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RESEARCH ARTICLE

Population genetic structure of *Iris ensata* on sky-islands and its implications for assisted migration

Yue-E Xiao[1,2] · Kai Jiang[1,2] · Xin Tong[1] · Yong-Hong Hu[2] · Xiao-Yong Chen[1]

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Abstract Due to global warming since the Last Glacial Maximum, many plant populations have retreated to mountain tops, i.e., sky-islands, which are isolated by low-elevation barriers and inhospitable habitats. Under projected environmental changes, these populations may decline and face local extinction. *Iris ensata* Thunb. populations at the species' most southern distribution range are in this position. We used eight polymorphic nuclear microsatellite markers and three fragments of chloroplast genome to analyze population structure of six extant sky-island populations of *I. ensata*. A total of 83 alleles were found across 192 individuals from six populations. High levels of intra-population genetic diversity ($H_E = 0.578$, $H_O = 0.608$) of *I. ensata* were detected. Moderate but significant levels of genetic differentiation were also found among the populations ($F_{ST} = 0.133$, $P < 0.001$). Mantel test showed no isolation-by-distance pattern ($r = 0.339$, $P = 0.161$). Assignment analysis classified all individuals into five groups, and four populations were dominated by only one group. Five chloroplast DNA (cpDNA) haplotypes were found, and one was shared by all populations. Two populations contained a private haplotype. Long-term fragmentation, but relatively large population sizes and restricted gene flow among populations, contributed to the above patterns. Under projected habitat changes, the studied populations are at risk of local extinction. We identified three populations that had a high priority of *ex situ* conservation to be the source populations for the future assisted migration. Number of individuals, how to select individuals, and a potential recipient site were discussed.

Keywords Assisted migration · Genetic contribution · *Iris ensata* · Microsatellites · cpDNA haplotypes · Sky islands · Tianmu Mts

Introduction

The globe has experienced an increase in temperature since the Last Glacial Maximum (LGM), especially over the past century when anthropogenic carbon dioxide emissions was accelerated. Rising temperatures drive species range shift poleward or toward higher elevations (Parmesan et al. 1999; Lenoir et al. 2008). Therefore, species at high latitudes are expected to decline, which has been observed in both plants and animals (Foden et al. 2007; Moore and Huntington 2008), and may eventually become extinct if the increase in temperature continues. Upward shifts in range limits along elevation gradients have also been found in tropical and temperate species. Climate change is likely to bring about habitat loss and declines in population sizes, threatening the species living at high elevations (Colwell et al. 2008; Moritz et al. 2008). This situation is especially serious for plants in montane wetlands, which will retract persistently due to mesophytization succession and this process will be accelerated by increasing temperature without corresponding increase of precipitation.

An approach to conserve these species/populations at high elevations is assisted migration, i.e., intentional human-mediated movement of individuals and populations (Aitken and Whitlock 2013). Assisted migration has

✉ Xiao-Yong Chen
xychen@des.ecnu.edu.cn

[1] School of Ecological and Environmental Sciences, Tiantong National Field Station of Forest Ecosystems, East China Normal University, Shanghai 200241, China

[2] Shanghai Chenshan Plant Science Research Center, Shanghai Chenshan Botanical Garden, Chinese Academy of Sciences, Shanghai 201602, China

proven to be effective in plants. For example, it had successfully helped *Lambertia orbifolia* C.A. Gardner and *Torreya taxifolia* Arn. escape lethal diseases in their native ranges (Vitt et al. 2010). Assisted migration is increasingly accepted (Vitt et al. 2010; Hewitt et al. 2011), although there is a concern about the risk of introduced species/populations becoming invasive in the new environments (e.g., Ricciardi and Simberloff 2009). To achieve a successful assisted migration, both ecological and social criteria have been proposed for decision-making (Richardson et al. 2009; Vitt et al. 2010). Population genetic information is the key for properly determining the source populations or individuals (Li et al. 2005). Unfortunately, very little genetic information, such as levels of inbreeding and genetic drift, had been obtained in most endangered species.

Iris ensata Thunb. (Iridaceae) is a perennial monocot species that grows in wetlands. It is diploid ($2n = 24$) and is the only wild ancestor of all ornamental Japanese iris cultivars (or named as Hanashobu) (McEwen 1990). *Iris ensata* flowers from June to July (Xiao et al. 2010) and is pollinated by bumblebees (Inoue et al. 2008). It is partly self-incompatible, and inbreeding depression has been observed in selfed offspring (Xiao et al. unpublished data). The seeds have corky taste and are dispersed by water currents (Arnold 2000; Meerow et al. 2007). *Iris ensata* also has strong clonal growth abilities. However, the slanting rhizomes are short, usually less than 10 cm, and thus the shoots generally clump together forming a phalanx-like structure.

Iris ensata is mainly found in northeastern China, far eastern Russia, Korea and Japan (Zhao et al. 2000). However, Tianmu Mountains of subtropical China, more than 700 km south of the main distribution range, contain several populations of *I. ensata*. In Tianmu Mts, *I. ensata* grows in montane wetlands on several mountains that are highly isolated as sky-islands by low-elevation inhospitable habitats, such as broadleaved forests. Those sky-islands are likely to be ice-age relics of mountain top refugia that were created as cool-climate species shifted upward following post-glacial temperature rising (McCarty 2001). Under projected mesophytization succession, temperature rising and anthropogenic disturbance, they will become less suitable for *I. ensata*. Given the limited space, this species may become locally extinct in the next few decades. Assisted migration seems to be the only way to conserve the isolated remnants.

In this study, we combined polymorphic nuclear microsatellite markers and chloroplast DNA sequencing to analyze genetic structure of the sky-island populations of *I. ensata*. Those populations have experienced long-term fragmentation, so we expected low within-population and high among-population genetic variation. Our specific aims were: (1) to test the impacts of long-term natural fragmentation on population genetic structure and (2) to determine the population(s) with priority for conservation. We then tried to propose potential strategies for the assisted migration.

Materials and methods

Study sites and field sampling

Based on extensive surveys, we found six *I. ensata* populations extant in different montane wetlands in the Tianmu Mts, Zhejiang Province, China (Fig. 1; Table 1). The six populations were close to the mountain tops within an altitudinal range between 950 and 1550 m, and the horizontal distance between the populations ranged from 12.4 to 80.9 km. A total of 192 individuals were collected from those populations. Fresh leaves were collected from different individuals with a minimal interval of 5 m to avoid sampling the same clone, and then were dried in silica gel. All vouchers were deposited at the Herbarium of Chenshan Botanical Garden (CSH).

DNA extraction, PCR amplification, nSSR genotyping and cpDNA sequencing

Total genomic DNA of each sample was extracted from about 30 mg dried leaves using the DNA Plant Kit (Tiangen, Shanghai, China) according to manufacturer's instructions. DNAs were dissolved in a 100 ml TE buffer for storage.

Samples were genotyped using nine polymorphic microsatellite primers (*IE48*, *IE84*, *IE118*, *IE204*, *IE236*, *IE278*, *IE312*, *IE418* and *IE723*) developed for *I. ensata* (Xiao et al. 2012). The forward sequence of each primer pair was labeled with a fluorescent dye. PCR protocols and genotyping methods were described in the previous study (Xiao et al. 2012). The genotypes were determined using an ABI PRISM 310 Genetic Analyzer and Gene Mapper v4.0 (Applied Biosystems, Foster City, CA).

After screening performance and polymorphism of 45 fragments of the chloroplast genome (data not shown), we amplified and sequenced partial *matK* gene (Wilson 2004) and two intergenic spacer (IGS) regions *psbJ-petA* and *petL-psbE* (Shaw et al. 2007). A total volume of 50 µl PCR reaction systems contained ddH_2O, 1 × buffer (Mg^{2+} free), 2.5 mM $MgCl_2$, 2.5 µM of each dNTP, 0.5 µM of each primer, 2 U of Taq polymerase (Sangon, Shanghai, China) and 20 ng DNA. PCRs were conducted on a Mastercycler pro Thermal Cycler (Eppendorf, Hamburg, Germany). The procedure was performed with an initial denaturing for 5 min at 94 °C followed by 35 cycles of

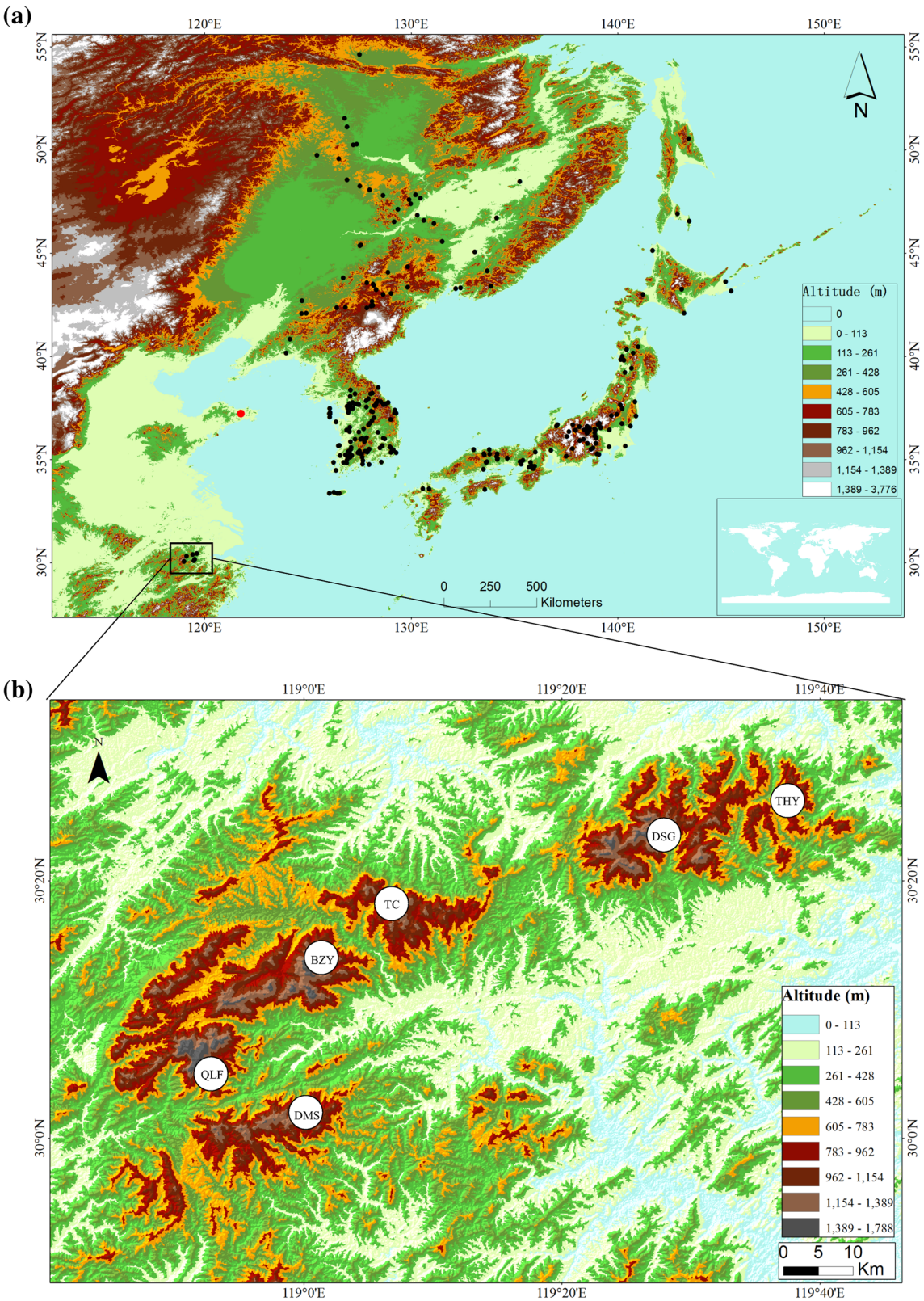


Fig. 1 **a** The present distribution of *Iris ensata* in East Asia (http://www.gbif.org; http://www.kahaku.go.jp; www.cvh.org.cn; http://www.nature.go.kr). *Black dots* indicate reported distribution sites. The *red dot* indicates the site of local extinction in Kunyu Mts of Shandong Peninsula. **b** Geographic distribution of six *I. ensata* populations in the southern distribution range. Damingshan (DMS), Qingliangfeng (QLF), Baizhangya (BZY), Tianchi (TC), Dashigu (DSG) and Taihuyuan (THY). (Color figure online)

Table 1 Characteristics of *Iris ensata* in all six populations from the Tianmu Mts, including location, population size, altitude, voucher number, sample size, statistics of genetic diversity based on nSSR data, allelic coancestry

Population	Location	Latitude and longitude	Habitat area (m^2)	Altitude (m)	Voucher #	N	MLG	MLL	A	A_P	A_R	H_O	H_E	F_{IS}	Modal F
DMS	Damingshan, Lin'an, Zhejiang	30.056°E, 118.995°N	400	1320	IE-DMS1, 2	24	24	24	5.25	3	5.25	0.635	0.661	0.039	0.109
QLF	Qingliangfeng, Lin'an, Zhejiang	30.092°E, 118.882°N	1500	1550	IE-QLF1, 2, 3	30	30	30	5.50	9	5.03	0.563	0.488	−0.156	0.203
BZY	Baizhangya, Lin'an, Zhejiang	30.165°E, 119.524°N	1000	1250	IE-BZY1, 2	36	36	36	5.25	4	4.80	0.569	0.552	−0.032	0.146
TC	Tianchi, Lin'an, Zhejiang	30.302°E, 119.119°N	800	1300	IE-TC1, 2, 3	31	31	31	5.75	4	5.50	0.661	0.611	−0.084	0.079
DSG	Dashigu, An'ji, Zhejiang	30.396°E, 119.464°N	2000	1250	IE-DSG1, 2, 3	47	46	46	6.38	5	5.48	0.625	0.648	0.040	0.083
THY	Taihuyuan, Lin'an, Zhejiang	30.436°E, 119.624°N	550	950	IE-THY1, 2, 3	24	23	24	4.13	2	4.13	0.599	0.516	−0.165	0.296

N sample size; *MLG* number of multi-locus genotypes; *MLL* number of multi-locus lineages; *A* mean number of alleles per locus; A_P number of private alleles; A_R alleles richness per locus; H_O observed heterozygosity; H_E expected heterozygosity; F_{IS} fixation index; *F* probability of allelic coancestry

30 s at 94 °C (denaturing), 30 s at 50 °C (annealing) and 45 s at 72 °C (elongation), and ending with 10 min at 72 °C. After checked on a 1.2 % agarose gel electrophoresis, purified products were bi-directionally sequenced by standard methods on the ABI 3731 automated sequencer (Applied Biosystems, Foster City, USA). Haplotype sequences were deposited in GenBank with accession numbers KP900847- KP900853.

Data analyses

Because *I. ensata* is a clonal plant, we first identified whether the ramets that had been collected from the same genets. GENECLONE v2.0 was used to test for clonality in each population by determining the multi-locus genotypes (MLGs) (Arnaud-Haond and Belkhir 2007). Identical MLGs in two observed ramets can either belong to two ramets of the same genet, or to two different genets. In this study, the MLGs were considered to be different multi-locus lineages (MLLs) if $P_{\text{sex}} > 0.01$ (Parks and Werth 1993).

We detected outlier loci using LOSITAN and the stepwise mutation model (Antao et al. 2008). Locus *IE204* showed a significant signal for selection, and thus was not used in the following analyses. The linkage disequilibria of eight loci in each population were tested by FSTAT (Goudet 1995). At the population level, genetic diversity was assessed using four parameters: mean number of alleles per locus (A), allele richness (A_R) (El Mousadik and Petit 1996), and observed (H_O) and expected heterozygosity (H_E) (Nei 1987). Two statistics A and A_R were calculated by FSTAT (Goudet 1995), and H_O and H_E within each population were calculated by TFPGA ver. 1.3 (Miller 1997). The relationship between genetic diversity and habitat area was tested using Spearman correlation analysis in *R* (http://www.r-project.org/). The fixation index (F_{IS}) and their biases from zero were assessed using FSTAT (Goudet 1995).

The overall and pairwise F_{ST} values between populations were calculated by FSTAT ver. 2.9.3.2 (Weir and Cockerham 1984). The relationship between genetic differentiation and spatial distance was analyzed by a Mantel test using the IBD software (http://www.bio.sdsu.edu/pub/andy/IBD.html). Calibrated genetic differentiation ($F_{ST}/(1\text{-}F_{ST})$) was used in the Mantel test because traditional genetic differentiation (F_{ST}) can be misleading (Rousset 1997). Genetic variance partitioning within and among populations was conducted by analysis of molecular variance (AMOVA) using GenAlEx ver. 6.41 (Peakall and Smouse 2006).

Bayesian clustering was used to detect population structure using software STRUCTURE 2.2 (Pritchard et al. 2000), which assigned all individuals into *K* clusters based

on their allele frequencies. The optimal number of clusters (K) was determined using ΔK as proposed by, Evanno et al. 2005 which is the value of second-order rate of change of log probability with respect to K. To describe the relationships between clusters, a neighbor-joining (NJ) tree (Saitou and Nei 1987) was constructed by applying the matrix of allele-frequency divergence among clusters using STRUCTURE.

We determined the relative contributions of drift versus gene flow to shaping the genetic structure across the studied region by using the likelihood approach implemented in the program 2MOD program (Ciofi et al. 1999). The drift model assumes that populations are fully isolated without migration between them and that their divergences were determined by genetic drift alone after they were separated from an ancestral panmictic population. In the gene flow model, it is assumed that the gene frequencies within populations are determined by a balance between gene flow and drift. A total of 100,000 iterations were run and the first 10 % were discarded as burn-in. The convergence was confirmed using Tracer version 1.4.8 (Drummond and Rambaut 2007). The probability (p) of modeling is based on the proportion of times that model was supported. The relative contributions of drift and gene flow in each population were determined from the parameter F.

The allele size permutation test by SPAGeDi version 1.2 (Hardy and Vekemans 2002) was used to decide whether the stepwise mutation model (SMM) or the infinite allele model (IAM) was applicable in each population using the Wilcoxon test. If the pR_{ST} value is not significantly lower than R_{ST}, then the IAM model is more applicable than the SMM model. The recent bottlenecks in the six *I. ensata* populations were tested using the program BOTTLENECK version 1.2.02 (Cornuet and Luikart 1996). The Wilcoxon test was used and three mutation model scenarios (IAM, SMM and TPM) were simulated.

To determine which populations were of high priority for assisted migration, we evaluated the contribution by each population to the whole genetic diversity or the total allelic richness of the six populations using the contribution diversity approach. This method is based on the diversity and distinctiveness (Lu et al. 2007), and it has successfully been used to evaluate species diversity contribution in the studied areas (e.g., Miloslavich et al. 2010). We calculated the genetic contribution of each population based on either gene diversity or allelic richness using software PGCA v1.0.

The relationship between allele accumulation and the number of sampled individuals was analyzed to determine how many individuals should be collected for conservation. Allele accumulation curves and the number of alleles for a certain number of sampled individuals were analyzed by SPECACCUM in package 'vegan' (Oksanen et al. 2008). The sample size for effective collection was gained when 95 % of the potential alleles being collected.

For the cpDNA datasets, the CLUSTALW program combing with manual adjustment was used to obtain multiple alignments of all sequences (Thompson et al. 1994). An unrooted network of haplotypes was constructed using TCS 1.21 (Clement et al. 2000).

Results

Genetic diversity

At the regional level, eight nSSR loci showed high levels of polymorphism in *I. ensata* (Table 1). The number of alleles per locus ranged from 7 (*IE118* and *IE278*) to 14 (*IE312* and *IE723*) and a total of 83 alleles were revealed by the eight loci. Based on the eight loci, 190 MLGs and 191 MLLs were found in the 192 samples from the six Tianmu populations. In population Dashigu (DSG), two samples sharing the same MLG and MLL (Psex >0.01) may belong to the same genet, and thus only one of the two samples was used in the our analyses.

At the population level, the mean number of alleles per locus (A) varied from 4.13 (Taihuyuan, THY) to 6.38 (DSG) (Table 1). The number of private alleles per population (A_P) ranged from 2 (THY) to 9 (Qingliangfeng, QLF) and the allelic richness (A_R) was between 4.13 (THY) and 5.50 (Tianchi, TC). H_E ranged from 0.488 (QLF) to 0.661 (Damingshan, DMS), and H_O was between 0.563 (QLF) and 0.661 (TC). Spearman correlation analysis indicated that genetic diversity levels (A, A_R, A_P, H_E, H_O) were not related to habitat size. The F_{IS} values were not significantly biased from zero, and four of them were negative (Table 1).

Genetic differentiation

Overall genetic differentiation among the *I. ensata* populations ($F_{ST} = 0.133$) was moderate and remarkably significant ($P < 0.001$). All pairwise F_{ST} values between populations were significant, which indicated the occurrence of genetic differentiations among the six populations (Data not shown). The lowest pairwise F_{ST} value (0.041) was between populations DSG and TC, and the highest pairwise F_{ST} value (0.281) was found between populations QLF and THY. Population QLF showed the greatest divergence from the other populations, and F_{ST} values ranged from 0.157 to 0.281. There was however very large genetic differentiation between population QLF and its nearest two populations, i.e., DMS and BZY ($F_{ST} = 0.214$ and 0.227, respectively). The Mantel test showed no significant correlation between genetic differentiation and geographical distances of populations ($r = 0.339$, $P = 0.161$) (Fig. 2).

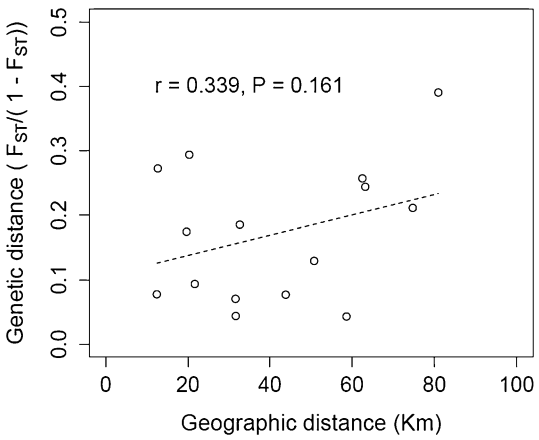


Fig. 2 The relationship between the geographic distances and genetic distances among six populations of *Iris ensata* in the Tianmu Mts

The AMOVA results indicated that there were significant genetic differentiation among the six populations ($P < 0.001$) (Table 2). A total of 87 % of the genetic variance was present at intra-population level, and only 13 % existed among populations.

Bayesian analysis of genetic structure demonstrated that the model had the highest ΔK value when K was 5 (Fig. 3a), indicating that the most probable number of groups was five. Four populations (QLF, THY, BZY and DMS) had relatively independent clusters C1, C2, C3 and C4, respectively, with a small number of mixed genotypes (Figs. 3b, 4a). In contrast, the other two populations (DSG and TC) were admixed by the five clusters, especially population TC (Figs. 3b, 4a). Furthermore, the NJ tree of five clusters (Fig. 4a) showed that the cluster C1 has the greatest differentiation.

Among 46 individuals of six *I. ensata* populations, five haplotypes (H1–H5) was identified based on four polymorphic sites detected across 3236 aligned positions of three cpDNA fragments. All haplotypes were differentiated by only one or two mutational steps. No-star pattern of haplotype network was detected (Fig. 4b). The most common haplotype H2 was shared by all populations, likely to be the ancestral haplotype. Two haplotypes H4 and H5 were private in populations QLF and THY, respectively (Fig. 4b).

The relative importance of drift versus gene flow

At the regional level in Tianmu Mts, the gene flow–drift equilibrium model was strongly favored over the drift model ($P = 1$), indicating a balance between genetic drift and gene flow. Furthermore, F values in populations THY (0.296) and QLF (0.203) were higher than those in the other four populations (from 0.079 to 0.146), which indicated that genetic drift probably had a relatively greater influence in the populations THY and QLF (Table 1).

Bottleneck evidence

The pR_{ST} values for all loci except locus *IE118* were not significantly lower than R_{ST}, suggesting that the IAM model was more applicable than the SMM model. The IAM model suggested that there were significant signs of recent bottlenecks in populations DMS and DSG (Table 3).

Populations for conservation priority

Based on gene diversity, population DMS showed the highest contribution (Fig. 5a). However, its contribution to the total number of alleles was smaller than the average (Fig. 5b), probably due to its low uniqueness. The contribution of population QLF to allelic richness was greatest because it had the highest uniqueness. Population DSG had a relatively higher contribution, based on the gene diversity and allele richness results (Fig. 5). Two populations, BZY and THY, showed negative Ct and Crt values, which coincided with their lowest levels of allelic richness. As gene diversity is affected by the number of alleles and allelic frequencies, high gene diversity may involve fewer alleles but more even allelic frequencies. Thus, allelic richness is a better measure when selecting populations for conservation. Given such considerations, we suggested that populations QLF, DSG and TC should be a high priority for *I. ensata* conservation in Tianmu Mts.

The resampling procedure demonstrated that allele number was sharply increased when the sampling size was small, but such trend became flattened when the sampling size rose above 50 (Fig. 6). When the sampling size reached 126, 95 % of the total alleles (79) would be collected.

Discussion

Genetic diversity in *I. ensata* populations on sky-islands

Negative F_{IS} values, though not significantly biased from zero, were recorded in four of the six studied *I. ensata*

Table 2 Analysis of molecular variance (AMOVA) within and among populations of *Iris ensata* in the Tianmu Mts

Source of variation	d.f.	Sum of squares	Percentage of variation	Statistic (P value)
Among populations	5	124.12	13	0.133 ($P < 0.001$)
Within populations	376	877.96	87	
Total	381	1002.08	100	

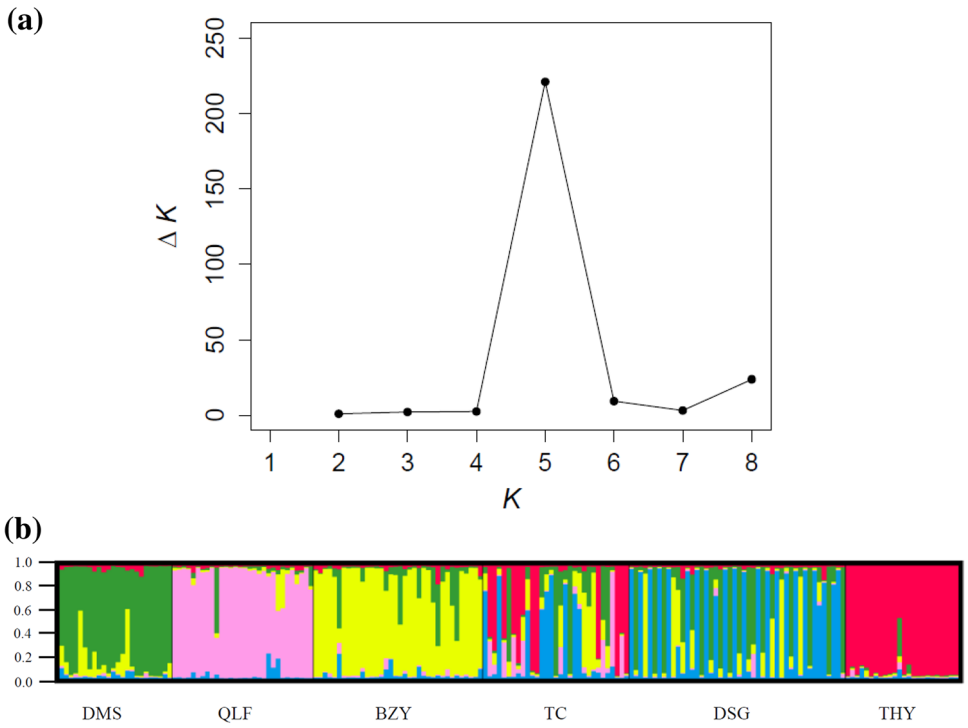


Fig. 3 STRUCTURE analysis of 191 individuals collected from six *Iris ensata* populations in the Tianmu Mts. **a** The distribution of $\triangle K$. **b** The proportion of the membership coefficient of each individual in six populations of *Iris ensata* for each of the inferred clusters defined using Bayesian clustering

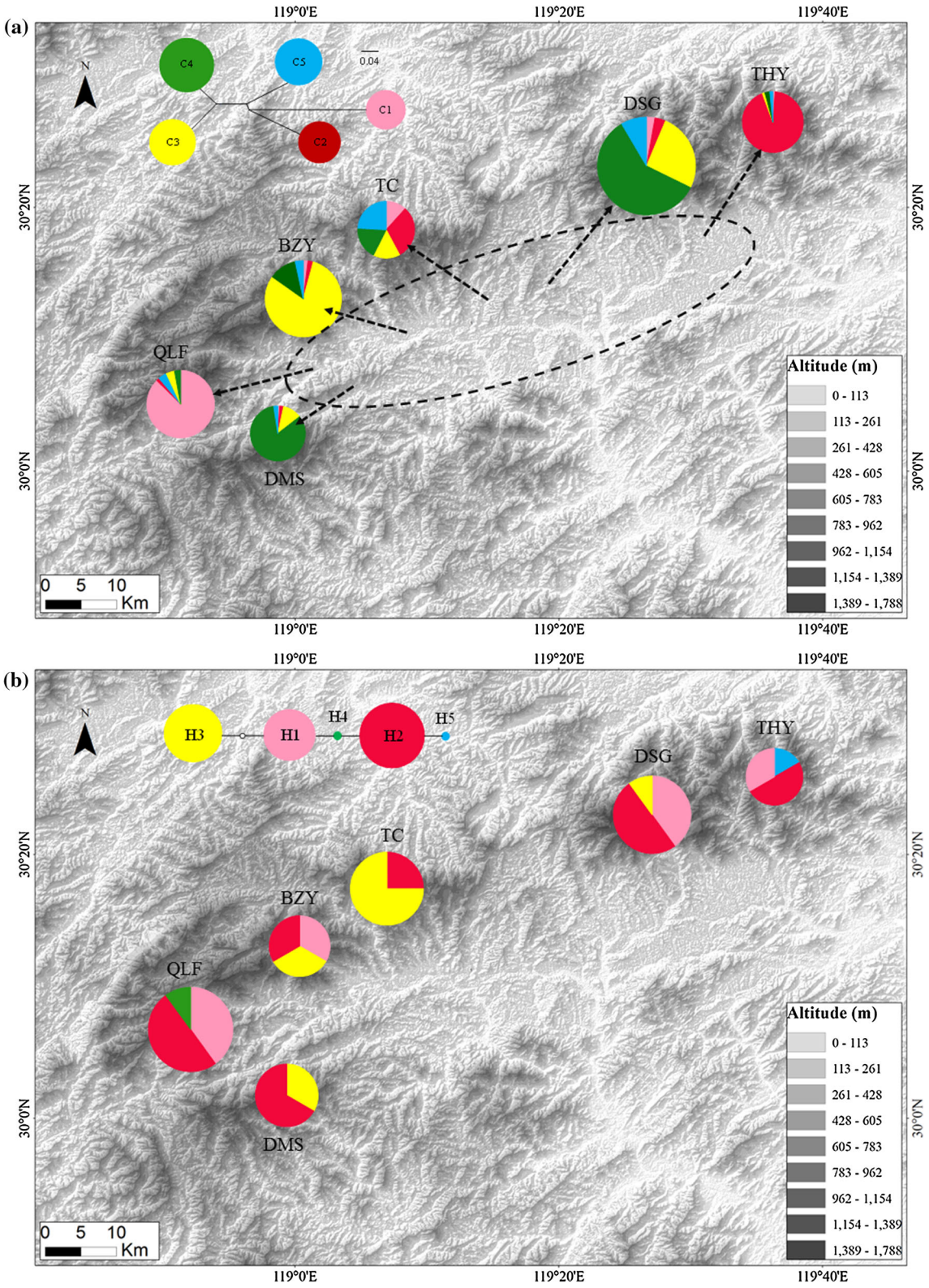
populations, which indicated that *I. ensata* was a predominantly outcrossing species. This result was consistent with our hand-pollination experiments, which revealed partial self-incompatibility and significant inbreeding depression in selfed offspring (Yue-E Xiao, unpublished data).This trend has also been observed in other irises, e.g., *Iris hexagona* (Meerow et al. 2007).

Populations inhabiting sky-islands generally have a long history of habitat fragmentation, and thus are assumed to have reduced genetic diversity, a common characteristic of fragmented populations. Indeed, reduced levels of genetic diversity have been observed in plants and animals on sky-islands. For example, a significant negative relationship between elevation and genetic diversity was observed in *Oxalis alpina* Rose ex R. Knuth in the Sonoran desert sky-islands (Pérez-Alquicira et al. 2010). In *Heptacodium miconioides* Rehder, an endangered shrub locating sky islands in eastern China, no cpDNA polymorphism but high RAPD diversity were observed (Lu et al. 2006). In the present study, we however revealed high levels of genetic diversity in populations of *I. ensata*, which is similar to other irises (Meerow et al. 2007).

The relatively high genetic diversity in the sky-island populations of *I. ensata* suggested that those populations were less impacted by natural habitat fragmentation. Although the sky-islands are not very large (400–2000 m^2) (Table 1), they could harbor moderate to large effective population sizes of *I. ensata* because of high density ($\sim$60 shoots/m^2). Therefore, genetic drift had not decreased within-population genetic diversity. This was supported by the evidence of restricted gene flow among the populations and a balance between gene flow and drift (see below). Therefore, although the populations suffered from a long history of natural fragmentation, they have been able to maintain moderate to high genetic diversities within the populations.

The relatively high genetic diversity in the isolated populations of *I. ensata* may also relate to its life history, such as clonality and outcrossing. Plants of outcrossing mating system generally have high within-population genetic diversity (Hamrick and Godt 1996). Clonal propagation can increase the longevity of genets, and alleviate the negative consequences of habitat fragmentation. The combination of genet longevity and the outcrossing breeding system may maintain genetic diversity in fragmented populations (Chen 2000; Brzosko et al. 2002). Thus, *I. ensata* seems to have survived previous environmental changes well. However, as the montane wetlands continuously shrink by the accelerating mesophytization succession and thus population sizes decline, genetic diversity of the studied populations is expected to decrease.

Retention of genetic diversity is essential to the long-term persistence of species (reviewed in Bouzat 2010). The populations inhabiting 'sky islands' might be particularly prone to losing diversity by bottlenecks due to rapid habitats changes (Jump and Peñuelas 2005). Recent bottleneck effects were detected in populations DMS and DSG. Genetic drift had not significantly decreased the


(a)
119°0'E
119°20'E
119°40'E
30°20'N
30°0'N
N
C4
C5
0.04
C1
C3
C2
THY
DSG
TC
BZY
QLF
DMS
Altitude (m)
0 - 113
113 - 261
261 - 428
428 - 605
605 - 783
783 - 962
962 - 1,154
1,154 - 1,389
1,389 - 1,788
0 5 10
Km
(b)
H3
H1
H4
H2
H5
THY
DSG
TC
BZY
QLF
DMS

◀**Fig. 4** **a** Geographic distributions of the five nSSR genetic groups detected by STRUCTURE analysis for *Iris ensata* populations in Tianmu Mts. The relationships between clusters were shown by a neighbor-joining tree. The average distance (expected heterozygosity) between individuals in each cluster is represented by the proportional sizes of *circles* with different color. The sizes of *circles* are proportional to the sample size in each population. The *ellipse* shows the possible location of glacial refugium, and *arrows* suggest possible post-glacial colonization routes. **b** Geographic distribution of the five chloroplast haplotypes of *I. ensata* populations in Tianmu Mts. The TCS network of haplotypes was shown. The sizes of *circles* are proportional to the sample size in each population

Table 3 Results of recent bottlenecks tests in six *Iris ensata* populations in the Tianmu Mts under three mutation models

Populations	IAM	TPM	SMM
DMS	0.004**	0.074	0.844
QLF	0.461	0.074	0.020*
BZY	1.000	0.641	0.055
TC	0.461	0.945	0.195
DSG	0.027*	0.945	0.074
THY	0.383	0.547	0.020*

* $P < 0.05$, ** $P < 0.01$

genetic diversity, leading to no significant relationship between genetic diversity and habitats size in *I. ensata*. Population DMS, with the smallest habitat size, had the highest expected heterozygosity. In fact, populations DMS and DSG have been developed for tourism over the past two decades, and areas of montane wetlands dramatically decreased. Furthermore, some tourists approached the mountaintop and removed plants for ornamental reasons. Although no detailed record, size of DMS population was expected to have been dramatically decreased. Thus, excluding anthropogenically negative impacts is crucial to prevent loss of genetic diversity in the studied populations.

Genetic differentiation and gene flow

The six extant *I. ensata* populations in the study region showed considerable genetic differentiation. They were clustered into five distinct groups and four populations had hardly any genotype admixture. Although most variation was partitioned within populations, a significant and moderate fraction (13 %) was partitioned among populations in *I. ensata*. Despite the small geographical range of the studied populations, the level of genetic differentiation ($F_{ST} = 0.133$) of *I. ensata* was relatively high compared to other studies with similar geographic ranges, and life histories (insect-pollination, outcrossing), even under long-term fragmentation ($\sim$7200 years) (e.g., Liu et al. 2013).

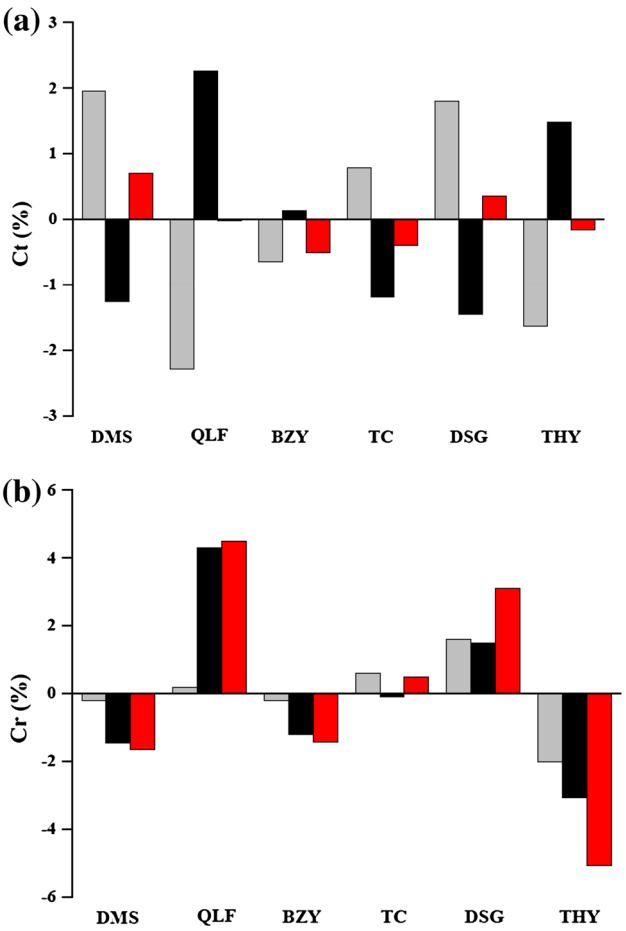


Fig. 5 Genetic contribution (*red columns*) of each *Iris ensata* population in Tianmu Mts based on gene diversity (Ct) (**a**) and allelic richness (Crt) (**b**). The *gray* and *black columns* represented the contribution to diversity within each population (Cs or Crs) and its differentiation (Cd or Crd), respectively. (Color figure online)

The relatively high differentiation was probably caused by long-term isolation and restricted gene flow. During the Ice Ages, *I. ensata* survived in southern or low-altitude refugia. Then, in a similar manner to many cool-temperate species (McCarty 2001), *I. ensata* migrated northward after the LGM, and some populations in the subtropics were driven upward and became isolated as sky-island populations. The sky-islands were surrounded by low-lying barriers that prevented the migration of *I. ensata*. Over a long period of time, gene flow among sky-island populations was quite small, which was balanced by the small drift impact in moderate and large populations. *Iris ensata* is pollinated by the bumblebee *Bombus trifasciatus*, which is commonly found at high altitudes (>1000 m) in Tianmu Mts. Field studies have shown that the foraging distance of bumblebees ranges from several hundred meters (Osborne et al. 1999) to 2.2 km (Kreyer et al. 2004). Therefore, the minimum isolation between *I. ensata* populations, i.e.,

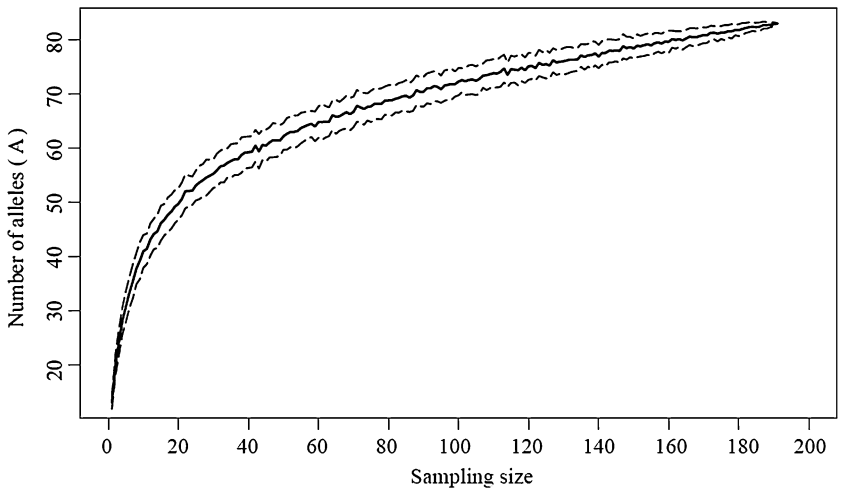


Fig. 6 Relationship between number of alleles and sampling size

12.4 km, is enough to restrict direct among-population migration by bumblebees. In addition, the winged seeds of *I. ensata* are dispersed by water currents (Arnold 2000), making it unlikely that seeds have been dispersed between populations on different mountaintop wetlands.

The strong genetic differentiation between adjacent populations is unlikely to be explained only by the present entire separation among populations, but ought to tightly link with population history. Here, based on the coancestry of cpDNA haplotypes, we proposed a tentative scenario. A large ancestral population with explicit substructure existed in Tianmu Mts during the LGM, and the mountaintops in TC and DSG were near the central of this large ancient population. There were moderate gene flow and some admixtures among different subpopulations, leading to an even distribution of genes from different subpopulations in the center (Fig. 4a). After LGM, individuals from the central population immigrated into the mountaintops in TC and DSG, while the other extant habitats were colonized by different subpopulations. Therefore, the historical metapopulation dynamics is expected to be mainly responsible for the current genetic differentiation pattern, irrespective of the geographic distance between populations. Further, the founding population size must be quite large in each extant population so that high level of genetic diversity were still retained under inter-population isolation and long-term genetic drift.

In combination with previous studies (DeChaine and Martin 2005; Marlowe and Hufford 2008), our findings suggested that restricted gene flow seems to be a common characteristic of plant populations inhabiting sky-island systems. High elevation populations of *Sedum lanceolatum* Torr. within the Rocky Mountains could not come into contact throughout the paleo-climatic fluctuations by moving up and down slopes, so there was little opportunity for inter-population gene flow, resulting in high levels of genetic differentiation (DeChaine and Martin 2005). Isolation on interglacial sky-islands limited migration among populations of *Synthyris dissecta* Rydb. in different ranges of the Northern Rocky Mountains, whereas allopatric fragmentation resulted in the formation of the Olympic endemic *S. lanuginose* (Piper) Pennell & J. W. Thomp. (Marlowe and Hufford 2008).

Implications for assisted migration

For threatened populations, in situ conservation is the conservation priority. In situ conservation should also be the first option for the threatened *I. ensata* populations. The six studied populations were located in montane wetlands of different mountains (Fig. 1). Because of the accelerating mesophytization, these wetlands will be replaced by bushes and forests, and become unsuitable for *I. ensata* growing. It is likely that they will become locally extinct over the next few decades, even without human disturbance, such as tourism and plant collecting for ornamental reasons. Thus, assisted migration seems to be the only method available for conserving these populations.

Assisted gene flow is a type of assisted migration that moves individuals and populations within a species range, and is thought to have lower ecological risks (Aitken and Whitlock 2013). However, other populations of *I. ensata* are more than 700 km north of the studied populations (Fig. 1), and long-distance inter-population translocation in this case may result in serious outbreeding depression, given the positive relationship, though not significant, between genetic distance and spatial distance. Additionally, as Aitken and Whitlock (2013) pointed out, only if the source populations were previously adapted to the same climate conditions as the target population is now experiencing, would assisted gene flow effectively enhance adaptation to the new climate conditions. The climates of

other *I. ensata* habitats are much more northern than the studied populations, and thus those populations are unlikely to have adapted to the projected climate warming. Therefore, assisted gene flow that moves individuals or populations adapted to warmer temperatures does not seem to be feasible in our studied populations, and other assisted migration approaches that move individuals to other areas seems unavoidable in the face of climate warming.

For assisted migration, given limited resources, we have to determine which population(s) should be source populations, or which is the most important and should have the highest translocation priority. With regards to conserving genetic variation, populations with high diversities and high specificities should have a high conservation priority. Our genetic contribution results suggested that population QLF has the highest priority for conservation followed by populations DSG and TC. These three populations had highest number of alleles or allelic richness and contained 67 % of the private alleles. The population QLF had the largest number of private alleles and thus the highest genetic uniqueness. Thus these three populations are of high priority for in situ and *ex situ* conservation. When we conduct assisted migration, these three populations can be the source populations. High genetic diversity in the three populations also suggested that genetic problems, such as inbreeding and genetic drift, can be easily avoid in the assisted migration populations. These genetic issues should be incorporated at an early stage into the design of assisted migration strategies, and also into the post-release monitoring. These recommendations have also been highlighted in the IUCN species reintroduction guidelines (IUCN/SSC 2013).

When the source population(s) have been identified, the next question is how many and which individuals should be collected for the assisted migration. In plant species, seeds are frequently used in assisted migration, and some seed collection strategies have been developed (Vitt et al. 2010). For a target species, a minimum of 3000 seeds, with an optimal target of 30,000, should be collected across a minimum of 50 maternal plants if 95 % of the genetic diversity is to be captured (Vitt et al. 2010). For clonal plants, ramet transplanting is frequently used in relocation. Genotyping individuals in source populations provides information that can be used to avoid collecting ramets of the same genet (Krauss et al. 2002). In our *I. ensata* populations, 191 genets were found in the 192 samples, taken at an interval of 5 m, which suggested that an interval of 5 m for ramet collection is enough to collect all the different genotypes. Therefore, we suggest that seeds and/or ramets should be collected from individuals that are separated from each other by at least 5 m. Furthermore, to effectively conserve the allelic richness, at least 126 individuals should be collected among the six populations, and collection should concentrate on populations with more private alleles. Practically we will obtain seeds or ramets from the three preferentially transported populations (QLF, TC and DSG).

Selecting a suitable recipient site is also a critical issue for successfully assisted migration. Reintroduction to a site of local extinction due to human disturbance is preferred if the human impacts can be removed because such a reintroduction will have the least risk of introducing invasive species (IUCN/SSC 2013. *Iris ensata* was found two decades ago in Kunyu Mts, Shandong Peninsula, which is about 650 km north of Tianmu Mts (http://frps.eflora.cn/frps/Iris%20ensata). However, no individual was found there during our extensive surveys in recent years, most likely due to human disturbance, such as plant harvesting for ornamental purposes, habitat loss, and tourism development. The climate conditions in Kunyu Mts are suitable for *I. ensata*. Thus wetlands in Kunyu Mts are ideal recipient sites for assisted migration after habitat improvement and reconstruction in this region. Nevertheless, more background information is still necessary to fulfill a successful assisted migration, including making a GIS-based habitat profile composed of protection level, soil conditions, hydrology conditions and vegetation type of the recipient sites (Vitt et al. 2010).

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