demographic expansion and spatial expansion were estimated using the methods of Schneider & Excoffier (1999) using Arlequin version 3.0 (Excoffier et al., 2005). The sum of squared deviations (SSD) between the observed and expected mismatch distributions were computed, and *P*-values were calculated as the proportion of the simulations that produced a larger SSD than that of the observed. The raggedness index (*HRag*) and its significance were also calculated to quantify the smoothness of the observed mismatch distribution. If the sudden expansion model was not rejected, then the expansion parameter (τ) was converted to an estimate of time (T, in number of generations) since expansion began using $T = \tau/2u$ (Rogers & Harpending, 1992; Rogers, 1995), where u is the neutral mutation rate for the entire sequence (namely, haplotype) per generation. The value for u was calculated as $u = \mu kg$, where μ is the substitution rate in s/s/y, k is the average sequence length of the particular DNA region (here 1761 bp; see the Results section) and g is the generation time in years (i.e., age of first reproduction). The value for g was approximated as three years based on Xun Gong's observations for *L. tongolensis*, cultivated at the Kunming Botanic Garden. The rate of nucleotide substitution was the same as mentioned above for estimating TMRCA. A parametric bootstrap approach (Schneider & Excoffier, 1999) with 1000 replicates was used to obtain 95% confidence intervals around τ and test the observed mismatch distributions' fit to the sudden expansion model for *L. tongolensis*.

2 Results

2.1 Chloroplast DNA variation

The aligned cpDNA *trn*Q-5′*rps*16 data matrix was 953 bp in length and contained 12 polymorphic sites that resulted in nine haplotypes. For the *trn*L-*rpl*32 region, the aligned length was 808 bp, with eight haplotypes derived from nine polymorphic sites. The sequences of nine *trn*Q-5′*rps*16 and eight *trn*L-*rpl*32 haplotypes have been submitted to the GenBank databases under the accession numbers HM024728–HM024736 and HM024720–HM024727, respectively.

The total length of the combined *trn*Q-5′*rps*16 and *trn*L-*rpl*32 dataset was 1761 bp. With the combination of the two cpDNA fragments, 12 different haplotypes, H1 through H12 (Tables 1, 2) were identified for 140 samples of *L. tongolensis* based on 21 polymorphic sites detected (Table 2). Including sequences from *L. tongolensis* and three unique sequences from *L. nelumbifolia* and *L. hodgsonii*, the overall alignment was 1836 bp in length (the fully aligned data matrix is available upon request).

The results showed that the total haplotype diversity (Hd) and the total nucleotide diversity (π) for the whole dataset were estimated to be 0.814 and 0.00174, respectively. Among the 14 populations, haplotype diversity (Hd) ranged from 0 to 0.778, and nucleotide diversity (π) ranged from 0 to 0.00277 (Table 1). Notably, the population (population 1) at Wumeng Mountain had no variation. However, all populations (populations 2–7) from the Yalongjiang watershed had more than one haplotype and high genetic diversity (Table 1). Of the seven populations in the Jinshajiang region, five (populations 8, 10–12, 14) carried a single haplotype; two (populations 9, 13) included two or more haplotypes.

2.2 Geographical distribution of cpDNA haplotypes

The distribution of 12 haplotypes (H1–H12) among the 14 sampled populations is shown in Fig. 1, and the number of individuals per haplotype within each population is detailed in Table 1. Eight out of the 14 populations (57.1%) were polymorphic for haplotype variations, including two populations in the Jinshajiang group (populations 9, 13) and all six populations in the Yalongjiang group (populations 2–7). The remaining five populations in the Jinshajiang group were fixed for one of three haplotypes (H2, population 8; H3, population 14; H5, populations 10–12). In addition, population 1 in the Wumeng group was fixed with haplotype H7. The haplotype distribution displayed a geographic structure between the Jinshajiang and Yalongjiang groups with the exception of two shared haplotypes, H5 and H2. Of them, H5 was the dominant haplotype (32.14%), and was shared among four populations (populations 9–12) of the Jinshajiang group and three populations (populations 5–7) of the Yalongjiang group. Another shared haplotype was H2 (17.14%), which was the third

Table 2 Variable sites from the aligned sequences of the two chloroplast DNA spacers in the 12 haplotypes (H) of *Ligularia tongolensis*. All sequences are compared to the reference haplotype H1

H	Variable sites																					No. of individuals	Frequency (%)
	*trn*Q-5′*rps*16												*trn*L-*rpl*32										
	1	4	4	5	5	6	6	7	8	8	8	9	1	2	3	4	4	4	5	5	6	NA	NA
	0	2	4	5	5	6	9	7	2	4	9	0	5	2	0	2	8	8	6	7	8	NA	NA
	9	9	8	4	5	6	7	7	1	1	9	4	6	9	8	4	0	7	4	7	5	NA	NA
H1	A	T	–	A	A	T	–	G	C	T	T	G	C	C	–	C	A	C	T	C	A	2	1.43
H2	·	·	·	·	·	·	·	·	·	C	·	·	·	·	·	·	·	A	·	·	·	24	17.14
H3	·	A	GTT	·	·	·	·	·	·	C	·	·	·	·	·	·	·	A	·	·	·	13	9.29
H4	·	·	GTT	·	·	C	TTA	·	·	C	·	·	·	·	AATAT	·	T	A	·	·	C	10	7.14
H5	·	·	GTT	C	T	·	·	·	·	C	·	·	·	·	·	·	·	A	·	·	·	45	32.14
H6	·	·	GTT	C	T	·	·	·	·	C	·	·	·	A	·	·	·	A	·	·	·	9	6.44
H7	T	·	GTT	C	T	·	·	T	T	C	·	·	·	·	·	·	·	A	·	·	·	28	20.00
H8	T	·	GTT	C	T	·	·	T	T	C	C	·	G	·	·	·	·	A	·	·	·	2	1.43
H9	T	·	GTT	C	T	·	·	T	T	C	·	·	·	·	·	T	·	A	·	·	·	1	0.71
H10	T	·	GTT	C	T	·	·	T	T	C	·	·	·	·	·	·	·	A	·	A	·	1	0.71
H11	T	·	GTT	C	T	·	·	T	T	C	·	A	·	·	·	·	·	A	A	·	·	4	2.86
H12	T	·	TTT	C	T	·	·	T	T	C	·	·	·	·	·	·	·	A	·	·	·	1	0.71

·, character states are the same as haplotype H1; –, deletion. NA, not applicable.

dominant haplotype and was shared between the two geographic groups (Jinshajiang, populations 8, 13; Yalongjiang, populations 2, 7). In the Jinshajiang group, three unique haplotypes were found. H1 was found only in population 13 for two individuals. H3 was distributed between population 13 and 14. H12 was distributed in population 9 only for one individual (Fig. 1, Table 1). In the Yalongjiang group, however, the pattern of haplotype distribution was more complex than that in the Jinshajiang group. Six haplotypes (H4, H6, H8, H9–H11) were found exclusively within the Yalongjiang group. H4 was distributed in three populations (populations 2–4) and H6 was distributed in two populations (populations 6, 7). H8 and H9 were found in population 2 for two and one individuals, respectively, H10 was found in population 3 for one individual, and H11 was found in population 5 for four individuals. The second dominant haplotype, H7 (20%), was shared between the Yalongjiang (populations 3–5) and Wumeng groups (population 1), and was fixed with the population of Wumeng Mountain. There was no shared haplotype between Jinshajiang watershed and Wumeng Mountain groups.

2.3 Phylogenetic analysis

All haplotypes of *L. tongolensis* and the sequences of *L. nelumbifolia* and *L. hodgsonii* were included in the phylogenetic analysis. The result showed that all *L. tongolensis* haplotypes formed one monophyletic group (61% bootstrap support) (Fig. 2), indicating that all of these divergent haplotypes were derived within *L. tongolensis* itself. Then *L. nelumbifolia* and *L. hodgsonii* were defined as outgroups to carry out intraspecific analyses. In the combined cpDNA dataset, a total of 16 characters were parsimony informative. An unweighted maximum parsimony analysis of *L. tongolensis* with outgroups generated a single most parsimonious tree consisting of 36 steps (consistency / retention index = 0.857/0.929). The monophyly of all

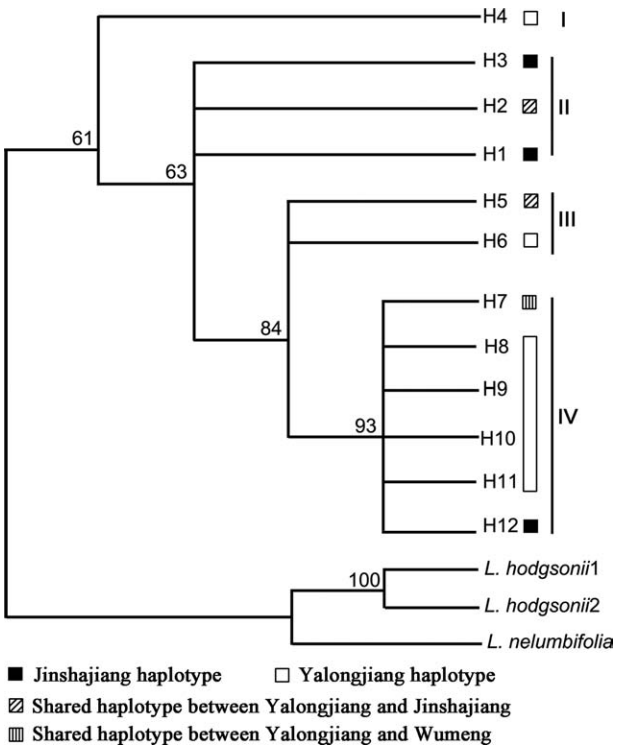

Fig. 2. Single most parsimonious tree for chloroplast DNA haplotypes of *Ligularia tongolensis* (H1–H12). Clades I –IV are indicated. Numbers on the branches indicate the bootstrap values from the maximum parsimony (1000 replicates) analyses.

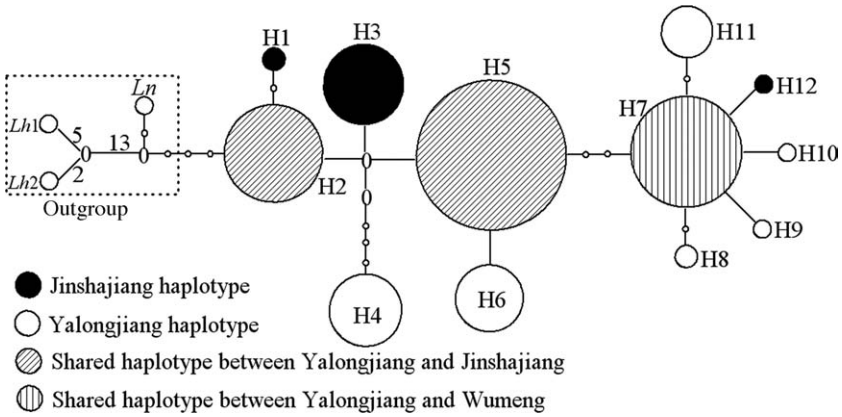

Fig. 3. Statistical parsimony network of the 12 chloroplast DNA haplotypes (H1–H12) of *Ligularia tongolensis* with one individual of *L. nelumbifolia* (*Ln*) and two individuals of *L. hodgsonii* (*Lh*1 and *Lh*2) as outgroups. The size of each circle is proportional to the haplotype frequency. Each solid line represents one mutational step that interconnects two haplotypes. The small open circles indicate inferred intermediate haplotypes not detected in this investigation. The numbers above or beside the line represent the mutational steps. 0 represents hypothetical ancestral nodes that were not detected or became extinct in populations.

ingroup haplotypes recovered four subclades (I–IV). However, the four clades did not form a clear pattern associated with the geographic groups among the Jinshajiang, Yalongjiang, and Wumeng groups. Hence, this topology suggested a paraphyletic structure of haplotypes from the three geographic groups. A parsimony network (Fig. 3) with one individual of *L. nelumbifolia* (*Ln*) and two individuals of *L. hodgsonii* (*Lh*1 and *Lh*2) as outgroups showed a similar structure to the phylogenetic tree. The three haplotypes (*Ln*, *Lh*1, and *Lh*2) of outgroups were connected to a missing haplotype by 2, 18, and 15 mutational steps, respectively. The dominant haplotype H5 and the third dominant haplotype H2, both of which were shared between Jinshajiang and Yalongjiang groups, were connected to an interior missing haplotype by one mutational step. Both H5 and H2 were interior in the center of the network. The second dominant haplotype H7, which was shared between the Yalongjiang and Wumeng groups, was connected to haplotype H5 by three mutational steps. Five haplotypes located in exterior positions were directly linked to haplotype H7 with one or two mutations away from haplotype H7. With these relative positions, we can characterize the genealogy as star-like.

2.4 Population and phylogeographic analysis

Total genetic diversity ($Ht = 0.862$) across all populations was much higher than the average intrapopulation diversity ($Hs = 0.349$), and, consequently, the population differentiation across the entire range of the species was high, with $Gst = 0.595$ and $Nst = 0.614$. The permutation test showed that Nst was higher than Gst, but not significantly ($P > 0.05$). This result indicated that phylogeographic structure was present, but not significant.

Based on the regional grouping (Jinshajing, Yalongjiang, and Wumeng groups), a hierarchical AMOVA analysis partitioned 20.26% of the variation among groups, 42.63% among populations within groups, and 37.11% within populations (Table 3). For the total dataset, 59.65% of variation existed among populations, 40.35% within populations, and a significant genetic differentiation was detected ($Fst = 0.597$). Pairwise AMOVA analysis between geographic groups was estimated. There were 13.97%, 60.00%, and 6.35% of variation in Jinshajiang group vs. Yalongjiang group, Jinshajiang group vs. Wumeng group, and Yalongjiang group vs. Wumeng group, respectively (Table 3). Correspondingly, the significant genetic differentiation was detected between three pairwise tests of three geographic groups. The genetic differentiation (Fst) was 0.946, 0.582, and 0.437 between Jinshajiang and Wumeng groups, Jinshajiang and Yalongjiang groups, and Yalongjiang and Wumeng groups, respectively (Table 3).

The Mantel test showed that there was no significant pattern of isolation by distance in *L. tongolensis*, as the matrix of geographic distances did not significantly correlate to Fst for the whole dataset ($r = -0.291$, $P = 0.964$).

2.5 Coalescence analysis and population demography

In this study, coalescent analysis carried out with Beast software indicated the time to TMRCA for *L. tongolensis* approximated 297 ka BP.

Mismatch distribution analyses were executed for *L. tongolensis*. The SSD and raggedness index

Table 3 Hierarchical AMOVA for 14 populations of *Ligularia tongolensis* based on data from two chloroplast DNA spacers

Regional grouping of populations	Source of variation	d.f.	SS	VC	PV	*F*st
Total	Among populations	13	130.779	0.942	59.65	0.597**
	Within populations	126	80.300	0.637	40.35	NC
Jinshajiang vs. Yalongjiang	Among groups	1	22.792	0.230	13.97	0.582**
	Among populations within groups	1	87.531	0.727	44.25	NC
	Within populations	117	80.300	0.686	41.77	NC
Jinshajiang vs. Wumeng	Among groups	1	29.036	1.243	60.00	0.946**
	Among populations within groups	6	43.714	0.717	34.64	NC
	Within populations	72	8.000	0.111	5.36	NC
Yalongjiang vs. Wumeng	Among groups	1	10.983	0.130	6.35	0.437**
	Among populations within groups	5	43.817	0.762	37.36	NC
	Within populations	63	72.300	1.148	56.29	NC
Jinshajiang vs. Yalongjiang vs.Wumeng	Among groups	2	43.248	0.348	20.26	0.629**
	Among populations within groups	11	87.531	0.732	42.63	NC
	Within populations	126	80.300	0.637	37.11	NC

d.f., degree of freedom; *F*st, genetic differentiation index; NC, not calculated; PV, percentage of variation; SS, sum of squares; VC, variance components.
**$P < 0.0001$.

(*HRag*) values under both the sudden expansion model (SSD = 0.027, $P > 0.05$; *HRag* = 0.091, $P > 0.05$) and the spatial expansion model (SSD = 0.018, $P > 0.05$; *HRag* = 0.091, $P > 0.05$) did not reject an expansion event. Furthermore, a recent demographic expansion for *L. tongolensis* was supported by a unimodal distribution of mismatch analysis (Fig. 4). The expansion parameter (τ) was 4.400 with a confidence interval of 0.688–8.045. Applying a cpDNA substitution rate of 1.52×10^{-9} s/s/y, the expansion ages for *L. tongolensis* is estimated as 274 (43–501) ka BP.

3 Discussion

3.1 Genetic variation and population differentiation

Genetic diversity and differentiation are important when estimating population structure and constructing phylogeographic patterns of species. In this research, two cpDNA non-coding regions, *trn*Q-5′*rps*16 and *trn*L-*rpl*32 intergenic spacers, revealed a considerable level of haplotype diversity (*H*d = 0.814) and high level of total genetic diversity (*H*t = 0.862) in *L. tongolensis* compared to other herbaceous plants (Chen et al., 2008; Wang et al., 2008; Fehlberg & Ranker, 2009). This result is consistent with previous ISSR-based studies (Wang et al., 2007). We also detected a low level of nucleotide diversity ($\pi = 0.00174$) for *L. tongolensis* in comparison with similar studies, such as *Primula secundiflora* ($\pi = 0.00495$) (Wang et al., 2008), *Metagentiana striata* ($\pi = 0.014$) (Chen et al., 2008), and *Encelia farinosa* ($\pi = 0.00628$) (Fehlberg & Ranker, 2009). The high level of haplotype diversity and low level of nucleotide diversity in *L. tongolensis* may be a consequence of homoplasy or may result from rapid population growth of an ancestral population with a low effective population size (Abramson, 2007).

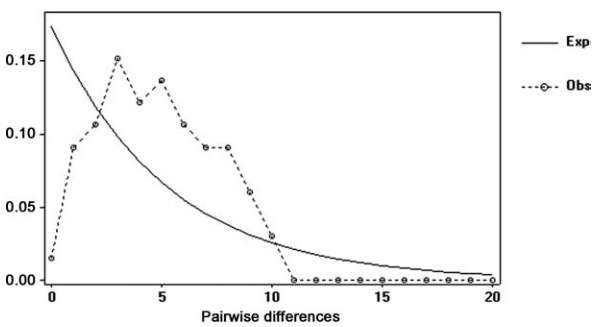

Fig. 4. Mismatch distributions of chloroplast DNA haplotypes based on pairwise sequence differences as a function of the frequencies of occurrence for *Ligularia tongolensis*. Along the *x* axis are the numbers of pairwise nucleotide differences between haplotypes; their frequencies are along the *y* axis. The dashed line represents observed values (Obs), and the solid line shows expected values (Exp) under a model of sudden (stepwise) population expansion (Rogers & Harpending, 1992).

Despite a high level of cpDNA diversity detected at the species level, haplotype variation within populations of *L. tongolensis* was low (*H*s = 0.349), and resulted in a significant differentiation detected among populations (*G*st = 0.595, *N*st = 0.614). AMOVA analysis gave similar evidence. For the total dataset, 40.35% of variation were partitioned within populations, and a significant genetic differentiation was detected (Table 3). When three groups were considered, only 37.11% of variation were partitioned within populations with a genetic divergence value of *F*st = 0.629 (Table 3). High differentiation of populations in *L. tongolensis* is similar to some herbaceous plants distributed in the HMR and neighborhood (Chen et al., 2008; Wang et al., 2008). Low genetic diversities within populations, along with high levels of population differentiation, indicated that gene flow was limited and populations of this species are strongly isolated from each other. In fact, the distributional areas of *L. tongolensis* are isolated and

fragmented by high mountains and deep valleys, which were created by several uplifts of the QTP (Shi et al., 1998). The coalescence time of *L. tongolensis* was estimated as 297 ka BP and partly consistent with the conclusions of Liu et al. (2006). They thought the explosive radiation of *Ligularia* occurred between the early Miocene to the Pleistocene, which fell within the period of recent major uplifts of the QTP. Within each uplift new habitats were created and the old ones became fragmented. It was possible that the habitats of *L. tongolensis* were fragmented severely after speciation. Due to the fragmentation of ancestral populations, the species were isolated from each other, which consequently led to strong genetic differentiation and low intrapopulational genetic diversity across the species. Of the 14 populations studied, six populations (42.9%) were fixed, with no genetic variation. This fact has usually been interpreted as the consequence of strong bottlenecks or genetic drift associated with small effective population sizes for maternally inherited markers (Birky et al., 1989).

Thus, the present genetic structure of *L. tongolensis* was mainly shaped by the fragmentation of ancestral populations. This was further supported by the absence of IBD tests ($r = –0.291$, $P = 0.964$), which suggests that the differentiation had not occurred in accordance with the isolation by distance model. Differentiation in *L. tongolensis* appears to be associated with historical events. In fact, the genetically similar populations (7, 8, and 13; 1, 3–5) are geographically distant, which might reflect a footprint of historical, rather than contemporary, gene flow and slow genetic drift.

Comparing Yalongjiang with Jinshajiang groups, only 13.97% of genetic diversity was distributed between the two groups (Table 3), which indicated either a relatively high gene flow or shared ancestral polymorphism (relicts of ancestral polymorphism). The small slippery seeds of *L. tongolensis* with pappi are undoubtedly wind dispersed. However, the Shaluli Mountain between Yalongjiang and Jinshajiang groups has an altitude of 4500–5834 m (Zhou et al., 2005; Xu & Zhou, 2009), which is a natural barrier for seed exchange. The detected high gene flow likely represented either ancient migration events or relicts of ancestral polymorphism (Whitlock & McCauley, 1999) due to the fragmentation of ancestral populations. This was further supported by both the IBD tests (see above) and the two shared interior dominant haplotypes (H2 and H5) between Jinshajiang and Yalongjiang groups.

3.2 Demographic history

In this study, 12 cpDNA haplotypes (H1–H12) of *L. tongolensis* were detected. The haplotype distribution displayed a geographic structure between the Jinshajiang and Yalongjiang groups, excepted in two shared haplotypes (H2, H5). In the parsimony network analysis with outgroups, both H5 and H2 were connected to an interior missing haplotype by only one mutational step. Furthermore, H5 and H2 were both dominant and interior in the center of the network (Fig. 3). With empirical evidence in support, the coalescent theory predicts that interior (older) haplotypes should be more common than tip (derived) haplotypes and are likely to represent ancestry (Donnelly & Tavaré, 1986; Crandall & Templeton, 1993). The presence of shared ancestral haplotypes in populations from the opposite areas of the HMR range (Jinshajiang and Yalongjiang) indicates that ancestral populations of this species may have had a continuous distributional range, which was then fragmented and isolated by the following tectonic events, such as the uplifts of the QTP. Our coalescence analysis estimated a coalescence time of *L. tongolensis* about 297 ka BP, some time within the period of recent uplifts of the QTP (Li et al., 2001). It is most likely that chloroplast polymorphisms existed across the entire range of *L. tongolensis* after speciation. With the uplifts of the QTP and the formation of Shaluli Mountain, populations within *L. tongolensis* had become strongly fragmented, resulting in the random allocations of the ancestral polymorphism between the two sides of Shaluli Mountain. So the present discontinuous distribution of shared haplotypes may be a relict of an ancestral/old polymorphism. Alternatively, long-distance dispersal could be a reason for the shared haplotypes (H2, H5) between the Jinshajiang and Yalongjiang groups, as the seeds of *L. tongolensis* are undoubtedly wind dispersed. However, considering the high elevation of Shaluli Mountain (4500–5834 m) (Zhou et al., 2005; Xu & Zhou, 2009) running north and south between the two regions, which results in a natural barrier for gene flow from east to west, the ancestral/old haplotype rationale is the most likely explanation for the discontinuous distribution of shared haplotypes. Furthermore, the phylogenetic analysis recovered four clades (Fig. 2). However, the four clades did not form a clear pattern associated with the geographic groups among the Jinshajiang, Yalongjiang, and Wumeng groups. The haplotypes from these regions were intermingled, showing a paraphyletic structure of haplotypes from the three geographic groups. This paraphyletic structure was also supported by the results of Wang et al. (2007) based on ISSR analysis. These phylogenetic patterns inferred from two different markers might indicate an incomplete lineage sorting with insufficient time to occur in *L. tongolensis* following population divergence. However, the permutation test using Permut showed a presence of phylogeographic

structure, but it was not significant: closely related haplotypes were not distributed in the same or neighboring region. The lack of association between the genealogical relationships of haplotypes and their geographical distribution might also be a product of incomplete lineage sorting of polymorphism, caused by fragmentation of ancestral populations, that is, the relict of ancestral haplotypes shared between regions.

The Quaternary climatic fluctuations are considered to have played a key role in the distribution of flora and fauna (Yang et al., 2006; Gao et al., 2007; Liu et al., 2007; Yang et al., 2008; Yuan et al., 2008). Based on direct dating of the glacial deposits using electron spin resonance, combined with glacial landforms and the weathering differences of the tills, Xu et al. (2005) asserted that Shaluli Mountain has probably experienced four major glaciations. The timings of these glaciations were dated as 571 ka BP, 135 ka BP, 48–43 ka BP, and 19–16 ka BP. Using *in situ* analysis with the cosmogenic isotope ^{10}Be for the roche moutonnée, Wang et al. (2006) gave a similar estimate of three glaciations that occurred in the southern part of Shaluli Mountain during the Quaternary, which were dated as 421–766 ka BP, 130–160 ka BP, and 15 ka BP. Like other studies, the Quaternary glaciation also had a significant effect on *L. tongolensis*. The presence of two dominant haplotypes in two specific regions (Jinshajiang and Yalongjiang) implies that the populations in these regions might have originated from two different sources of refugia (Banu et al., 2010). Because only the high-elevational regions were disjunctly covered by ice sheets during the glacial stages and the glacier of each mountain system did not connect (Shi et al., 1998; Shi et al., 1999; Xu et al., 2005; Wang & Ge, 2006), *L. tongolensis* could have survived between the two sides of Shaluli Mountain where it was ice-free. Mismatch distribution analyses under both the sudden expansion model and the spatial expansion model did not reject an expansion event. Furthermore, star-like genealogies of plastid DNA also suggested an expansion event (Fig. 3). Using the substitution rate of 1.52×10^{-9} per site per year for cpDNA, *L. tongolensis* was deduced to have experienced a population expansion, with expansion ages dated as 274 ka BP. The expansion time fits within the timescale of the Quaternary interglaciation developed at Shaluli Mountain (Xu et al., 2005; Wang et al., 2006). During the Quaternary glaciation and interglaciation, it is possible that *L. tongolensis* within each region experienced frequent retreat/expansion along the altitude and latitude gradients. However, the details of this expansion require further explanation in light of recent reconstructions of spatial forest biome changes of palaeovegetation in China (Ni et al., 2010). The vegetation type of the distributional range for *L. tongolensis* was cool mixed forest. According to the palynological data, cool mixed forest was reduced greatly during the Last Glacial Maximum in the HMR, then expanded its range in the Holocene (Ni et al., 2010). Assuming similar forest biome shifts for earlier glacial/inter-glacial cycles, *L. tongolensis* might experience frequent retreat/expansion with the cool mixed forest. This repeated retreat/expansion would result in the loss of some rare haplotypes due to bottleneck effects and founder events. A severe bottleneck event would result in the extinction of some haplotypes, as evidenced by a large number of missing haplotypes in the genetic network (Fig. 3). Meanwhile, founder events, in particular, can lead to the local spread of a single haplotype, which may then be followed by migration and natural selection. We found that a relatively large number of local populations of *L. tongolensis* were fixed in one haplotype, including five populations of the Jinshajiang group (populations 8, 10–12, and 14) and one population of the Wumeng group (population 1). However, the existence of haplotype H7 in population 1 might be shaped by ancestral polymorphism and the bottleneck effect. It is possible that *L. tongolensis* might have continuous distribution range from Yalongjiang to Wumeng Mountain (population 1). Afterward, tectonic events and climatic oscillations led to the extinction of populations in lowland regions and only population 1 survived at Wumeng Mountain. Therefore, Wumeng Mountain might have also served as a refuge for *L. tongolensis*.

In conclusion, our results suggest that the tectonic events and Quaternary glaciation resulted in the current distribution and differentiation of *L. tongolensis*. Due to the uplifts of QTP, ancestral widespread populations were fragmented, resulting in the random allocation of polymorphism between Yalongjiang, Jinshajiang, and Wumeng group. After this divergence, this species survived in three isolated regions (Jinshajiang region, Yalongjiang region, and Wumeng Mountain) during subsequent climatic oscillations. In addition, expansion events were also detected for *L. tongolensis* during the interglaciation. However, it is still unknown how many species may show a phylogeographic history similar to that of *L. tongolensis.* For a better understanding of the factors that influenced the phylogeographic history of this region's flora, further work on species with a similar distribution pattern is necessary.

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