Phylogeography of *Iris loczyi* (Iridaceae) in Qinghai–Tibet Plateau revealed by chloroplast DNA and microsatellite markers

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Abstract

Quaternary climate oscillations and complex topography have tremendous effects on current distribution and genetic structure of species, and hence the Qinghai–Tibet Plateau (QTP), the largest plateau in the world, has become a hotspot for many phylogeographic studies. However, little is known about the phylogeographic pattern of herbaceous plants in QTP. Here, we investigate the genetic diversity, population structure and historical dynamics of *Iris loczyi*, using five chloroplast DNA (cpDNA) fragments and seven microsatellite markers. A total of 15 populations, and 149 individuals were sampled throughout the QTP. High genetic diversity was detected both in cpDNA (\(H_d = 0.820\)) and SSR (\(H_o = 0.689, \ H_e = 0.699\)). Ten cpDNA haplotypes and 163 alleles were identified. AMOVA and clustering analyses revealed obvious differentiation between regions. The \(N_{st} \), \(G_{st} \) and Mantel test showed significant phylogeographic structure of *I. loczyi*. The neutrality test and mismatch distribution analyses indicated that *I. loczyi* could not have undergone a historical population expansion, but population XS from the Qilian Mountain area could have experienced a local expansion. Bottleneck analyses indicated that *I. loczyi* had not experienced bottleneck recently. Based on cpDNA and SSR results, the Qilian Mountain area was inferred as a potential glacial refuge, and the southern Tibet valley was considered as a 'microrefugia' for *I. loczyi*. These findings provided new insights into the location of glacial refuges for the species distributed in QTP, and supplemented more plant species data for the response of QTP species to the Quaternary climate.

Keywords: Genetic diversity; *Iris loczyi*; population structure; Qinghai–Tibet Plateau; refuge.

Introduction

There is growing evidence that the Quaternary climate oscillations have had tremendous effects on current distribution and genetic structure of species, which may vary with latitude and topography (Hewitt 2000, 2003; Hewitt 2011), with those of the mountainous regions being the most interesting. The role of mountainous regions in nurturing biodiversity is widely recognized (Antonelli 2015; Spicer 2017; Hoorn et al. 2018; Rahbek et al. 2019a, b), which is fully reflected in the Himalaya, the Hengduan Mountains and now the Qinghai–Tibet Plateau (QTP) (Spicer et al. 2020). Among them, the QTP has attracted increasing interest of biogeographers due to its rugged landscape and rich species diversity (Wen et al. 2014; Favre et al. 2015; Hughes and Atkinson 2015).

The QTP is the youngest and largest plateau in the world covering most of Qinghai Province and Tibet of China, with a...
mean elevation of 4500 m (Zheng 1996), surrounded by several vast mountain ranges (Himalayan, Kunlun, Qilian, Karakoram and Hengduan Mountains) (Rowold et al. 2019). A common hypothesis for the rich of mountain biodiversity is uplift-driven diversity, which means that geological events created the conditions for speciation (Hoorn et al. 2013; Schwery et al. 2014; Favre et al. 2015; Hughes 2016; Lagomarsino et al. 2016). The uplift of the QTP was one of the most important geological events to have occurred since the Cenozoic (Spicer et al. 2003), with the formation of high mountains and deep valleys within the plateau (Li et al. 1995) that profoundly influenced the global climate, monsoon intensity and the distribution of species in the region (Raymo and Ruddiman 1992; Zhisheng et al. 2001). Therefore, QTP has become a hotspot for many phylogeographic studies (Wen et al. 2014), and it may display divergent characteristics in how the Quaternary history (climate oscillations and geological events) affected the current distribution and genetic structure of plants.

Previous studies have revealed two major patterns of plants response to glacial climate change on the QTP: one is that species survived in a refuge on the QTP during the Last Glacial Maximum (LGM), then expanded during the postglacial periods (Wang et al. 2009b, c); the other is surviving in multiple refugia during the LGM throughout the current distribution (Opennooth et al. 2010; Wang et al. 2010). However, they have mainly focused on woody plants or alpine herbs, and little is known about the response of dense perennial herb species growing in sunny environments to glacial climatic oscillations.

Iris loczyi (Iridaceae) is a dense perennial herb with knobby rhizomes, linear leaves, tough texture. The flower stem is short, and the base is often surrounded by lanceolate membranous sheath-like leaves. Flowers pale purple, outer segments oblanceolate or narrowly obovate, and inner segments oblongate. The capsule is obovate to cylindrical, reddish brown. Flowering from May to June, fruiting from July to September (Zhao 1985; Mathew 1989). This species would be expected to occur only in Xinjiang and western Tibet in China (Flora of China online). However, we found its distribution in eastern Tibet and a large area in Qinghai Province when we collected field samples. Iris loczyi is mainly distributed in sunny alpine meadows and desert gravel, and exists in the form of communities, with an altitude of more than 2000 m. Iris loczyi has strong adaptability to the harsh environment of the QTP, so it is an important genetic resource for studying the evolutionary history of species on the QTP under climate change and complex terrain conditions.

Here, we conducted a phylogeographic study for I. loczyi, employing five chloroplast fragments and seven microsatellite markers. This is also the first phylogeographic study of Iris species distributed in QTP by combining chloroplast and nuclear markers. The aims of this study are to (i) estimate the genetic diversity and phylogeographic structure of I. loczyi, (ii) infer the potential LGM refugia of I. loczyi and determine whether it occupied a single refugium or multiple refugia.

Materials and Methods

Plant sampling and DNA extraction

A total of 149 I. loczyi leaf samples were collected from 15 populations distributed in eastern Tibet and Qinghai Province, covering most of the geographic range of I. loczyi across QTP (Fig. 1). One hundred and thirteen individuals of 11 populations were collected from the Qilian Mountain area, and 36 individuals of four populations from the southern Tibet valley (Table 1). The distance between sampled individuals was ~60 m in the natural populations. Voucher specimens are deposited in the Herbarium of Northeast Normal University (NENU). Iris goniocarpa was chosen as an outgroup. Total genomic DNA was extracted using the PlantGen DNA Kits (TianGen, USA).

Chloroplast DNA amplification and sequencing

We amplified and sequenced five chloroplast fragments selected from 56 primer pairs collected from previous research [see Supporting Information—Table S1], namely trnS-trnG, trnS-trnFM, rpl20-rps12, psal-accD and trnH-psbA (Demesure et al. 1995; Hamilton 1999; Shaw et al. 2007). The PCR amplification was performed in a 30-µL reaction volume containing 20–50 ng template DNA, 10× PCR buffer (Mg2+), 10 mM dNTP mixture, 0.1 µM forward and reverse primers and 2.5 U Taq DNA polymerase (TaKaRa, Dalian, China). PCR amplification was performed under the following conditions: 5 min predenaturation at 95°C; followed by 35 cycles of 30 s denaturation at 94°C, 30 s annealing at 48°C (47°C for trnH-psbA), 60 s elongation at 72°C and final elongation step of 10 min at 72°C. All PCR products were detected by electrophoresis on 1.5% agarose gels and sequenced with the ABI3730 sequencer (Shenggong Biological Engineering Co., Ltd, Shanghai, China).

Microsatellite genotyping

A total of seven microsatellite markers were used in this study [see Supporting Information—Table S2], three (IEST5, IEST8 and IEST12) of which were selected from previously published SSR markers in iris laevigata (Sun et al. 2012) and were detected to be polymorphic in I. loczyi, with the remaining four (IK2, IK6, IK12 and IK13) were selected from primers independently developed by our team. PCR were performed in volume of 20 µL containing 20–50 ng DNA, 10× buffer (Mg2+), 10 mM dNTP mixture, Bovine Serum Albumin, 0.1 mM forward and reverse primer and 2.5 U Taq DNA polymerase. PCR amplification was performed under the same conditions as chloroplast DNA (cpDNA), except for the annealing temperature [see Supporting Information—Table S2]. The amplified fragments were separated on an ABI 3730 DNA sequence (Applied Biosystems) capillary electrophoresis instrument, and the sizes were assessed using GENE Mapper 3.7 (Applied Biosystems). To reduce score error, allelic size determination was performed twice.

Genetic diversity and structural analyses

For cpDNA data, the sequences of each primer pairs alignment were performed in MAFFT 7.222 (Kazutaka and Daron 2013) and edited manually in BioEdit 7.0.1 (Hall 1999). The concatenate of sequences were performed by Perl script. Genetic diversity parameters, including the number of polymorphic sites (S), number of haplotypes (h), haplotype diversity (Hd) and nucleotide diversity (π), were calculated using DnaSP 5.10 (Rozas et al. 2003). The program PermutCpSSR 2.0 (Hartnup et al. 2011) was used for evaluating differentiation for ordered alleles (Np) and unordered alleles (Gp). To determine the best grouping for all populations, Spatial Analysis of Molecular Variation (SAMOVA) analyses (Dupanloup et al. 2002) were performed with K varying from 1 to 10. Based on the results of SAMOVA, analyses of molecular variance (AMOVA) were performed in Arlequin 3.11 (Excoffier et al. 1992), to study the genetic variation partitioned within and among populations/groups. The correlation between genetic (pairwise Fst values) and geographical distances was detected using Mantel test in Arlequin, with 1000 randomizations.

The haplotype network was constructed using TCS 1.21 (Clement et al. 2000) and the fix connection limit was set as
200 steps with gaps = missing. Bayesian phylogenetic tree of haplotypes was built using MrBayes 3.2.6 (Ronquist et al. 2012) under the GTR substitute model. Two Markov chain Monte Carlo (MCMC) searches were run for 500 000 generations each. Trees were sampled every 1000 generations, and the first 25 % was discarded as burn-in.

For SSR data, genetic diversity parameters consisting of mean number of alleles (\(N_a\)), inbreeding coefficient (\(F_{is}\)), observed heterozygosity (\(H_o\)) and expected heterozygosity (\(H_e\)) were calculated using Genepop (http://www.genepop.curtin.edu.au) (Raymond and Rousset 1995) and Popgene (Yeh and Boyle 1996). AMOVA analyses were implemented in Arlequin, and gene flow between population pairs was estimated using Wright’s method, \(N_m = (1 – F_{ST})/4F_{ST}\) (Wright 1931). STRUCTURE 2.3.3 (Pritchard et al. 2000) was used to detect population structure. Six independent simulations were run for each \(K (K = 2–8)\) with \(1 \times 10^5\) burn-in, followed by \(1 \times 10^5\) MCMC steps. STRUCTURE HARVESTER (Earl and Vonholdt 2012) was used to determine the optimal \(K\) value by delta \(K\) (\(\Delta K\)). Principal coordinate analysis (PCoA) was carried out based on population genetic distance in GenAIEx 6.5 (Peakall and Smouse 2012). The UPGMA phylogenetic tree of populations was constructed using Population 1.2.3 (http://bioinformatics.org/project/?group_id=84) based on Nei’s standard genetic distance between populations.

Historical population dynamics analyses

To further evaluate the evidence of historical population dynamics, the neutrality test was performed using DnaSP based on cpDNA and the values of Tajima’s \(D\), Fu and Li’s \(D\) and Fu and Li’s \(F\) (Fu and Li 1993) were calculated. Mismatch distribution analyses (MDA) were performed in Arlequin, with 1000 parametric bootstraps used to test the goodness of fit based on the sum of squared deviations (SSD) and Harpending’s raggedness index (\(H_{Rag}\)) (Rogers and Harpending 1992; Harpending 1994). In addition, the bottleneck was detected using BOTTLENECK 1.2.02 (Cornuet and Luikart 1997) based on SSR data, under the infinite allele model (IAM) and two-phased model of mutation (TPM). One thousand iterations were run. Sign test and Wilcoxon sign-rank test were used to analyse the number of loci with heterozygosity excess and whether the heterozygosity excess was significant.

Results

Genetic diversity and population structure based on cpDNA

The total alignment length of cpDNA sequence was 3445 bp, containing 108 polymorphic sites (Table 1). Ten haplotypes were identified. The total haplotype diversity (\(H_d\)) and nucleotide
Table 1. Sample information and genetic diversity parameters for 15 populations of *I. loczyi*. QH = Qinghai Province; S = number of polymorphic sites; h = number of haplotypes; $H_s$ = haplotype diversity; $\pi$ = nucleotide diversity; $N_a$ = allele number; $F_{is}$ = inbreeding coefficient; $H_e$ = observed heterozygosity; $H_o$ = expected heterozygosity. The codes of Group I–III represent the grouping of STRUCTURE analyses.

| Code | Location                  | Size | cpDNA | nSSR |
|------|---------------------------|------|-------|------|
|      |                           |      |       |      |
|      |                           |      | $S$   | $h$  | $H_s$ | $\pi$ | Haplotypes | $N_a$ | $F_{is}$ | $H_s$ | $H_o$ |
| Qilian Mountain area |                          |      |       |      |      |        |        |      |        |      |      |
| Group I |                         |      |       |      |      |        |        |      |        |      |      |
| XS     | Xishan Bay, QH           | 18   | 1     | 2    | 0.111 | 0.00004 | H6, H7 | 7.143 | 0.228   | 0.556 | 0.693 |
|        |                           |      |       |      |      |        |        |      |        |      |      |
| Group II |                        |      |       |      |      |        |        |      |        |      |      |
| BS     | Baishugou, QH           | 9    | 0     | 1    | 0.000 | 0.00000 | H5    | 5.857 | −0.044  | 0.762 | 0.732 |
| JM     | Jiangmu Mountain, QH     | 10   | 79    | 3    | 0.688 | 0.00934 | H3, H4, H6 | 18.429 | 0.098   | 0.743 | 0.828 |
| KH     | Bitter Sea, QH           | 10   | 0     | 1    | 0.000 | 0.00000 | H4    | 6.714 | 0.120   | 0.657 | 0.742 |
| LY     | Laiyang Village, QH      | 10   | 0     | 1    | 0.000 | 0.00000 | H4    | 6.000 | −0.105  | 0.829 | 0.754 |
| LS     | Lanzhouilong, QH         | 10   | 0     | 1    | 0.000 | 0.00000 | H1    | 6.286 | 0.056   | 0.714 | 0.754 |
| QB     | Qingbao River, QH        | 10   | 0     | 1    | 0.000 | 0.00000 | H1    | 6.286 | 0.176   | 0.614 | 0.738 |
| LY     | Lyi Village, QH          | 8    | 0     | 1    | 0.000 | 0.00000 | H4    | 6.714 | −0.155  | 0.893 | 0.781 |
| SZ     | Qingzhiu Town, QH        | 10   | 0     | 1    | 0.000 | 0.00000 | H2    | 6.143 | −0.055  | 0.771 | 0.738 |
| TJ     | Tianjun County, QH       | 10   | 1     | 2    | 0.533 | 0.00018 | H1, H4 | 6.571 | −0.081  | 0.786 | 0.730 |
| ZD     | Gonghe County, QH        | 8    | 0     | 1    | 0.000 | 0.00000 | H4    | 6.571 | 0.081   | 0.685 | 0.741 |
| Southern Tibet valley |                  |      |       |      |      |        |        |      |        |      |      |
| Group III |                     |      |       |      |      |        |        |      |        |      |      |
| JC     | Jishang Town, Tibet      | 10   | 1     | 2    | 0.533 | 0.00018 | H9, H10 | 3.857 | −0.075  | 0.557 | 0.520 |
| SY     | Shayinxema, Lhasa, Tibet | 8    | 0     | 1    | 0.000 | 0.00000 | H8    | 3.714 | 0.039   | 0.571 | 0.582 |
| YH     | Lhasa Yanzhi Lake, Tibet | 8    | 0     | 1    | 0.000 | 0.00000 | H8    | 4.000 | 0.025   | 0.554 | 0.567 |
| YR     | Yangre Village, Tibet    | 10   | 0     | 1    | 0.000 | 0.00000 | H8    | 4.857 | −0.073  | 0.643 | 0.599 |
| Total  |                          | 149  | 108   | 10   | 0.820 | 0.00981 | H1–H10 | 5.873 | 0.014   | 0.689 | 0.699 |

Genetic diversity and population structure based on SSR

A total of 163 alleles at seven SSR loci were revealed across 149 individuals from 15 *I. loczyi* populations (detailed genotype data were listed in Supporting Information—Table S3). Genetic diversity parameters for each locus were estimated [see Supporting Information—Table S4]. The total number of alleles per locus ranged from 14 (IEST8 locus) to 39 (IK6 locus) with a mean of 23.29. The observed heterozygosity ($H_o$) ranged from 0.4898 (IK2 locus) to 0.9664 (IK13 locus) with a mean of 0.6817 while expected heterozygosity ($H_e$) ranged from 0.7135 (IEST12 locus) to 0.9259 (IK6 locus) with a mean of 0.8602. Diversity parameters for each population were listed in Table 1. The number of alleles ($N_a$) ranged from 3.714 (SY) to 7.429 (JM) with a mean of 5.873. The $H_s$ ranged from 0.554 (YH) to 0.893 (JM).
with an average value of 0.689, the \(H_e\) ranged from 0.520 (JC) to 0.808 (JM) with an average value of 0.699. The inbreeding coefficient (\(F_is\)) ranged from −0.155 (RY) to 0.228 (XS) with an average value of 0.014. Population JM had the highest genetic diversity (\(N_a = 7.429, H_o = 0.743\)), which was consistent with the result of cpDNA. The genetic diversity of the Qilian Mountain area is higher, compared with the southern Tibet valley.

AMOVA analyses showed that the majority of genetic variation (80.82\%) existed within populations (Table 2). A similar result was observed for grouping by STRUCTURE, where 74.96\% genetic variation resided within populations. Estimates of gene flow between population pairs indicated that gene flow between populations was moderate/frequent (\(N_m > 1\)) in the Qilian Mountain area, and moderate in the southern Tibet valley, but there was low (\(N_m < 1\)) gene flow between populations in the two regions [see Supporting Information—Table S5], which were consistent with AMOVA analyses.

STRUCTURE analyses revealed that the optimal \(K\) value was 3 (Fig. 3A), suggesting that the 15 populations were divided into three groups, which was consistent with the results of SAMOVA analyses based on cpDNA, population XS from the Qilian Mountain area was grouped separately (Group I), the remaining 10 populations in this region were grouped (Group II), except that the four populations from the southern Tibet valley were clustered into one group (Group III). In addition, the results suggested the existence of gene flow from population XS to JM (Fig. 3B). The same grouping was found in the PCoA analysis (Fig. 3C). In the UPGMA tree, all populations were grouped into three major clades (Fig. 3D), which were consistent with the results of STRUCTURE and PCoA analyses, and the population relationships showed a correlation with geographic distance.

### Historical population dynamics

The neutrality test showed that Tajima’s \(D\) \((1.65602, P > 0.02)\) was non-significant positive value while Fu and Li’s \(D\) \((2.61466, P < 0.02)\) and Fu and Li’s \(F\) \((2.61708, P < 0.02)\) were both significant positive values (Table 3), indicating that *I. loczyi* could not have undergone a historical population expansion. Analyses of MDA did not support the expansion hypothesis either, where the mismatch distribution curve showed a multimodal distribution (Fig. 4A). In a separate test of the three groups, Group I presented a non-significant negative value of Tajima’s \(D\) \((-1.16467, P > 0.1)\), and the mismatch distribution curve was unimodal with the non-significant SSD and \(H_{Rag}\) \((P > 0.05; Table 3; Fig. 4B)\), indicating that the population from Group I (XS) could have experienced population expansion. By contrast, Group II and population JC were non-significant negative or positive values of Tajima’s \(D\) \((-2.13501, 1.30268, P > 0.02)\), and MDA analyses showed multimodal (Fig. 4C and D), suggesting that these populations could not have experienced expansion (the group of SY, YH and YR could not be analysed for neutrality test and MDA because these populations contained only one haplotype). Bottleneck analyses showed insignificant heterozygosity excess \((P > 0.05 both in Sign test and Wilcoxon sign-rank test; see Supporting Information—Table S6)\), and the allele frequency presented a typical L-shaped distribution (Fig. 5), indicating that *I. loczyi* had not experienced bottleneck recently.
In this study, the five cpDNA fragments in I. loczyi displayed a high degree of genetic diversity ($H_d = 0.820$; Table 1), similar to other herbaceous species distributed in QTP, such as Stellera chamaejasme ($H_d = 0.834$) (Zhang et al. 2010), and species from Rhodiola sect. Trifida ($H_d = 0.923$) (Li et al. 2018). Additionally, the degree of genetic diversity was higher than the average level ($H_d = 0.670$) of 170 angiosperm species (Petit et al. 2005) and other Iris species, such as Iris dichotoma ($H_d = 0.725$) (Zhang et al. 2020).

SSR data also revealed a high genetic diversity ($H_o = 0.689$, $H_e = 0.699$), which was generally higher than other studied herbaceous plants in the QTP, e.g. Rheum tanguticum ($H_o = 0.342$, $H_e = 0.515$) (Chen et al. 2009), Elymus sibiricus ($H_o = 0.269$, $H_e = 0.181$) (Ma et al. 2008) and Hordeum vulgare ($H_o = 0.126$) (Wang et al. 2009a).

### Discussion

#### Genetic diversity and population structure

In this study, the five cpDNA fragments in I. loczyi displayed a high degree of genetic diversity ($H_d = 0.820$; Table 1), similar to other herbaceous species distributed in QTP, such as Stellera chamaejasme ($H_d = 0.834$) (Zhang et al. 2010), and species from Rhodiola sect. Trifida ($H_d = 0.923$) (Li et al. 2018). Additionally, the degree of genetic diversity was higher than the average level ($H_d = 0.670$) of 170 angiosperm species (Petit et al. 2005) and other Iris species, such as Iris dichotoma ($H_d = 0.725$) (Zhang et al. 2020). SSR data also revealed a high genetic diversity ($H_o = 0.689$, $H_e = 0.699$), which was generally higher than other studied herbaceous plants in the QTP, e.g. Rheum tanguticum ($H_d = 0.342$, $H_d = 0.515$) (Chen et al. 2009), Elymus sibiricus ($H_d = 0.269$, $H_e = 0.181$) (Ma et al. 2008) and Hordeum vulgare ($H_d = 0.126$) (Wang et al. 2009a).

| Source of variation | cpDNA VC | cpDNA PV | Fixation index | nSSR VC | nSSR PV | Fixation index |
|---------------------|----------|----------|----------------|---------|---------|----------------|
| All populations     | 55.649   | 92.11    | $F_{ST} = 0.921^*$ | 0.584   | 19.18   | $F_{ST} = 0.192^*$ |
| Within populations  | 4.766    | 7.89     | 2.460          | 74.96   | 3.044   |
| Total               | 60.416   | 79.99    |                |         |         |
| By groupings ($K = 4$ for cpDNA, $K = 3$ for SSR) |          |          |                |         |         |
| Among groups        | 88.542   | 91.14    | $F_{CT} = 0.911^*$ | 5.24    | 15.96   | $F_{CT} = 0.160^*$ |
| Among populations within groups | 3.845    | 3.96     | $F_{SC} = 0.447^*$ | 0.298   | 9.08    | $F_{SC} = 0.108^*$ |
| Within populations  | 4.766    | 4.91     | $F_{ST} = 0.951^*$ | 2.460   | 74.96   | $F_{ST} = 0.250^*$ |
| Total               | 97.153   | 77.19    |                |         |         |

Table 2. Analyses of molecular variance (AMOVA) of I. loczyi populations based on cpDNA and nSSR. VC = variance component; PV = percentage of variation; $F_{CT} =$ correlation of haplotypes within groups relative to total; $F_{SC} =$ correlation within populations relative to groups; $F_{ST} =$ correlation within populations relative to total. *P < 0.01.

Figure 3. (A) Plot showing delta $K$ values for different $K$ values tested in STRUCTURE analyses, and $K = 3$ was the optimal value. (B) STRUCTURE clustering results based on $K = 3$. The population and grouping codes were placed at the bottom. (C) Principal coordinate analyses (PCoA) clustering results. The dotted ellipse represents the grouping. (D) The UPGMA phylogenetic tree of 16 populations in I. loczyi. The vertical lines on the right represent the grouping.
as well as the mean diversity values of perennial plants ($H_o = 0.63$, $H_e = 0.68$) (Nybom 2004). *Iris* loczyi can either reproduce asexually or sexually, and can inbreeding or outcrossing. The special reproductive system provides the possibility for its high genetic diversity. Population dynamics analyses showed that *I. loczyi* had not experienced population expansion and bottleneck recently, so stable population dynamics is also an important factor for maintaining high genetic diversity. Based on different evidences, some other researchers have suggested that the uplift of mountains was responsible for higher genetic diversity (Xu et al. 2010; Qiu et al. 2011). Therefore, the orogenic process at QTP can be considered as an essential factor. Theoretically, plant species with a widespread geographical distribution often possess higher genetic diversity, compared with species confined to a restricted distribution (Spicer et al. 2003; Yoichi and Tomaru 2014; Levy et al. 2016; Chung et al. 2018). Compared with the above species, *I. loczyi* has a relatively large geographical distribution in QTP; therefore, this could be another possible factor for the high genetic diversity. The AMOVA analyses of cpDNA showed that the main source of variation originated among populations/groups, while it originated within populations in SSR, the reason was probably due to the fact that the seed flow was limited to the population or the surrounding area by the influence of gravity. And, previous studies have revealed that outcrossing, clonal and long-lived species exhibit relatively high variation within populations (Wróblewska et al. 2003).

The differentiation values calculated by AMOVA analyses showed a significant genetic differentiation between *I. loczyi* populations ($F_{ST} = 0.921$ for cpDNA, $F_{ST} = 0.192$ for SSR; Table 2), and the results of the Mantel test also indicated a significant correlation ($r = 0.49; P < 0.01$) between genetic and geographic distances. The geographical distribution of haplotypes indicated that there was no shared haplotype between the southern Tibet valley (H8–H10; Fig. 1) and the Qilian Mountain area (H1–H7); furthermore, the haplotype network showed that the haplotypes in the two regions were far from each other (Fig. 2A). Low gene flow ($N_m = 0.814$ for SSR) was identified at the level of all populations. Estimates of gene flow between population pairs suggested that there was moderate/frequent gene flow between populations within regions, but low gene flow

| Groups      | Neutrality tests | Mismatch distribution |
|-------------|------------------|-----------------------|
|             | Tajima’s D       | Fu and Li’s D         | Fu and Li’s F | SSD (P) | $H_{obs}$ (P) |
| Group I     | -1.16467         | -1.49949              | -1.61172      | 0.00010** | 0.61728**     |
| Group II    | -2.13501         | 2.35006*              | 0.57725       | 0.04718** | 0.10606**     |
| Population JC | 1.30268         | 0.80424               | 1.02604       | 0.03032** | 0.28889*      |
| Total       | 1.65602          | 2.61466*              | 2.61708*      | 0.06706** | 0.06021**     |

Figure 4. Mismatch distribution of the number of pairwise nucleotide differences for cpDNA data in (A) overall populations, (B) Group I, (C) Group II and (D) population JC. The column and the solid curve represent observed values and simulated values, respectively.
In the past decade, the response of species on the QTP and its adjacent areas to Quaternary climate oscillations has been extensively studied (Oppoenoorth et al. 2009; Wang et al. 2010; Gao et al. 2012; Ren et al. 2016). In this study, the neutrality test and MDA analyses both suggested that I. loczyi did not experience large-scale population expansion (Table 3; Fig. 4), and only a few populations had experienced local range expansion (Group I). The populations in the Qilian Mountain area and the southern Tibet valley were far apart from each other, and neither of them had experienced expansion events. Therefore, a single glacial refuge can be ruled out; that means, I. loczyi might have survived in situ in multiple refugia on the QTP during the LGM. Compared with the southern Tibet valley, the Qilian Mountain area has a higher genetic diversity, with H4 having the highest frequency (31.5%). So, the Qilian Mountain area might have been a refuge of I. loczyi during the LGM. On the other hand, the Bayesian phylogenetic tree of haplotypes showed that haplotypes (H8–H10) from the southern Tibet valley were resolved as a sister group to the haplotypes H1–H5 from the Qilian Mountain area. STRUCTURE analyses showed that the populations from the southern Tibet valley were clustered into a separate group. In addition, H8 was an endemic haplotype confined to the southern Tibet valley with high frequency (17.4%). The distribution of populations with high genetic diversity and frequency of private haplotypes indicated the existence of ‘microrefugia’ throughout the distributional range (Li et al. 2018). Thus, the southern Tibet valley might have been a ‘microrefugia’ for I. loczyi in LGM.

As the world’s largest and highest plateau, the southern and the south-eastern edges of the QTP, especially the Himalaya and Hengduan Mountains, were considered as the biodiversity hotspots (Myers et al. 2000; Mittermeier et al. 2004, 2011) and important refugia for most of the plant species (Yang et al. 2008; Wang et al. 2009b; Zhang et al. 2012; Khan et al. 2014), while the inner and northern parts of the QTP were much less studied. The northern part of QTP is bounded by the Kunlun Mountains and the Qilian Mountains while the characteristic topographic features of the inner region are parallel mountain ridges incised by a deep river valley. The distribution of I. loczyi in QTP occupies two different geographical regions, the Qilian Mountain area and the southern Tibet valley, that both were considered as the refugia of I. loczyi in the glacial period. These findings provide new insights into the location of glacial refuges for the species distributed in QTP, and the study of historical population dynamics supplements more plant species data for the response of QTP species to the Quaternary climate oscillations.

Supporting Information

The following additional information is available in the online version of this article—

Table S1. Fifty-six pairs of chloroplast universal primer related information. The primers in bold are used in this study.

Table S2. Characteristics of seven microsatellite markers for I. loczyi.

Table S3. Summary of SSR genotype data.

Table S4. Genetic diversity information of seven microsatellite loci.

Table S5. Nm values between population pairs of I. loczyi based on SSR loci.

Table S6. Sign test and Wilcoxon sign-rank test to evaluate I. loczyi for mutation drift equilibrium under different models.
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Conflict of Interest
None declared.

Contributions by the Authors
H.X. and M.S. designed the research and collected samples; G.Z. and Y.H. analysed the data; Y.H. and H.W. performed the experiment and collected the data; Z.W. prepared the figures and tables; G.Z. wrote the draft manuscript; H.X. and M.S. revised the draft manuscript. All the authors read and approved the final manuscript.

Data Availability
All cpDNA haplotype sequences of Iris loczyi and the sequences of the outgroup I. goniocarpa used in this study are available from NCBI (https://www.ncbi.nlm.nih.gov/). The GenBank accession numbers are MZ398313–MZ398362, MZ405099–MZ405102. SSR genotype data are available as a supplementary file.

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