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CHLOROPLAST DNA PHYLOGEOGRAPHY OF THE CHINESE ENDEMIC ALPINE QUILLWORT *ISOETES HYPSOPHILA* HAND.-MAZZ. (ISOETACEAE)

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We examined chloroplast *trnL* intron sequence variation within *Isoetes hypsophila*, a Chinese endemic alpine quillwort species. Sequence data were obtained from 46 individuals in six populations throughout the natural distribution of the species. Sequences appeared to have neutral evolution (Tajima's criterion $D = -1.66453$, $0.1 > P > 0.05$; Fu and Li's test $D^* = -1.92897$, $0.1 > P > 0.05$; $F^* = -2.17823$, $0.1 > P > 0.05$). Eleven haplotypes were identified in *I. hypsophila*. A relatively high level of haplotype diversity ($h = 0.817$) and a low level of nucleotide diversity ($\pi = 0.0045$) were detected in *I. hypsophila*. Indirect estimates of gene flow based on cpDNA variation revealed significant genetic differentiation between the ZD1 population in Yunnan Province and the populations from Sichuan Province. High genetic differentiation between Yunnan and Sichuan populations was consistently indicated by both hierarchical analyses of molecular variance (AMOVA; $G_{ST} = 0.5011$) and the structure of a neighbor-joining tree. From nested clade analysis, restricted gene flow with isolation by distance was inferred to be responsible for the current spatial genetic structure of *I. hypsophila*.

Keywords: alpine quillwort, cpDNA *trnL* intron sequence, *Isoetes hypsophila*, nested clade analysis, phylogeography.

Introduction

Patterns of genetic and geographical structure of natural populations have been strongly influenced by both intrinsic factors, such as migratory capabilities and mating system, and extrinsic factors, including habitats and historical events (e.g., glaciations and vicariance; Ge et al. 2002). To determine the roles of historical events in shaping the present spatial genetic structure of a species, phylogeographical methods, such as the nested clade analysis (NCA), are commonly used. These techniques, combined with the information about the spatial distribution and the genetic structure of populations, can be used to test alternative phylogeographical hypotheses (Templeton and Sing 1993). In addition, the events that have shaped the current spatial genetic structure can be temporally ordered by such analyses, allowing an evolutionary history of the species to be inferred (Templeton 2004).

During the past 2 decades, the phylogeographic history of a broad spectrum of organisms, including animal and plant taxa, has been reconstructed (Trewick et al. 2000; Huang et al. 2004). However, there are, as yet, few published phylogeographic analyses of free-sporing land plants (Rumsey et al. 1996; Trewick et al. 2002; Su et al. 2005*a*, 2005*b*). Because these species are expected to have high levels of gene flow because of their abundant, very small, wind-dispersed haploid spores, which are capable of long-distance dispersal (van Zanten 1978), they might be considered to be of little utility for phylogeographical studies (Hooper and Haufler 1997; Maki and Asada 1998; Schneller et al. 1998). However, several studies have revealed interpopulation diversity and evidence of restricted gene flow in ferns (Li and Haufler 1999; Ranker et al. 2000; Pryor et al. 2001). In addition, many free-sporing land plants produce water-dispersed as well as wind-dispersed spores. For example, dispersal of *Isoetes* spores is often accomplished via detached sporophylls (Small and Hickey 1997). Under this form of dispersal, changes in topography that split a continuous water system into several isolated systems could limit gene flow among populations, which would increase the potential for the development of spatial structure (Liu et al. 2004).

Isoetes L. is a genus of heterosporous lycopsids characterized by a strongly reduced plant body (Pigg 2001). A previous survey of the distribution of diploid and polyploid species of *Isoetes* indicated that this pattern of distribution of *Isoetes* in East Asia is correlated with the geological history and geographical changes that have occurred in this region (Liu et al. 2004). In particular, changes of altitude (e.g., uplifting of the Qinghai-Tibet Plateau) are to a large extent responsible for the modern distribution pattern of *Isoetes* in East Asia (Liu et al. 2004). *Isoetes hypsophila*, an alpine quillwort endemic to China, is a diploid species ($2n = 22$) distributed in shallow zones of plateau lakes or marshes of Sichuan and Yunnan provinces in China. In our recent field investigation, only six populations, including one population in Yunnan Province and five populations in Sichuan Province, were found in China. This species has been listed among the first category of the key protected wild plants (Yu 1999). The natural distribution area (with an altitude $\geq$3300 m) of this species belongs to the first (the Qinghai-Tibet Plateau) and second (the

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Table 1

Sample Size (*n*) and Locations of Sampled Populations of *Isoetes hypsophila*

Population	Locality	Latitude (N), longitude (E)	Altitude (m)	*n*
ZD1	Zhongdian, Yunan	27°49′55.1″, 99°42′01.3″	3300	8
DC1	Daocheng, Sichuan	29°16′10.5″, 100°03′04.9″	4220	8
DC2	Daocheng, Sichuan	29°26′48.5″, 100°12′46.5″	4400	8
DC3	Daocheng, Sichuan	29°19′35.9″, 100°06′47.0″	4330	8
DC4	Daocheng, Sichuan	29°14′18.8″, 100°02′49.7″	4050	8
DC5	Daocheng, Sichuan	29°26′43.8″, 100°04′37.5″	4320	6

Note. Species-level $n = 46$.

Yunnan-Guizhou Plateau) steps of the Chinese stepped land features (Zhang et al. 2000). Integrating the evidence from molecular data and the existing fossil records, Liu et al. (2004) proposed that *I. hypsophila* is a relict in high-altitude regions. Small and discontinuous populations of *I. hypsophila* were reported (Liu et al. 2005). Thus, *I. hypsophila* appears to be an ideal organism for investigating the distributional changes of an alpine plant on the Qinghai-Tibet and Yunnan-Guizhou plateaus.

Chloroplast DNA (cpDNA) noncoding intergenic spacers have been frequently used to survey population genetic variation and phylogeographic patterns of plants (Cannon and Manos 2003; Honjo et al. 2004; Ikeda and Setoguchi 2007). Their uniparental inheritance and rapid, nearly neutral evolution is well suited for reconstructing intraspecific phylogeographical patterns (Ferris et al. 1998). In this study, we analyzed the genetic structure of the cpDNA *trnL* intron haplotypes and their distribution through the entire range of *I. hypsophila* to infer whether there is spatial genetic structure among populations and to determine what factors are involved in shaping its population genetic structure.

Material and Methods

Plants

Forty-six individuals from the six extant populations of *Isoetes hypsophila* in China were included in the study (table 1). The distance between plants collected within an individual population was at least 5 m. Approximately 5 g of fresh leaves per plant was collected from each plant and immediately dried with silica gel.

DNA Extraction, PCR Amplification, and Sequencing

Total genomic DNA was isolated from 0.5 g of silica-dried leaf tissue following the procedure described by Fu et al. (2003). PCR was performed in a reaction volume of 50 μL, containing 0.25 mM of each dNTP, 2.5 μL of 10× Taq buffer (10 mM Tris-HCl [pH 8.3], 1.5 mM $MgCl_2$, and 50 mM KCl), 1 mM of each primer, 2 U Taq polymerase (Tian Yuan Biotech), and 60 ng of DNA template. The primers of Taberlet et al. (1991) were used to amplify the *trnL* (UAA) intron of cpDNA. Primers were synthesized by Shanghai SBS Biotech. Amplification of genomic DNA was performed on a PTC-100 thermocycler (MJ Research), initiated with 4 min at 94°C, followed by 35 cycles of 1 min at 94°C, 1 min annealing at 55°C, 2 min extension at 72°C, and a final extension cycle of 7 min at 72°C. The size of PCR products was determined by agarose electrophoresis. All PCR products were purified from an agarose gel using a PCR product purification kit (Shanghai SBS Biotech). The purified PCR products were sequenced in both directions by standard methods on an ABI 377 automated sequencer at the Beijing Genomics Institute, Chinese Academy of Sciences.

Data Analysis

Sequences were aligned using ClustalX (Thompson et al. 1997). The alignments were then adjusted manually. CpDNA haplotypes were determined from nucleotide substitutions and indels. Haplotype diversity (h), nucleotide diversity per site (π; Nei and Tajima 1983), tests of neutrality, including Tajima's (1989) D and Fu and Li's (1993) D^* and F^*, and the determination of their associated significance were performed using DnaSP, version 4.0 (Rozas et al. 2003).

The nonparametric analysis of molecular variance (AMOVA) was performed using squared Euclidean distances (Excoffier et al. 1992). Variance was apportioned to the following components: among individuals within population, among populations within region (Yunnan and Sichuan), and between regions. Genetic analysis was performed with ARLEQUIN, version 2.001 (Schneider et al. 2000). Patterns of geographical subdivision and gene flow were also estimated hierarchically with DnaSP, version 4.0. Gene flow within and among populations was estimated using the expression $F_{ST} = 1/(1 - 2\,Nm)$, where N is the female effective population size and m is the female migration rate.

The phylogeny of the haplotypes was inferred by the neighbor-joining method. The neighbor-joining tree was constructed according to Kimura's (1980) two-parameter model using MEGA 3 (Kumar et al. 2004). Confidence in the nodes was tested by performing 1000 bootstrap replicates (Felsenstein 1985).

Table 2

Nucleotide Diversity (π), Haplotype Diversity (h), and the Distribution of cpDNA Haplotypes in Each Population of *Isoetes hypsophila*

Population	π	Haplotype no.	h	Haplotypes
ZD1	.0009	2	.429	C, D
DC1	.0052	3	.715	A, J, K
DC2	.0031	4	.786	A, B, H, J
DC3	.0051	5	.786	A, E, F, G, I
DC4	.0005	2	.250	A, I
DC5	.0024	2	.600	G, J
Species level	.0045	11	.817	

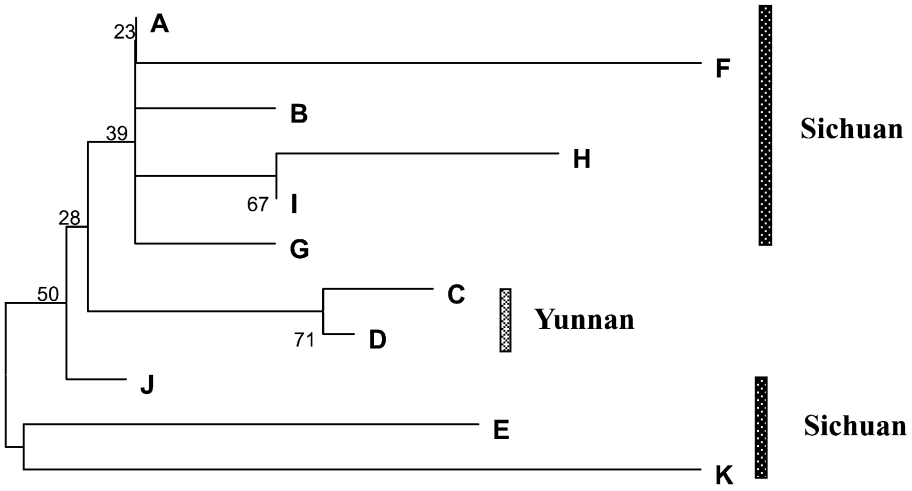

Fig. 1 Neighbor-joining tree of *Isoetes hypsophila* based on sequences of haplotypes of the *trnL* intron of cpDNA. Numbers above branches indicate the bootstrap values of 1000 replicates.

Pairwise differences between DNA haplotypes were also calculated using MEGA 3 (Kumar et al. 2004). These were used to construct a minimum spanning network in a hierarchical manner with the aid of MINSPNET (Excoffier and Smouse 1994). We further subjected data to NCA. Clade distances (D_c) and nested clade distances (D_n) were defined based on geographical locations of samples in the nesting cladogram and were estimated as described by Templeton et al. (1995). Differences between interior (ancestral) and tip (recent) D_c and D_n were calculated. Permutation tests (replicated randomly 10,000 times) were conducted separately for each level of the nested cladogram using GeoDis, version 2.5 (Posada et al. 2000). Inferences about the historical processes that were likely to be responsible for observed patterns of clade structure were made following the methods of Templeton et al. (1995).

Results

Genetic Diversity

The length of the intergenic spacer between the *trnL* intron varied from 492 to 494 bp (GenBank accession nos. EF405969 to EF405979). The length after multiple alignments of the sequences was 494 bp. Within this sequence, there were 20 nucleotide substitutions and two indels (two insertions were found at sites 10 and 399). Eleven haplotypes were identified. Haplotype diversity and nucleotide diversity were estimated as 0.817 and 0.0045, respectively (table 2). Across all populations, haplotype diversity varied from 0.250 (DC4) to 0.786 (DC2 and DC3), and nucleotide diversity ranged from 0.0005 (DC4) to 0.0052 (DC1; table 2). Sequence variation demonstrates nonsignificant deviation from expectations of neutrality as estimated by both Tajima's criterion ($D = -1.66453$, $0.1 > P > 0.05$) and Fu and Li's tests ($D^* = -1.92897$, $0.1 > P > 0.05$; $F^* = -2.17823$, $0.1 > P > 0.05$).

Phylogeny of cpDNA Haplotypes

Haplotypes from the ZD1 population in Yunnan Province constituted a group, but haplotypes from five Sichuan populations (DC1~5) were not monophyletic (fig. 1). No haplotypes were shared between the two provinces (Sichuan or Yunnan). Haplotype A was widely distributed over Sichuan, except for the DC5 population (table 2).

Phylogeography of Isoetes hypsophila

The F_{ST} values between populations of the Sichuan Province ranged from 0. 0.023 to 0.356, and the inferred *Nm* values ranged from 0.905 to 21.239. The F_{ST} values between Sichuan regional populations and the ZD1 population in Yunnan Province ranged from 0.582 to 0.857, and the inferred *Nm* values ranged from 0.083 to 0.359 (table 3). Hierarchical AMOVA showed that considerable variation (50.11%) occurred between the two regions, with 9.04% and 40.84% between populations within a region and within a population, respectively ($P < 0.001$; table 4).

In the haplotype network, 10 clades, 1-1 to 1-10, were identified from the minimum spanning network (fig. 2). All tip haplotypes (B, C, E, F, H, and K), except for haplotype G, were unique to a particular population, while the haplotypes A, I, and J of the interior nodes were widespread (table 2). Geographic structure was significant for the two-step clade 2-2 (table 5). Restricted gene flow with isolation by distance was inferred from Templeton et al.'s (1995) inference key to be responsible for the present-day patterns of genetic diversity.

Table 3

Pairwise Comparisons of *Nm* (above Diagonal) and F_{ST} (below Diagonal) between Populations of *Isoetes hypsophila* Based on cpDNA *trnL* Intron Sequence

	1 ZD1	2 DC1	3 DC2	4 DC3	5 DC4	6 DC5
1	.000	.316	.251	.359	.083	.186
2	.613	.000	3.665	1.951	.866	2.586
3	.666	.112	.000	13.389	5.910	5.182
4	.582	.204	.036	.000	21.239	3.376
5	.857	.336	.078	.023	.000	.905
6	.729	.162	.088	.129	.356	.000

Table 4

Analysis of Molecular Variance for Populations of *Isoetes hypsophila* Based on cpDNA *trnL* Intron Sequences Data

Source of variation	df	SSD	Variance component	Variation (%)
Among regions	1	13.521	.87422	50.11
Among populations within regions	4	7.632	.15773	9.04
Within populations	40	28.500	.71250	40.84

Note. SSD = sum of squares of deviations.

Discussion

Many previous studies of plant phylogeography have emphasized the importance of climatic oscillations during the Quaternary ice age on plant distribution (Hewitt 2004; Ikeda and Setoguchi 2007). During glacial episodes, plant species may retreat into refugia, while during interglacial periods, they expand out of these refuges to become more widely distributed (Taberlet et al. 1998; Hewitt 2000). However, these patterns do not explain the differentiation found in *Isoetes hypsophila*. Extant populations of *I. hypsophila* occur only at high elevations (on the Qinghai-Tibet and Yunnan-Guizhou plateaus with altitudes ≥3300 m). Retreat of this species into high alpine refugia could explain the pattern of genetic differentiation in *I. hypsophila*. However, this explanation seems unlikely because it would require dispersal to a higher altitude. Because the spores of *I. hypsophila* are dispersed by flowing water, they are almost always likely to disperse from higher to lower altitudes. It has been suggested that migrating waterfowl could have had an effect on the dispersal of spores of *Isoetes* species (Taylor and Hickey 1992). However, if this were the case, a regular distribution of species along the migration line should exist. This has, however, not been found on Mainland China (Liu et al. 2004). There is no strong evidence to support the idea that the *I. hypsophila* populations distributed in high altitude resulted from water bird distribution of spores. In addition, during glacial periods, the plant species commonly needed to retreat into warmer refugia instead of harsher environments.

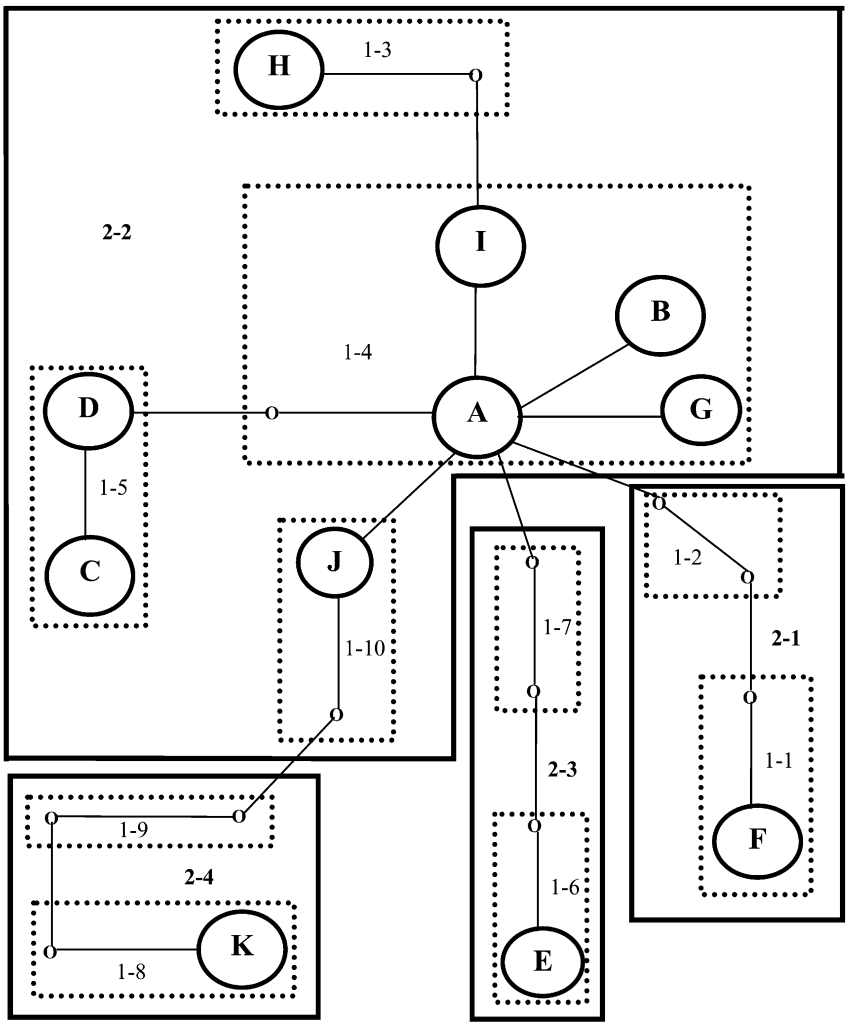

Fig. 2 Nested clades of 11 haplotypes of cpDNA of *Isoetes hypsophila*. One-step (1-1 to 1-10) and two-step (2-1 to 2-4) clades are indicated.

Table 5

Results of Nested Clade Analyses of the Geographical Distance of cpDNA Haplotypes of *Isoetes hypsophila*

Nested clades and haplotypes	Type of geographical distance: Within clade (D_c)	Type of geographical distance: Nested clade (D_n)	Permutational χ^2 statistic	Probability
Clade 1-4			18.4018	.0870
A (interior)	8.9167	9.7869		
B (tip)	6.6916	8.1045		
J (tip)	4.3718	10.4554		
I (interior)	5.8464	6.8159		
$I - T$	3.4305	−.2150		
Clade 2-2			.0000	.0000
1-3 (tip)	.0000	15.1721		
1-4 (interior)	8.4420	9.0466		
1-5 (tip)	10.2592	10.6035		
1-10 (interior)	11.1243	11.8014		
$I - T$	.1393	−.2341		
Total clade			19.7143	.0860
2-1 (tip)	.0000	27.0488		
2-2 (interior)	51.7617	50.4320		
2-3 (tip)	.0000	27.0488		
2-4 (tip)	.0000	19.6742		
$I - T$	51.7617*[a]	27.0705		

Note. $I - T$ = difference between interior (ancestral) and tip (recent) D_c and D_n.

[a] D_c or D_n values significantly larger than expected at the 5% level based on 1000 permutations.

An alternative explanation is that the extant populations of *I. hypsophila* are relicts of a formerly widespread Pliocene distribution. In this study, 11 haplotypes from 46 individuals of *I. hypsophila* were detected at the cpDNA *trnL* intron locus. In the minimum spanning network of cpDNA haplotypes in *I. hypsophila*, the haplotypes A, I, and J of the interior nodes were widespread. The indirect estimates of gene flow based on cpDNA variation revealed significant genetic differentiation between the ZD1 population in Yunnan Province and the populations from Sichuan Province. High genetic differentiation between Yunnan and Sichuan was consistently indicated by AMOVA, which revealed that 50.11% of variation was partitioned between regions ($P < 0.001$). The NCA suggested that the present-day distribution of *I. hypsophila* populations, as well as their genetic structure, could be explained by restricted gene flow with isolation by distance.

A possible explanation for the process is that the ancestors of *Isoetes* could have reached the Qinghai-Tibet region before the beginning of the uplift of the Qinghai-Tibet Plateau (Eocene of lower Tertiary; Liu et al. 2004). During this period, *I. hypsophila* would have been widely distributed in this area. Subsequently, however, this area became fragmented by the uplift of the Qinghai-Tibet Plateau. The ancestral haplotypes A, G, I, and J were likely to represent the relicts of the widely distributed plants. With the Qinghai-Tibet Plateau uplift, the populations presumably became separated by high mountains, which probably imposed significant barriers to gene flow between populations. For example, the extant ZD1 population, which is distributed on the Yunnan-Guizhou Plateau, and the populations from Sichuan (DC1~5) are separated by several high mountains (altitude ≥5200 m). This restricted dispersal led to the evolution of the geographically restricted haplotypes B, C, D, E, F, H, and K from the common ancestral haplotypes A, G, I, and J. An alternative possibility is that the ancestral haplotypes in the ZD1 population were the result of long-distance dispersal from Sichuan during the uplift of the Qinghai-Tibet Plateau. This hypothesis would explain the absence of the ancestral haplotypes in the ZD1 population as a result of a population bottleneck.

Relatively high gene flow between populations of *I. hypsophila* within Sichuan Province was inferred in this study. The lack of correspondence between phylogeny and geography in the neighbor-joining tree suggests the regular occurrence of gene flow among these populations as well. However, we do not believe that there is at present extensive gene flow between populations of *I. hypsophila* in Sichuan because the current habitat is highly fragmented and the inherent migratory capabilities of sporophytes and gametophytes are limited; instead, we suggest that high inferred *Nm* values are likely to represent historical migration events. Before the uplift of the Qinghai-Tibet Plateau, the populations of *I. hypsophila* probably grew in marsh habitats with continuous a water system, and the water flow might have acted as a dispersal agent for spores as well as for sporelings, allowing gene flow to occur over long distances. However, the subsequent changes in topography would have split a continuous water system into isolated small ones and thus limited gene flow among the extant populations. Although AMOVA analysis revealed a low between-population differentiation among populations within Sichuan region (9.04%), the low between-population, within-region variation is probably skewed downward by the fact that only one population was sampled in Yunnan. In addition, more than half of the haplotypes are unique to a single population, indicating that substantial differentiation among the five Sichuan populations should be recognized. The limited gene flow among the extant *I. hypsophila* populations in Sichuan Province could be responsible for the substantial genetic differentiation.

Petit et al. (2003) suggested that glacial refuge areas harbor a large fraction of intraspecific diversity and that plant populations in refuge areas have high genetic divergence and uniqueness rather than a high number of haplotypes. In this study, a high genetic divergence between Sichuan and Yunnan populations as well as a substantial genetic differentiation among Sichuan populations of *I. hypsophila* was revealed. In addition, several haplotypes unique to a single sampled population (less than half were found to be shared among the six sampled populations) were also detected in this study. Petit et al.'s (2003) criteria for locating regions of glacial refugia encourage us to regard the current *I. hypsophila* distribution region as a glacial refuge. The refuge might not be the result of retreats during a glacial period (Shi et al. 1998); instead, it might demonstrate long-term persistence of populations through uplift and glacial periods.

The Tethys retreat and Himalayas-Hengduanshan Mountains uplift (the Qinghai-Tibet Plateau) are believed to have dramatically influenced the natural environment of East Asia and also the evolutionary history of East Asia flora (Coleman and Hodges 1995; Sun 2002). This research supports this view by demonstrating that factors such as vicariance and historical migrations likely contributed to the current distribution of genetic variation in *I. hypsophila*. To obtain a better understanding of the factors that have shaped the evolutionary history of

the species on the Qinghai-Tibet and Yunnan-Guizhou plateaus, more phylogeographic studies are required on a wide range of species endemic to this area.

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