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Genetic diversity of the critically endangered *Ferula sinkiangensis* K.M.Shen (Apiaceae) and the implications for conservation

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Abstract: Research on the genetic diversity and structures of endangered plant species with small population sizes is a prerequisite for developing reasonable conservation strategies. Ferula sinkiangensis K.M.Shen is a critically endangered herbaceous perennial and monocarpic medical plant growing in semiarid steppe ecosystems with a small population size within the Yili Valley in Xinjiang, China. Unfortunately, the lack of genetic information has limited our ability to initiate effective conservation strategies for the species. In this study, 14 polymorphic microsatellite primers of F. sinkiangensis were developed and characterized using Illumina HiSeq paired-end reads from genomic DNA. Primers were used to assess the genetic diversity and structure of the species in the Yili Valley. A total of 92 alleles were detected across 14 microsatellite loci from 65 specimens of F. sinkiangensis. Moderate to high levels of genetic diversity (He = 0.446, I = 0.821, PPB = 92.86%) were found within the species. Our results contradict the general hypothesis that rare and endangered species with small population sizes and narrow ranges of distribution exhibit low levels of genetic diversity. The moderate to high levels of genetic diversity within the species can be explained by the mating system, life history traits, and human activities in recent years. Several reasonable conservation and reintroduction strategies are proposed, including maintenance of the plant's natural habitats, seeds or seedlings collection for germplasm storage, and artificial breeding of the species.

Key words: Ferula sinkiangensis, genetic assessment, microsatellite, small population size, medicinal plant

1. Introduction

The genetic diversity of a population or species is the foundation of evolutionary change (Darwin, 1859) and provides a genetic basis for adaptation to environmental change (Markert et al., 2010). The genetic analysis helps us to understand the genetic variation within and between individuals and populations. This information is useful to identify management units for conservation efforts and to detect population-level effects of habitat loss, fragmentation, and isolation (Palsbøll et al., 2007; Allendorf et al., 2013). For rare and endangered species, genetic diversity assessment within and between populations is an essential step for formulating appropriate management strategies for natural populations (Frankham et al., 2002; Palsbøll et al., 2007; Gordon et al., 2012; Allendorf et al., 2013; Ottewell et al., 2015; Frankham et al., 2017).

In general, the genetic diversity of plant populations is determined by population size, mating system, genetic drift, gene flow, and evolutionary and life history (Loveless and Hamrick, 1984; Hamrick and Godt, 1996; Leimu et

The genus Ferula L. (Apiaceae) consists of more than 170 perennial herbaceous taxa, mainly distributed in the

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al., 2006; Dong et al., 2007), and human factors (Qiao et al., 2010; Wu et al., 2015). In particular, anthropogenic activities, including overexploitation of natural resources, habitat degradation, land reclamation, and overgrazing, are responsible for reductions in population size (Chiang et al., 2006; Luan et al., 2006; Qiao et al., 2010; Wu et al., 2015). For rare and endangered species with small population sizes and narrow ranges of distribution, it has been hypothesized that a relatively low level of genetic diversity reduces species fitness (Young et al., 1996; Markert et al., 2010) because species with low genetic diversity are more susceptible to genetic drift, founder effect, and inbreeding depression (Nei et al., 1975; Hamrick et al., 1992; Hamrick and Godt, 1996; Frankham, 1997; Nybom, 2004). However, some endangered species (Zawko et al., 2001; Wu et al., 2015; Feng et al., 2019) and some common species with small population size (Luan et al., 2006; Schou et al., 2017) have moderate to high genetic diversity.

Mediterranean region, Central Asia, and northwest China (Pimenov and Leonov, 1993). In this genus, many species are used as medical resources (Shen, 1987; Mahendra and Bisht, 2012). In China, there are 26 Ferula spp., of which seven are endemic (She and Watson, 2005). Ferula sinkiangensis K.M.Shen is an herbaceous perennial and monocarpic plant growing in semiarid steppe ecosystems, and the species is endemic to Xinjiang, China (She and Watson, 2005). This species grows for about 7 years before flowering and fruiting (Shen, 1987). F. sinkiangensis was reported from Yining, Nileke, and Manashi counties in Xinjiang, and it has been widely used as a medicinal herb in China (Shen et al., 1975). Owing to its narrow range of distribution and important medicinal value, F. sinkiangensis has been listed as a critically endangered (CR) species (Wang and Xie, 2004; Qin et al., 2017) and an important wild medicinal plant used to treat stomach disorders in Xinjiang District for centuries (Zhang et al., 2019). Despite these listings, the distribution of F. sinkiangensis is further shrinking, likely due to global climate change, overexploitation, habitat degradation, land reclamation, and overgrazing in recent years. At the same time, lack of enough conservation awareness from the government and local people and insufficient basic research limit the successful conservation of the species although a small nature reserve has been established in Yining county. A recent report suggested that F. sinkiangensis has lost its ground in Manashi county, and the population size in Yining county has been reduced to 1/20th of its size in the 1980s (Huang et al., 2012).

With the availability of diverse molecular markers, such as allozyme, amplified fragment length polymorphism (AFLP), random amplified polymorphic DNA (RAPD), inter-simple sequence repeat (ISSR), single nucleotide polymorphisms (SNP) and simple sequence repeat (SSR) analysis, the genetic diversity of several rare and endangered species has been widely studied (Hamrick and Godt, 1989; Nybom, 2004). The best molecular markers suited for detecting genetic variability should be relatively easy to implement, inexpensive, and polymorphic enough. As a codominant and polymorphism genetic marker, microsatellites (SSRs) have been proven to be a reliable technique, capable of detecting the genetic diversity and structures of rare and endangered plants (Yang et al., 2015; Nisar et al., 2017; Bilgen et al., 2019). In order to implement reasonable conservation strategies and promote effective management practices, the genetic diversity of F. sinkiangensis was researched. The aims of this study were to develop and characterize microsatellite markers for F. sinkiangensis, to measure and analyze the genetic diversity of F. sinkiangensis, and to provide some recommendations for the conservation of the species.

2. Materials and methods

2.1. Plant sampling

In 2017–2018, a total of 65 specimens of *F. sinkiangensis* were collected from Yili Valley. Within the valley, 61 specimens were sampled from the Yining locality (YN, $43^{\circ}44'N$, $82^{\circ}06'E$) and 4 specimens were sampled from the Nileke locality (NLK, $43^{\circ}41'N$, $82^{\circ}18'E$; just 4 specimens were found in 2017 and were lost in 2018 at this locality). All fresh plant samples were dried in silica gel and then stored at –20 °C until DNA extraction.

2.2. DNA extraction, libraries, and isolation of microsatellite loci

All 65 genomic DNA samples were extracted using the Plant Genomic DNA Isolation Kit (Tiangen, Beijing, China) following the manufacturer's instructions. DNA sequencing libraries were constructed from one sample from the YN locality according to the method used by Li et al. (2019). After screening the raw sequences and removing redundant sequences, the remaining sequences were assembled using PANDAseq version 2.9. Then the microsatellite loci were detected by 4 software programs (MISA, http://pgrc.ipkgatersleben.de/misa/misa.html; MREP, http://mreps.univmlv.fr/; SSRIT, http://archive.gramene.org/db/markers/ ssrtool; and TRF, http://code.google.com/p/highssr/). Primer3 was used to design primers for the sequences detected by the 4 software programs simultaneously (Rozen and Skaletsky, 2000).

2.3. Amplification and sequencing

Fifty pairs of primers were randomly selected to amplify 15 samples from the YN locality. The amplification used the method of Schuelke (2000), based on the use of a forward SSR-specific primer with the M₁₃ universal primer sequence (TGTAAAACGACGGCCAGT) at the 5' end labeled with 6-FAM. For DNA amplification, the SimpliAmp Thermal Cycler (Thermo Fisher Scientific, Waltham, MA, USA) was used with initial denaturation at 95 °C for 5 min followed by 10 cycles at 95 °C for 30 s (denaturation), at 58 °C for 30 s (annealing), and at 72 °C for 30 s (extension), then followed by 32 cycles at 95 °C for 30 s (denaturation), at 55 °C for 30 s (annealing), and at 72 °C for 30 s (extension), with 5 min of final extension at 72 °C. The PCR reactions were run with 20 µL of reaction mixture containing 30 ng of template DNA, 0.5 mM dNTPs, 2 µL of 10X PCR buffer (TransGen, Biotech), 10 pmol of each primer, and 0.5 U of Taq DNA polymerase. The PCR products were checked on a 1.0% agarose gel. The primer set that had a successful amplification was used for all collected samples. The PCR products were genotyped on an ABI3730 automatic sequencer using mixed molecular size markers (10ulLIZ500: 1000ulhi-di, Applied Biosystems, USA). GeneMapper 4.0 (Applied Biosystems, USA) was used to score the genotypes. In addition, all of the 14 newly developed polymorphic microsatellite loci in F. sinkiangensis

were assessed for cross-amplification with the closely related species *F. tadshikorum* collected from Tajikistan and *F. fukanensis* collected from Fukang, Xinjiang.

2.4. Data analysis

To assess the level of genetic diversity within each locality and SSR locus, the number of alleles (A), effective alleles (Ne), observed heterozygosity (Ho), expected heterozygosity (He), Shannon information index (I), polymorphism information content (PIC), and percentage of polymorphic loci (PPB) for all 65 specimens were calculated using GenALEx 6.5 (Peakall and Smouse, 2012) and CERVUS v 3.0.7 (Kalinowski et al., 2007). Nei's gene diversity index (h), heterozygote deficiency within populations (F_{1s}), and F-statistics values $(F_{1S}, F_{1T}, and F_{ST})$ within each locus across all localities were calculated using FSTAT 1.2 (Goudet, 1995). The Hardy-Weinberg equilibrium at each locus of all the localities was evaluated with 1000 randomizations and adjustment of P-values was done using sequential Bonferroni correction in the web version of GENEPOP (Raymond and Rousset, 1995; Rousset, 2008). BOTTLENECK 1.2.02 was used to analyze population bottlenecks with 10,000 replicates (Piry et al., 1999). The analyses were performed for the stepwise mutation model (SMM) and the two-phase model (TPM). The significance level was estimated by the Wilcoxon signed rank test (Cornuet and Luikart, 1996). In addition, tests for shifted or normal L-shaped distribution of allele frequencies were performed (Luikart et al., 1998).

3. Results

3.1. DNA libraries and microsatellite loci

More than 4.4 million reads were obtained based on Illumina HiSeq paired-end reads, with a modal read length of 300 bp. After cleaning and assembling, about 1.40 million sequences were selected that were larger than 300 bp and used to detect SSRs. Based on MISA, MREP, SSR, and TRF, 88,738, 140,607, 25,022, and 655,644 loci were detected, respectively (Figure). Among them, there were 3033 microsatellite loci detected by the 4 software programs simultaneously (2 units repeat, 2432; 3 units repeat, 403; and 4 units repeat, 198) (Figure). Fourteen of the 50 primer pairs showed clear polymorphism and amplification in *F. sinkiangensis*. These primers were used in this study to analyze genetic diversity (Table 1). In addition, most of the 14 primer pairs were effective for closely related species *F. tadshikorum* and *F. fukanensis*, except one (FS14) in *F. fukanensis* (Table 2).

3.2. Overall microsatellite diversity

A total of 92 alleles were detected from 65 specimens of *F. sinkiangensis* using the 14 microsatellite loci. The mean number of alleles (*A*) was 6.571 for all loci, ranging from 3 (FS40, FS45) to 14 (FS13), and the mean number was 6.000 and 2.571 for the YN and NLK localities, respectively. The mean *Ne* was 2.250 for all loci, ranging from 1.025 (FS2) to 4.790 (FS39), and the number was 2.231 and 2.269 for

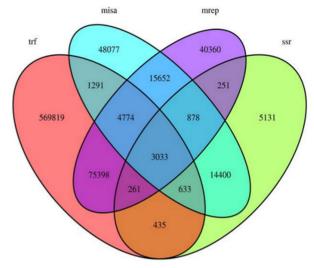


Figure. Wayne diagram of microsatellite loci of *F. sinkiangensis* detected by 4 software programs.

the YN and NLK localities, respectively. The mean Ho was 0.437 (0.433 for YN and 0.440 for NLK), which was lower than He, 0.446 (0.461 for YN and 0.500 for NLK) (Tables 1 and 3). The I value was 0.821 (0.917 for YN and 0.725 for NLK). PPB was 100.00% and 85.71% for YN and NLK, respectively (Table 3). Three loci (FS2, FS21, and FS40) were lowly polymorphic (PIC < 0.2), 4 loci (FS1, FS9, FS14, and FS45) were moderately polymorphic ($0.2 \le PIC$ ≤ 0.5), and 7 loci (FS6, FS12, FS13, FS18, FS22, FS36, and FS41) were highly polymorphic (PIC > 0.5) (Table 1). Five loci (FS1, FS9, FS22, FS36, and FS41) showed a significant departure from Hardy-Weinberg equilibrium with the deficits of heterozygotes after Bonferroni corrections (P < (0.001) (Table 1). Within-population genetic diversity (h_s) was 0.485, and the total genetic diversity (h_r) was 0.517. The inbreeding level (F_{IS}) for the YN locality was 0.068, and for the NLK locality it was 0.140.

3.3. Genetic bottlenecks

The genetic bottleneck test showed a recent bottleneck at the YN locality (Table 4). The excess of heterozygosity test showed a highly significant excess at the YN locality (TPM method (P = 0.005) and SMM method (P < 0.001)). The YN locality showed a normal L-shaped allele frequency distribution, and the NLK locality showed a shifted allele frequency distribution.

4. Discussion

4.1. Polymorphic and highly informative 14 microsatellite loci for *F. sinkiangensis*

Due to their codominant, hypervariable, and reliable scoring characteristics, SSR markers have high efficiency in population genetic studies (Yang et al., 2015; Nisar et al.,

Locus	Primer (5'-3')	Repeat motif	Size (bp)	A	Ne	Но	Не	PIC	GenBank accession no.
FS1	F:AGCCTTGGTAGAAGCTCAGA R:TGCTACAAGATGACTTCACGACA	(AAG) ₁₀	254-338	5	1.734	0.891	0.527	0.416***	MK920973
FS2	F:ACAATCACCTACCTTCCCGG R:TCTTGGTGAGCATGGCCATT	(AAT) ₈	156–282	4	1.025	0.031	0.047	0.046	MK920974
FS6	F:AAGCGCTTCTTGGGAGGC R:CCTCACTTTGGGCAGCCATA	(ATT) ₁₁	115–267	8	2.598	0.785	0.674	0.619	MK920975
FS9	F:CAGAGGAGGAGGCTGTTGC R:AGACTCATAGCTGCTCGTAGA	(GTT) ₁₀	252-429	5	2.362	0.908	0.531	0.418***	MK920976
FS12	F:ACAAAAGGGTGGATGAGATGGT R:TTGATTAGGTCAACTGCTGTCC	(TGG) ₇	105-310	9	3.388	0.585	0.591	0.559	MK920977
FS13	F:ATGCACTTCTTGGGAGGCAA R:TCAGACCTTCTATTTACACAGCA	(TTA) ₉	111–296	14	4.790	0.538	0.717	0.67	MK920978
FS14	F:GCTCGTAACCCTAGCAGCAA R:GGACACATCCTCATAATACTTTGCA	(TTG) ₇	250-329	8	1.708	0.234	0.393	0.363	MK920979
FS18	F:AGTCTATAAAGTGCATGCTCTCA R:TGCTTTAAAAGATGTTTGTGGAGT	(TATC) ₆	224-247	7	2.526	0.746	0.673	0.61*	MK920980
FS21	F:AGCCTCTCCAATGCCATCTC R:TCGCCCCATTCTTAAGACTGT	(ATTT) ₅	112-288	4	1.174	0.046	0.075	0.074	MK920981
FS22	F:ACTGTGAACAAACAAACGGATG R:GTGATAATTTGACCAGCGCCA	(AC) ₉	234-252	11	2.529	0.246	0.706	0.674***	MK920982
FS36	F:TGCAGGTAGATGGCAGTTTGT R:TGTTGTGCGTATGGGTTGGA	(GA) ₉	226-270	7	2.619	0.095	0.708	0.659***	MK920983
FS40	F:CCAACTCCATTTGTCGTTGCA R:GAATCCCCATTGCACCACCA	(GT) ₁₃	122-258	3	1.334	0.015	0.09	0.087	MK920984
FS41	F:AGAGTTGTGCTACCGGATGT R:AAACGGTCCATCAACGTCCA	(TA) ₉	262-311	4	2.557	0.862	0.678	0.617***	MK920985
FS45	F:ACCTAGTTGCCACCTCTCTTC R:TGGCAAAATCCATATATCGCACA	(TC) ₉	264-269	3	1.155	0.092	0.225	0.212	MK920986

Table 1. Characteristics of 14 microsatellite loci of *F. sinkiangensis*. GenBank accession numbers, primer sequences, repeat motifs, allele size range (bp), and genetic diversity index are given. Fragment sizes are based on 65 specimens from two localities.

A, number of alleles; *Ne*, effective number of alleles; *Ho*, observed heterozygosity; *He*, expected heterozygosity; *PIC*, polymorphic information content; P-values for Hardy–Weinberg equilibrium tests are given for each locus, *P < 0.05, **P < 0.01, ***P < 0.001.

2017; Bilgen et al., 2019). In this study, a set of microsatellite loci was developed to examine the population genetics of *F. sinkiangensis*. Among the explored loci, 14 of them were shown to be highly polymorphic and informative, and thus were selected to study genetic diversity (Table 1). It is noteworthy that these 14 primer pairs were also successfully amplified in *F. tadshikorum* and *F. fukanensis* (Table 2), indicating successful use for cross-amplification with closely related species in the future. In addition, many of the loci detected by the 4 software programs (Figure) can be used to assess cross-species amplification for future population genetics studies within the genus *Ferula*.

4.2. Moderate to high level of genetic diversity but small population size

A moderate to high level of genetic diversity in *E*. *sinkiangensis* was detected, in spite of the small population size and narrow geographic distribution. These results might indicate that the species can adapt to changing environmental conditions and has great evolutionary potential. This study showed that the genetic diversity of

Locus	Species						
Locus	F. tadshikorum	F. fukanensis					
FS1	+	+					
FS2	+	+					
FS6	+	+					
FS9	+	+					
FS12	+	+					
FS13	+	+					
FS14	+	-					
FS18	+	+					
FS21	+	+					
FS22	+	+					
FS36	+	+					
FS40	+	+					
FS41	+	+					
FS45	+	+					

Table 2. Results from cross-species amplification for Ferula tadshikorum and F. fukanensis.

F. sinkiangensis ($h_s = 0.485$, $h_r = 0.517$, *Ho* = 0.437, *He* = 0.446, *I* = 0.821, *PPB* = 92.86%) was lower than the genetic diversity of widespread species (He = 0.58) and higher than the genetic diversity of endemic species (He = 0.42) (Nybom, 2004). The genetic diversity of F. sinkiangensis was also higher than that of some other species of Ferula, such as *F. loscosii* (He = 0.125, $h_r = 0.152$) (Pérez-Collazos and Catalań, 2008), F. communis (Hpop = 0.320) (Rahali et al., 2016), F. communis complex (Hw = 0.263, h_r = 0.317) (Dettori et al., 2016), *F. arrigonii* (Hw = 0.317, $h_T =$ 0.336) (Dettori et al., 2013), and *F. asafetida* ($h_r = 0.34$, *I* = 0.51) (Tajbakht et al., 2018). Compared to other rare and endangered species, F. sinkiangensis showed a moderate to high level of genetic diversity, which was higher than Nuphar submersa (He = 0.42) (Shiga et al., 2017) and Ottelia acuminata var. jingxiensis (He = 0.441, I = 0.781) (Li et al., 2019), and lower than Ruta oreojasme (He =

0.687) (Meloni et al., 2015), *Vincetoxicum atratum* (He = 0.67) (Yamashiro et al., 2016), *Ammi seubertianum* (He = 0.66, I = 1.28), *A. trifoliatum* (He = 0.67, I = 1.35) (Vieira et al., 2018), and *Tapiscia sinensis* (He = 0.6904, I = 1.4368) (Zhou et al., 2016). The moderate to high level of genetic diversity within *F. sinkiangensis* contradicted the general hypothesis that small and narrowly distributed species have low levels of genetic diversity (Frankham, 1997; Leimu et al., 2006; Flight, 2010).

Several factors contribute to a high level of genetic diversity in rare and endangered plants, including speciesspecific factors (e.g., mating system, genetic bottleneck, evolutionary and life history) and anthropogenic factors such as overexploitation of natural resources, habitat degradation, land reclamation, and overgrazing (Loveless et al., 1984; Hamrick et al., 1996; Leimu et al., 2006; Dong et al., 2007; Qiao et al., 2010; Wu et al., 2015). It has also been suggested that selfing species usually have considerably lower levels of genetic diversity than outcrossing species (Hamrick and Godt, 1989; Nybom, 2004). Although there is not information on the mating system of F. sinkiangensis, outcrossing may be happening in the species because insect pollination has been observed in other species of the genus Ferula (Koul et al., 1993; Yaqoob and Nawchoo, 2016). Therefore, moderate to high levels of genetic diversity in F. sinkiangensis may be explained by the cross-pollination within the species. Research on the mating system of F. sinkiangensis is necessary in future. The overlapping of generations in F. sinkiangensis (perennial and monocarpic) is highly advantageous to retain genetic variation (Shen et al., 1975). In addition, a recent decline in the F. sinkiangensis population has been reported by scientists, local individuals, and literature records (Shen, 1987; Huang et al., 2012). Though F. sinkiangensis were described as a new species in 1975, the collection and utilization of Ferula resin started much earlier. In 1958, about 4000 kg of Ferula resins from F. sinkiangensis was collected by a local company and individuals (Shen, 1987), indicating that the species had a larger population in the 1950s. However, overexploitation (collecting Ferula resin before seeds mature, and land being reclaimed for farming) led to habitat and population size reduction

Table 3.	Genetic	diversity	of F.	sinkian	gensis	localities.
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Рор	Ν	Α	Ne	Ι	Но	Не	PPB
YN	60.571	6.000	2.231	0.917	0.433	0.461	100.00%
NLK	3.643	2.571	2.269	0.725	0.440	0.431	85.71%
Total	32.107	4.286	2.250	0.821	0.437	0.446	92.86%

A, number of alleles; *Ne*, effective number of alleles; *Ho*, observed heterozygosity; *He*, expected heterozygosity; *I*, Shannon's information index; *PPB*, percentage of polymorphic loci.

Locality	Wilcoxon signe (one-tailed P-v	Mode-shift test	
	ТРМ	SMM	
YN	0.005	< 0.001	Normal L-shape
NLK	0.050	0.053	Shifted

Table 4. Tests for mutation drift equilibrium and mode shift using BOTTLENECK.

in recent years (Shen, 1987; Huang et al., 2012). The surviving population of *F. sinkiangensis* harbors part of the genetic variation detected in this study. Compared to species factors, human factors might be dominant for *F. sinkiangensis*, similar to *Tupistra pingbianensis* (Qiao et al., 2010) and *Rhododendron protistum* var. *giganteum* (Wu et al., 2015).

In general, when mating among close relatives increases the population size decreases, resulting in a high inbreeding coefficient and inbreeding recession (Frankham et al., 2002). The inbreeding coefficients for the YN and NLK localities were positive ($F_{IS} = 0.068$ and 0.140, respectively), suggesting that there were excessive homozygotes in the species. The positive index of an inbreeding coefficient suggests the risk of inbreeding recession in rare and endangered *F. sinkiangensis*.

4.3 Conservation strategies

The results from the genetic diversity of endangered *F. sinkiangensis* provide valuable insight for the conservation and management of the species. The moderate to high level of genetic diversity in *F. sinkiangensis* contrasts with and challenges the general hypothesis that small and narrowly distributed species usually have a low level of genetic diversity. Our results also suggest that *F. sinkiangensis* might have great evolutionary potential and

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can adapt to various environmental conditions (Markert et al., 2010). Based on the results, it was inferred that not genetic factors but rather a sharp decline in the abundance of the species due to human-related factors, such as habitat degradation and fragmentation and overexploitation and land reclamation, may have contributed to the endangered status of the species. However, due to small population size and narrow geographic ranges, the species faces a growing risk of genetic drift and inbreeding recession (Nei et al., 1975; Hamrick et al., 1992; Hamrick and Godt, 1996; Frankham, 1997; Nybom, 2004). Therefore, to protect and increase viable populations of the species, some conservation strategies should be proactive. Here, in situ and ex situ conservation and restoration were suggested for simultaneous utilization. In situ conservation, which may maintain an appropriate, effective population size of F. sinkiangensis, can be achieved by expanding the existing protected areas and the reclamation and restoration of habitats destroyed by farmland expansion. For ex situ conservation, seeds or seedlings should be collected for germplasm storage and to maximize the protection of existing genetic diversity. In addition, artificial breeding should be encouraged for regression and population reconstruction. Finally, strengthening public outreach and conservation education should enable science-based conservation of rare and endangered species in China and other places.

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