Biochemical Systematics and Ecology 38 (2010) 236–242

Contents lists available at ScienceDirect

Biochemical Systematics and Ecology

journal homepage: www.elsevier.com/locate/biochemsyseco

Genetic diversity and structure of a traditional Chinese medicinal plant species, *Fritillaria cirrhosa* (Liliaceae) in southwest China and implications for its conservation

De-Quan Zhang [a,c], Lian-Ming Gao [a], Yong-Ping Yang [b,*]

[a] *Laboratory of Biodiversity and Biogeography, Institute of Tibetan Plateau Research at Kunming, Chinese Academy of Sciences, No. 132, Lanhei Road, Kunming, Yunnan 650204, PR China*
[b] *Institute of Tibetan Plateau Research at Kunming, Chinese Academy of Sciences, No. 132, Lanhei Road, Kunming, Yunnan 650204, PR China*
[c] *Graduate School of Chinese Academy of Sciences, Beijing 100039, PR China*

ARTICLE INFO

Article history:
Received 10 August 2009
Accepted 24 December 2009

Keywords:
Fritillaria cirrhosa
AFLP
Genetic diversity
Evolutionary lineage

1. Introduction

Assessment of the level and distribution of genetic diversity within a plant species contributes vital information regarding its evolutionary history and is critical to the development of effective conservation and management practice (Ge, 1997; Francisco-Ortega et al., 2000; Shah et al., 2008). Several aspects of conservation biology, such as loss of genetic diversity and restoration of threatened populations, can only be addressed by detailed population genetics studies (Hamrick and Godt, 1996). Loss of genetic diversity could lead to a decline in the ability of a species to cope with changing environments and demographic fluctuations, both in the short and long term (Milligan et al., 1994; Reisch et al., 2003). Generally, geographically widespread species tend to possess more genetic polymorphisms than species with restricted distribution; moreover, low levels of genetic diversity are shown as a common feature of rare plants (Karron, 1991; Hamrick and Godt, 1996; Li et al., 2002). However, some endangered species also showed high levels of genetic variation even within extremely narrow distributions (Kang and Chung, 2000; Nakagawa, 2004; Ellis et al., 2006).

With about 130 species, *Fritillaria* L. is the largest genus of the Family Liliaceae sensu, and is phylogenetically close to the genus *Lilium*. There are about 24 species generally recognized in China, out of which 15 species are endemic. *Fritillaria cirrhosa* is an endangered perennial herb, distributed mainly in SW China (Mt. Hengduan region) and the Eastern Himalayas of India, Bhutan and Nepal. It generally grows on alpine or subalpine meadows or with shrubs at altitudes of 3200–4600 m (Chen and Mordak, 2000). Populations of *F. cirrhosa* are scattered in isolated patches throughout its distribution range. This species can reproduce both sexually or asexually, but the former is dominant (Ma, 1996). It usually flowers from May to July, and its

* Corresponding author. Institute of Tibetan Plateau Research at Kunming, Chinese Academy of Sciences, No. 132, Lanhei Road, Kunming, Yunnan 650204, PR China. Fax: +86 871 5223398.
E-mail address: yangyp@mail.kib.ac.cn (Y.-P. Yang).

0305-1978/$ – see front matter
doi:10.1016/j.bse.2009.12.029

flowers generally have yellow petals with purple spots and developed nectaries. Although there are few reports about the breeding system for *F. cirrhosa*, we deduce that it is likely outcrossing according to field survey and breeding features of its closely related species (Amsberry and Meinke, 2002). The capsules mature in September or October, and contain 80–200 seeds per capsule with 1000-grain weight for 1.96 g (Chen et al., 1993; Ma, 1996). The bulbs of *F. cirrhosa* (Chuanbeimu) have been used as traditional Chinese medicine (TCM) for a long time in China. It is mainly used as antitussive, expectorant and antihypertensive drugs (Yan et al., 1999). Due to its strict habitat requirements, domestication and cultivation are extremely difficult. Therefore, majority of *F. cirrhosa* is still gathered from the wild. Due to over-harvesting, habitat fragmentation and over-grazing in the last decades, the wild populations and sizes of *F. cirrhosa* are rapidly decreasing and it is facing extinction. It was listed as a Class III protected species in the Red-list of China's National Protected Medicinal Plants (http://www.chinabiodiversity.com/feedback/intro_23.htm). Thus, an understanding of the genetic variation and structure of *F. cirrhosa* is important for effective conservation of this species in China.

Scientific approaches for conservation and utilization of plant resources require accurate assessment of the amount and distribution of genetic variation within and among populations (Han et al., 2007). Molecular marker techniques have been widely used to provide valuable information about the genetic diversity and structure of natural plant populations (Powell et al., 1996; Ouborg et al., 1999). The AFLP technique, developed by Zabeau and Vos (1993, 1995), is based on PCR amplification of a fraction of restriction fragments generated by the digestion of total DNA. Because a large number of polymorphic loci can be investigated in a single experiment, the AFLP technique has become one of the dominant methods in studies of genetic diversity, especially for species whose genomic sequence information required for markers is not available (Bensch and Akesson, 2005; Meudt and Clarke, 2007). It has been widely used for detecting genetic diversity and structure (Krauss, 2000; Tang et al., 2003; Liu et al., 2006a,b; Abbott et al., 2007) and phylogenetic relationships (Drossou et al., 2004; Gulbitti et al., 2007; Worley et al., 2009).

In this study, our main objectives were: (i) to quantify the level of genetic diversity within and among populations of the endangered species; (ii) to estimate the genetic differentiation under the pressure of human collecting activity; (iii) and to provide recommendations for sustainable utilization and conservation of the important traditional Chinese medicinal resources of *F. cirrhosa*.

2. Materials and methods

2.1. Sample collection

During 2005, leaf tissues of 159 individuals were collected from 9 wild populations of *F. cirrhosa* from SW China (Fig. 1 and Table 1). To avoid resampling from the same clone, distance between individuals was greater than 5 meters. Healthy, fresh leaves were sampled and dried by silica gel until DNA extraction. Vouchers were collected from each population and deposited at the herbarium of Kunming Institution of Botany, Chinese Academy of Science (KUN).

2.2. DNA extraction and AFLP analysis

Genomic DNA was extracted and purified using Universal Genomic DNA Extraction Kit ver.3.0, produced by TaKaRa Biotechnology (Dalian) Co. Ltd. The AFLP procedure was carried out according to Vos et al. (1995) with minor modifications. Genomic DNA was digested with *EcoRI* and *MseI* (New England Biolabs) at 37 °C for 3 h and then ligated to adapters at 16 °C for 10 h. PCR amplification was performed in two consecutive reactions. A total of 30 cycles of PCR pre-amplifications (30 s at 94 °C, 30 s at 56 °C and 60 s at 72 °C) were performed. The PCR products of the pre-amplification reaction were then used as template, after being diluted 10-fold in sterile water for selective amplification using a combination of AFLP primers.

From the 64 primer combinations provided by PE company, two pairs (EcoRI.1/MseI.11 and EcoRI.6/MseI.13) were selected (EcoRI.1, 5′-GACTGCGTACCAATTCACT-3′/MseI.11, 5′-GATGAG TCCTGAGTAACAG-3′; EcoRI.6, 5′-GACTGCGTACCAATTCAGC-3′/MseI.13, 5′-GATGAGTCCTGAGTA ACTC-3′). The PCR reactions of selective amplification were performed for one cycle at 94 °C for 30 s, 65 °C for 30 s and 72 °C for 60 s, followed by reduction of the annealing temperature at each cycle by 0.7 °C for 12 cycles; the annealing temperature was maintained at 56 °C for the remaining 23 cycles. The final PCR products were laded on to a 6% denaturing polyacrylamide gel and electrophoresed in 1 × TBE buffer for 1.5 h at 100 W, followed by silver staining and photographing (Wang et al., 2005).

2.3. Data analysis

Only bands that could be unambiguously scored across all the sampled populations were used in the analysis. AFLP profiles were scored for each individual as presence (1) or absence (0) of a specific band to form a data matrix. POPGENE 1.31 was used for analysis and measure of the genetic diversity parameters at population and species level (assuming Hardy-Weinberg equilibrium) (Yeh et al., 1999), including the percentage of polymorphic loci (*PPB*), Nei's (1973) gene diversity (H_E, namely expected heterozygosity) and total genetic diversity (H_T). Similarly, Shannon's index (S) is also calculated as estimating the genetic variation, with $S = -\sum p_i \log_2(p_i)$, where p_i is the frequency of a given AFLP band. To estimate the genetic divergence among populations, we also calculated the relative magnitude of genetic differentiation among populations ($G_{ST} = H_T - H_S/H_T$). Based on the island model, gene flow was inferred indirectly using Wright's (1951)

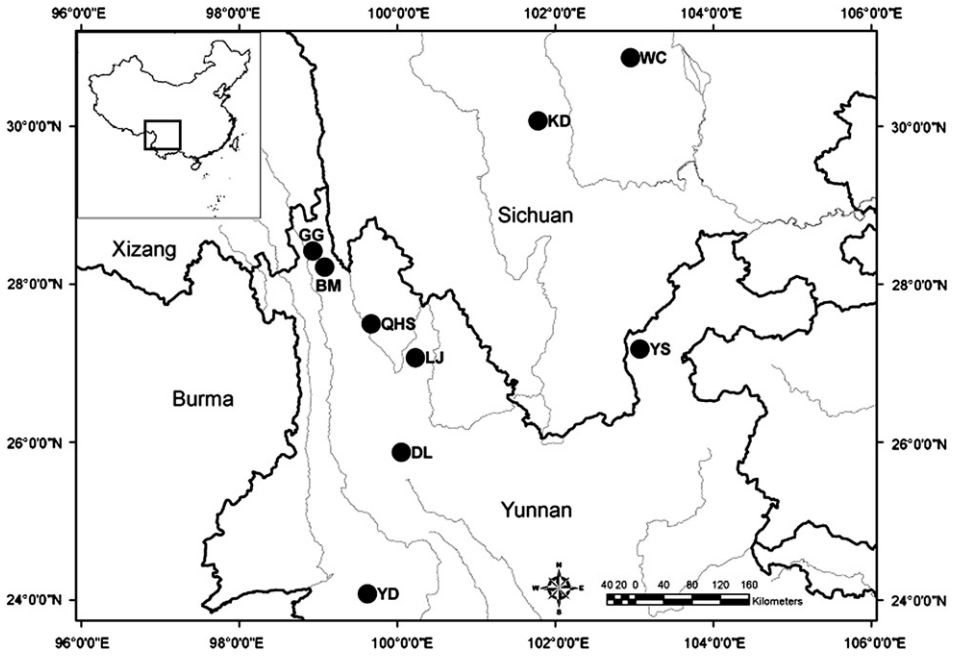

Fig. 1. Geographic locations of sampled populations of *F. cirrhosa* in SW China. Population abbreviations follow those in Table 1.

formula: $N_m = 0.25(1 - F_{ST})/F_{ST}$. Otherwise, Nei's genetic distance (D) was calculated for all pairwise combinations of populations.

In addition, an analysis of molecular variance (AMOVA) was applied to estimate variance components for AFLP phenotypes, partitioning the variations among populations and individuals using AMOVA 1.55 (Excoffier et al., 1992). The variance components were tested statistically by nonparametric randomization tests using 1000 permutations.

To explore the genetic relationships among all populations, a UPGMA (unweighted pair-group method using arithmetic average) dendrogram was constructed based on the matrix of Nei's genetic distance by the program TFPGA, version1.3 (Miller, 1997). In order to test the relationships between genetic distances (D) (Nei, 1972) and geographical distances (km) among populations, mantel test was performed using the same software (computing 1000 permutations).

3. Results

3.1. Genetic diversity

Two primer combinations for selective-amplification produced a total of 161 reproducible and unambiguous bands, ranging in size from 120 bp to 750 bp, in which 148 bands (91.93%) were polymorphic. The percentage of polymorphic loci (*PPB*) for a single population ranged from 44.72% (YS) to 84.47% (LJ) with an average of 74.95% (Table 2). Assuming Hardy–Weinberg equilibrium, the average gene diversity was estimated to be 0.2704 (H_E) at population level, and 0.3709 (H_T) at the species level. The Shannon's index was 0.4019 (S_{POP}) and 0.5414 (S_{SP}) at population and species level, respectively. Among the nine populations, population LJ exhibited the highest level of genetic diversity ($PPB = 84.47\%$, $H_E = 0.3275$ and $S_{POP} = 0.4806$),

Table 1
Locations and sample size of 9 populations of *F. cirrhosa*.

Code of population	Sample size	Locality	Position	Altitude (m)
GG	19	Gongga, Deqin, YN	28°29′N, 98°56′E	4500
QHS	19	Qianhushan, Zhongdian, YN	27°30′N, 99°40′E	3700
BM	18	Baimaxueshan, Deqin, YN	28°31′N, 99°01′E	4300
DL	19	Cangshan, Dali, YN	25°52′N, 100°03′E	3364
LJ	19	Yulongxueshan, Lijiang, YN	27°04′N, 100°14′E	3142
YD	16	Daxueshan, Yongde, YN	24°05′N, 99°37′E	3200
YS	10	Yaoshan, Qiaojia, YN	27°11′N, 103°04′E	3400
KD	20	Zheduoshan, Kangding, SC	30°04′N, 101°47′E	3941
WC	19	Balangshan, Wenchuan, SC	30°52′N, 102°57′E	3580

YN: Yunnan Province; SC: Sichuan Province.

Table 2
Analysis of genetic diversity for nine populations of *F. cirrhosa* detected by AFLP.

Population	Size	H_E	*S*	*PPB*	G_{ST}	*Nm*	Φ_{ST}
GG	19	0.2720(0.1788)	0.4112(0.2461)	81.99%			
QHS	19	0.2573(0.1754)	0.3935(0.2421)	81.99%			
BM	18	0.2958(0.1812)	0.4403(0.2488)	81.99%			
DL	19	0.2530(0.1927)	0.3792(0.2703)	73.29%			
LJ	19	0.3275(0.1759)	0.4806(0.2412)	84.47%			
YD	16	0.2422(0.2143)	0.3560(0.3027)	62.11%			
YS	10	0.1723(0.2108)	0.2520(0.3000)	44.72%			
KD	20	0.3069(0.1738)	0.4563(0.2391)	83.85%			
WC	19	0.3048(0.1882)	0.4481(0.2605)	80.12%			
Mean	17.8	0.2704(0.1887)	0.4019(0.2612)	74.95%			
Species level	159	0.3709(0.1408)	0.5414(0.1898)	91.93%	0.2771	0.6523	0.2553

H_E, Nei's (1973) genetic diversity; *S*, Shannon's information index; *PPB*, the percentage of polymorphic bands; G_{ST}, genetic differentiation between populations (Nei's); N_m: estimated gene flow; Φ_{ST}, genetic differentiation between populations estimated by AMOVA analysis.

whereas population YS showed the lowest variability ($PPB = 44.72\%$, $H_E = 0.1723$ and $S_{POP} = 0.2520$). Similarly, there was also relatively low genetic variation in the population YD (Table 2).

3.2. Genetic differentiation

Genetic differentiation among the nine sampled populations (G_{ST}, assuming Hardy-Weinberg Equilibrium) is 0.2771, showing that the majority of variation (72.29%) was distributed within populations, similar to results of AMOVA analysis ($\Phi_{ST} = 0.2533$) (Table 3). The overall level of inferred gene flow (N_m) was estimated as 0.6523 individuals per generation among populations, showing a relatively high migration rate between populations. Nei's genetic distance (*D*) between populations varied from 0.0747 (between population GG and QHS) to 0.3109 (between population YD and YS) (Table 4). The results of Mantel test indicated that there was a significant correlation between the genetic distance and the geographic distance between populations ($r = 0.4515$, $p = 0.006$).

The UPGMA tree based on Nei's (1972) genetic distance (*D*) was shown in Fig. 2. Population YD diverged first and and YS second on the UPGMA tree, and the remaining populations (GG, BM, QHS, KD, WC DL and LJ) from Mt. Hengduan region formed a clade which was divided into two clusters, one of which included the two southern populations (DL and LJ) and the other included the northern populations (GG, BM, QHS, KD and WC). A finescale spatial geographic pattern was reflected in the groupings of populations within the dendrogram (Fig. 2).

4. Discussion

4.1. Genetic diversity

It is proposed that high genetic diversity has a positive effect on the long-term persistence of species by increasing their ability to adapt to changing environmental conditions (Vrijenhoek, 1985). Accordingly, decreased genetic variation would affect population viability by reducing individual fitness (Hattemer, 1991). The population genetic analyses of the AFLP data revealed high levels of genetic diversity at population level and species level of *F. cirrhosa* from SW China (Table 2). Generally, overharvesting of wild resources might result in sharp decrease of the population numbers and sizes, with patches of local extinction, impacting the ability to adapt to changing environments and evolutionary potential. For example, random harvesting of American ginseng resulted in significant loss of genetic diversity (Cruse-Sanders et al., 2005). Moreover, Buchert et al. (1997) reported a 75% density reduction for *Pinus strobus* with a 25% decrease of total allele number, especially the rare or low frequency alleles. As an important medicinal plant, *F. cirrhosa* has been harvested for thousands of years, with the greatest pressure in the past 30 years. From our field survey and ethnobotanic investigation, we observed that the population number and size are rapidly decreasing due to overcollection. Over-harvesting might also result in loss of genetic variation in wild populations. However, these results do not indicate a loss in genetic diversity due to human collection of *F. cirrhosa*. Similar studies found this to be the case in other species as well (Nakagawa, 2004; Abbott et al., 2007).

Table 3
Analysis of molecular variance (AMOVA) within/among populations in *F. cirrhosa*.

Source of variance	*d.f.*	SSD	MSD	Variance component	Total variance (%)	*p*-value[a]
Among populations	8	1353.8415	169.230	8.2334	25.33%	<0.001
Within populations	150	3639.7686	24.265	24.2651	74.67%	<0.001

d.f., degree of freedom; SSD, sum of squared deviations; MSD, mean square deviations.
Significance tests after 1000 permutations.
[a] *P*-values represent the probability of having more extreme variance than the observed values.

Table 4
Geographic distance (km) (above diagonal) and Nei's genetic distance (below diagonal) among nine populations of *F. cirrhosa*.

Pop ID	GG	QHS	BM	DL	LJ	YD	YS	KD	WC
GG	****	138	44	341	209	479	461	343	416
QHS	0.0747	****	119	208	79	362	361	366	473
BM	0.0702	0.0861	****	327	180	476	419	307	424
DL	0.1194	0.1288	0.1379	****	170	177	349	523	610
LJ	0.1311	0.1381	0.0994	0.0812	****	342	288	358	454
YD	0.2892	0.2937	0.2655	0.2798	0.2476	****	502	699	787
YS	0.2828	0.2862	0.2558	0.2972	0.2139	0.3109	****	361	382
KD	0.1024	0.0909	0.0970	0.1236	0.0857	0.2762	0.2198	****	119
WC	0.1293	0.1240	0.0843	0.1496	0.1144	0.2307	0.2287	0.1205	****

Breeding system, seed dispersal and geographic distribution have been proven to be closely associated with genetic variation and their partitioning within and among populations (Hamrick and Godt, 1989). As a perennial outcrossing herb, seed dispersal by wind or water plays an important role in maintaining gene flow for *F. cirrhosa*. In this study, the mean genetic diversity within population ($H_E = 0.2704$) was similar to the average value of the outcrossing species ($H_E = 0.27$) and higher than that of the short-lived perennial herb ($H_E = 0.20$) based on RAPD data statistics (Nybom, 2004). However, another similar medicinal plant, *Paris polyphyllla* var. *yunnanensis*, showed very low genetic variation revealed by ISSR data ($H_E = 0.153$) (He et al., 2007). For such an endangered and overharvested plant, it is unusual for *F. cirrhosa* to possess relatively high genetic diversity at population ($H_e = 0.2704$, $S_{pop} = 0.4019$) and species levels ($H_t = 0.3709$, $S_{pop} = 0.5414$). It may be due to its evolutionary history and special life traits.

In the 9 populations sampled here, YS and YD showed relatively low levels of genetic variation, whereas the remaining populations exhibited relatively high levels of variability (Table 2). Both populations YS and YD are located at the margin of the distribution range. Populations located at the margins of the range that are separated spatially from central populations often have smaller population sizes and lower genetic variation than the central populations (Blows and Hoffmann, 1993; Liu et al., 2006a,b). In addition, smaller sample size in populations YS (10) and YD (16) could contribute to the low genetic variation of the two populations.

4.2. Genetic structure

The genetic differentiation of a plant population reflects interactions among a range of different processes in the long-term evolutionary history of a species, such as habitat fragmentation, genetic drift, mating systems and gene flow (Schaal et al., 1998; Templeton, 2006). In this study, the results of AFLP data using different methods (Nei's G_{ST} and AMOVA analysis) showed similar values of genetic differentiation among populations ($G_{ST} = 0.2771$, $\Phi_{ST} = 0.2533$, respectively), revealing a low level of genetic differentiation among the nine sampled populations of *F. cirrhosa* in SW China. This perennial herb only grows on alpine or subalpine meadows at an altitude of 3000–4500 m. The isolated and patchy distribution of wild populations of this species should show significant genetic structure among populations. The value of genetic differentiation detected among populations of *F. cirrhosa* was significant low compared to the average value for short-lived perennial species ($G_{ST} = 0.32$, $\Phi_{ST} = 0.41$) but similar to that of outcrossing plant species ($G_{ST} = 0.22$, $\Phi_{ST} = 0.27$) respectively (Nybom, 2004). The low level of population differentiation of *F. cirrhosa* might result from its outcrossing breeding system and frequent gene flow ($N_m = 0.6523$) between populations. Seeds of *F. cirrhosa* are light (1000-grain weight for 1.96 g) (Chen et al., 1993), which may enhance the gene flow by seed dispersal. The distribution of this species may have been continuous in the past and then recently fragmented, since it does not show significant genetic drift in its evolutionary history.

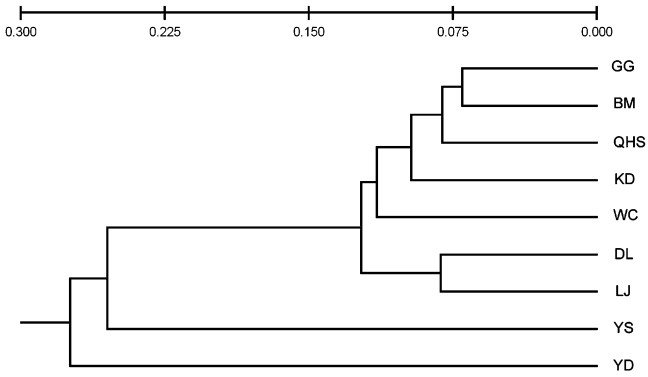

Fig. 2. UPGMA dendrogram based on Nei's (1972) genetic distance between the populations of *F. cirrhosa* sampled.

From Mantel test analysis, there was a significant relationship between the genetic distance and geographic distance among the nine populations ($r = 0.4515$, $p = 0.006$) of *F. cirrhosa*. The UPGMA tree also supported the "isolation by distance" (IBD) model. For example, populations BM and GG are geographically close and the genetic distance between them is relatively small (Table 4). The grouping of populations within the UPGMA corresponded well with the floristic division. The Yunnan Plateau region populations YD and YS diverged first on the dendrogram while the remaining populations (GG, BM, QHS, KD, WC DL and LJ) of Hengduan Mt. region formed a cluster. The current genetic diversity distribution pattern of *F. cirrhosa* populations might be due to an evolutionary event of vicariance from a single common ancestor through fragmentation of its original geographic range, and this vicariance could be explained by the different history of the floristic region (i.e. Yunnan Plateau region and Hengduan Mt. region respectively) in this region. However, more populations from Himalayas are needed for further study.

4.3. Implications for conservation and sustainable utilization

The maintenance of genetic variation is one of the major objectives in conserving endangered and threatened species (Hamrick and Godt, 1996). Knowledge of genetic variation within and among populations provides information essential in the formulation of appropriate management strategies for conservation (Milligan et al., 1994; Francisco-Ortega et al., 2000). Though *F. cirrhosa* maintains relatively high genetic diversity as revealed in this study, sharply decreasing numbers of wild populations and individuals remains a threat. Thus, the conservation status of the species is still urgent and serious. Similarly, many medicinal and economic plants are faced with the same problem of reasonable use and protection of their resources, such as *Taxus fuana, Paris polyphylla* and *Lamiophlomis rotata* (Liu et al., 2006a,b; He et al., 2007; Shah et al., 2008). For *F. cirrhosa*, attention should be focused on *in-situ* conservation in western Sichuan Province, such as WC and KD populations, which is the largest cultivation area of *F. cirrhosa* in China. The populations of Yunnan Plateau (DY and YS) are also important for conservation as they possess distinct genotypes compared to those from the populations of Mt. Hengduan. Cruse-Sanders et al. (2005) suggested that conserving a proportion of the mature individuals in populations is important to the protection of reproductive fitness and the evolutionary potential of the species. Although the effect of current harvesting of *F. cirrhos*a is limited for wild resource at the molecular level, we suggest a method of sustainable collection, as protecting a percentage of mature individuals is necessary and important for maintaining effective population size and evolutionary potential. As an important traditional medicinal plant, promoting domestication and cultivation of this wild resource are necessary both to satisfying market demand and protecting the wild resource. It was reported that *F. cirrhosa* has been successfully cultivated in similar habitats in Kangding county (Chen et al., 2003), which may decrease the harvest of its wild populations and contribute to protection of this important medical plant. Additional measures including proposing a collection guideline, formulating management regulation and promoting domestication and cultivation should be developed for conservation and sustainable use of *F. cirrhosa*.

Acknowledgements

We thank Mr. Hu Yun-Qian from Kunming Institute of Botany for providing experimental helps, and Dr. Yuan Yong-Ming for providing valuable comments of the manuscript. This study was supported by grants from the National Natural Science Foundation of China (NSFC 40930209 to H. Sun), MOST grant (2008BAD98B06), MAPs Traditional Knowledge Network in Southwest China and the Ford Foundation (1075-0729 and 1090-0448).

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