Biochemical Systematics and Ecology 37 (2009) 699–706

Contents lists available at ScienceDirect

Biochemical Systematics and Ecology

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

Genetic diversity within and among populations of the endangered and endemic species *Primula merrilliana* in China

Jian-Wen Shao [a,b], Wen-Long Chen [a], Yan-Qiu Peng [a], Guo-Ping Zhu [a,c], Xiao-Ping Zhang [a,c,*]

[a] College of Life Science, Anhui Normal University, Wuhu 241000, PR China
[b] State Key Laboratory of Systematic and Evolutionary Botany, Institute of Botany, Chinese Academy of Sciences, Beijing 100093, PR China
[c] The Key Laboratory of Conservation and Employment of Biological Resources of Anhui, Wuhu 241000, PR China

ARTICLE INFO

Article history:
Received 10 April 2009
Accepted 5 December 2009

Keywords:
Primula merrilliana Schltr.
Conservation
Genetic variation
ISSR

ABSTRACT

Primula merrilliana Schltr. is an endangered and narrowly-distributed endemic species of southern Anhui Province in China. In this study, the level of genetic variation and the pattern of genetic structure in six natural populations of *P. merrilliana* were assessed by using ISSR (inter-simple sequence repeats) markers. Based on ten primers, 137 clear and reproducible DNA fragments were generated, of which 109 were polymorphic. The statistical results indicated that there was a relatively low genetic diversity within populations, and a high genetic differentiation among populations ($G_{ST} = 0.53$, $\Phi_{ST} = 0.49$). The level of population genetic diversity was correlated to habitat type and the gene flow (N_m) was low with only 0.45. The unexpected genetic structure of *P. merrilliana* may be explained by limited gene flow that was caused by habitat fragmentation and limited seeds and pollen dispersal ability, self-compatible breeding system and biennial life form.

1. Introduction

Of the ca. 500 species in the genus *Primula* L. (Primulaceae), about 300 species are native to China, with the centre of diversity in western Sichuan, eastern Xizang, and northwestern Yunnan. Many species of *Primula* are cultivated for their attractive flowers as pot plants, in rock gardens, or in garden borders (Chen and Hu, 1990; Hu and Kelso, 1996). *Primula merrilliana* Schltr. is a very noticeable and interesting species in this genus. Taxonomically, this species is a peculiar representative of biennial herb with pinnatisect leaves and panpori pollens, which may leave us some invaluable clues to explore what really happened in phylogeny of the genus (Chen and Hu, 1990; Hu and Kelso, 1996; Hao et al., 2002). From aesthetic point of view, *P. merrilliana,* since it has delicate flowers (Fig. 1) and lasting blooming time, has high value for horticultural utilization (Shao et al., 2008b). However, during the last decades, human activities have caused a considerable decrease of the deciduous and latifolious forests, the number of populations and individuals has declined tremendously and is now restricted in the narrow areas of southern Anhui, China, in sporadic way (Fig. 1). *P. merrilliana* is also forced to survive in small and isolated patches, such as road verges, ditch banks and tilth-side under or at the edge of deciduous and latifolious forests between 50 and 1000 m a.s.l. (Chen and Hu, 1990). Furthermore, the results of our previous study showed that the natural populations of this herb showed a skewed distribution in pattern of a large number of very small populations, and the fecundity in these small populations had strongly reduced. Therefore, it is now classified as an endangered species and will be listed in the Chinese Plant Red Book (2nd edition) (Shao et al., 2008a). To reduce the chances of extinction of *P. merrilliana*,

* Corresponding author. Tel.: +86 553 3883539; fax: +86 553 3869571.
E-mail address: pinghengxu@sina.com.cn (X.-P. Zhang).

0305-1978/$ – see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bse.2009.12.001

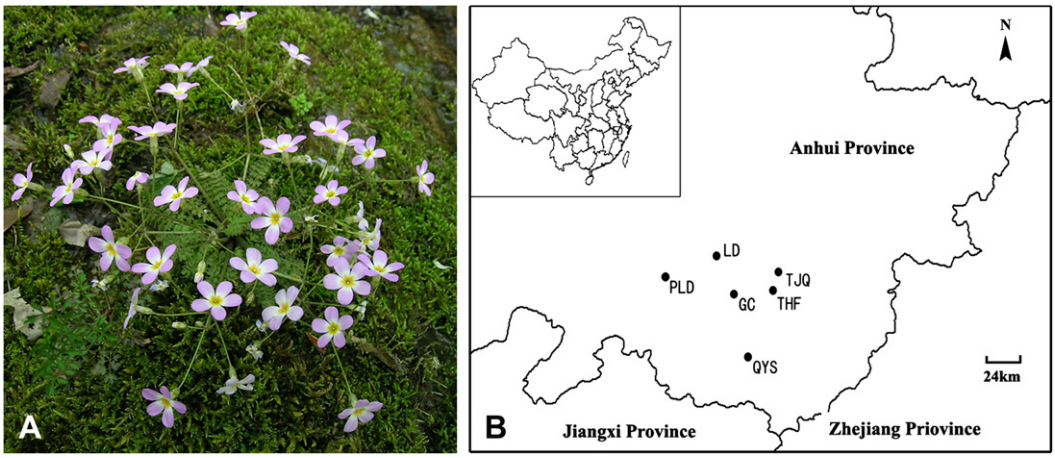

Fig. 1. The morphology of *Primula merrilliana* (A) and map showing locations of populations sampled in this study (B). Population codes are identified in Table 1.

actions should be taken to reinstate regeneration in the surviving wild populations and to establish new populations as soon as possible.

Understanding the genetic structure of existing natural populations is prerequisite for taking effective and efficient conservation practices, both for defining appropriate units for *in situ* conservation and for developing effective sample collection strategies for *ex situ* conservation (Barrett and Kohn, 1991; Schemske et al., 1994). The most species of *Primula* are small herbs characterized by the cross-fertilised distylous flowers which require insect pollinators (mainly *Bombus* spp. and/or *Bombylius* spp.) for legitimate pollination between mutually compatible morphs, and have no special features to facilitate seed dispersal (Woodell, 1960; Boyd et al., 1990; Washitani et al., 1995). Therefore, most of them are vulnerable to the change of environment and have become endangered. Recently, a large number of studies have been undertaken to assess the extent of genetic variation of the plant in this genus (Nan et al., 2002, 2003; Van Rossum et al., 2002, 2004; Van Rossum and Triest, 2003; Jacquemyn et al., 2004; Xue et al., 2004; Reisch et al., 2005; Ishihama et al., 2005; Van Rossum, 2008). However, almost all of these studies focus on the perennial species. Considering that life-history traits have remarkably effects on genetic diversity (Nybom and Bartish, 2000), is the genetic structure of biennial species *P. merrilliana* similar to those perennial herbs? Which specific site should be chosen to serve as an optimal source for *ex situ* collections? These fundamental questions, urgent for conservation strategies of *P. merrilliana*, remain unknown.

Neutral molecular markers such as inter-simple sequence repeats (ISSR) markers are well-established in population genetics and have proven to be useful tools for detection of plant population variability and differentiation (Zietkiewicz et al., 1994; Esselman et al., 1999; Qian et al., 2001). In the present study ISSR technique was used to investigate genetic variation in *P. merrilliana* among six populations throughout the entire distribution range of the species in order to provide baseline information for the development of conservation strategies for this endangered species.

2. Materials and methods

2.1. Population sampling

The geographic distribution of *P. merrilliana* is typically narrow, only occurring in the south of Anhui Province in China. During April 2005, 131 individuals were sampled from six natural populations in the distribution area of *P. merrilliana* (Fig. 1 and Table 1). These six populations varied in size and occurred in different habitats. Based on census estimates (Shao et al., 2008a) and field investigation, the population size (number of flowering individuals) ranged from 89 to more than 3000 and

Table 1
The location and size of sampled populations of *Primula merrilliana*.

Code	Loction	Latitude (N)	Longitude (E)	Altitude (m)	Population area (m^2)	Population size[a]
LD	Liudu, Shitai County	30°19′	117°51′	170–180	ca. 1100	970
PLD	Penlaidong, Shitai County	30°14′	117°03′	100–110	ca. 800	1693
QYS	Qiyunshan, Xiuning County	29°49′	118°02′	165–170	ca. 400	211
GC	Guocun, Taiping County	30°07′	117°57′	255–265	ca. 60	256
THF	Taohuafeng, Huangshang	30°06′	118°09′	710–810	ca. 500	89
TJQ	Tanjiaqiao, Taiping County	30°06′	118°10′	300–360	ca. 1200	>3000

[a] Number of flowering individuals.

the area ranged from ca. 60 to 1200 m^2 (Table 1). The population of PLD and QYS were located at the edge of deciduous and latifolious forests, while populations THF, TJQ, LD and GC occurred in the deciduous and latifolious forests. According to the size and area of populations, the number of samples collected ranged from 17 to 26, and the distance between adjacent samples was at least 3 m. Collected leaves of individual plants were dried directly with silica gel and stored at room temperature.

2.2. DNA extraction and ISSR–PCR amplification

Genomic DNA was extracted following the CTAB procedure (Doyle, 1991). DNA quality and quantity were checked on 1% agarose gel. Ten primers (Table 2) were selected from the 100 primers (UBC primer set no. 9, Biotechnology Laboratory, University of British Columbia) tested based on the clarity and reproducibility of the band patterns. The amplifications were performed in 15 μL reaction mixtures containing 25–30 ng genomic DNA, 10 mM Tris–HCl (pH 9.0), 25 mM KCl, 1.5 mM $MgCl_2$, 0.25 mM dNTPs, 1.0 U of Taq polymerase, 0.3 μM of primers. The reaction mixtures were overlaid with mineral oil. For each primer, amplifications were carried out in 96-well plates using the following program: an initial step of 5 min at 94 °C, followed by 40 cycles of 1 min at 94 °C, 45 s at 51 or 52 °C, and 2 min at 72 °C, and a final extension step of 5 min at 72 °C. The PCR products were electrophoresed on 1.5% agarose gels buffered with 0.5×TBE. A 100 bp DNA ladder (New England Biolabs, Beverly, MA, USA) was used as a size marker. A negative control reaction in which the template DNA was replaced by water was performed alongside every PCR amplification in order to verify the absence of contamination. DNA fragments were identified by image analysis software for gel documentation (Quantity one Version 3.6) following staining with ethidium bromide.

2.3. Data analysis

Only bands that were unambiguously scored across all samples were included in the analysis. Amplified fragments were scored for presence (1) or absence (0) of homologous bands. The resulting presence/absence data matrix of the ISSR phenotypes was analyzed using POPGENE version 1.31 (Yeh and Yang, 1999) to estimate genetic diversity parameters: the percentage of polymorphic bands (*PPB*), the effective number of alleles per locus (A_e), observed number of alleles per locus (A_o), expected heterozygosity (H_e). At the species level, genetic diversity measures (H_T: total population gene diversity; G_{ST}: coefficient of gene differentiation) and the level of gene flow (N_m) were measured using Nei's (1973) gene diversity statistics. Nei's unbiased genetic identity (I) and genetic distance (D) between populations were computed using the program (Nei, 1972). Genetic diversity was also estimated using Shannon's information measure (Lewinton, 1972). $H_o = -\sum P_i \log_2 (P_i)$, where P_i is the frequency of a given ISSR band. H_o was calculated at two levels: the average diversity within populations (H_{pop}), and the total diversity (H_{sp}). Then the proportion of diversity among populations was estimated as $(H_{sp}-H_{pop})/H_{sp}$. In addition, we used HICKORY version 1.0 (Holsinger and Lewis, 2003) to calculate a Bayesian estimate of within-population diversity (H_B; Zhivotovsky, 1999) under the *f*-free model, using default values for burn-in (50,000), sampling (250,000) and thinning (50). This Bayesian approach incorporates the effects of uncertainty about the magnitude of inbreeding (f) and hence does not require the assumption of Hardy–Weinberg equilibrium (HWE) for calculating allele frequencies from dominant ISSRs (Holsinger and Lewis, 2003).

To quantify the amount of rare ISSR markers, we computed frequency-down-weighted-marker values (DW) for each population (Schönswetter and Tribsch, 2005). Using ARLEQUIN version 2.0 (Schneider et al., 2000), we also calculated within-population divergence as the mean number of pairwise differences between ISSR haplotypes (π; Tajima, 1983). The analysis of molecular variance (AMOVA) was also used to partition the total phenotypic variance into within populations and among populations (Excoffier et al., 1992). AMOVA input files were created with the use of AMOVA-PREP 1.01 (Miller, 1998) using the Euclidean distance metric of Excoffier et al. (1992). All analyses were carried out using WINAMOVA version 1.55 (Excoffier et al., 1992; Excoffier, 1993). To examine the genetic relationship among populations, using TFPGA version 1.3 (Miller, 1997),

Table 2
Sequences (5′–3′) and reaction conditions of screened ISSR markers for *P. merrilliana*.

Primer	Sequence 5′–3′	Anneal temperature	No. of bands scored	No. of polymorphic bands
UBC814	$(CT)_8A$	51 °C	19	18
UBC835	$(AG)_7YC$	51 °C	19	14
UBC852	$(TC)_8RA$	51 °C	13	11
UBC853	$(TC)_8RT$	52 °C	11	9
UBC854	$(TC)_8RG$	51 °C	15	14
UBC856	$(AC)_8YA$	51 °C	13	11
UBC857	$(AC)_8YG$	51 °C	15	12
UBC860	$(TG)_8RA$	52 °C	11	6
UBC879	$(CTTCA)_3$	51 °C	11	8
UBC895	$(AG)_2$TTGGTAGCTCTTGATC	51 °C	10	6
Total			137	109

R = A/T; Y = C/G.

Table 3
Genetic variability of *P. merrilliana* detected by ISSR analysis.

Population	*N*	A_o	A_e	H_e	H_o	*PPB* (%)	H_B	DW	π
LD	26	1.40	1.18	0.11	0.17	40.15	0.17	18.23	18.25
PLD	23	1.52	1.26	0.15	0.23	51.82	0.21	22.45	22.73
QYS	22	1.55	1.24	0.15	0.24	54.74	0.22	21.23	26.60
GC	17	1.36	1.15	0.09	0.14	35.77	0.14	13.85	15.21
THF	22	1.44	1.21	0.13	0.20	43.80	0.18	18.93	20.78
TJQ	21	1.40	1.15	0.10	0.15	39.42	0.15	18.30	17.26
Population lever	–	1.44 (0.07)	1.20 (0.05)	0.12 (0.03)	0.19 (0.04)	44.28 (7.48)	0.18 (0.03)	18.83 (2.98)	20.14 (4.12)
Species lever	131	1.80	1.42	0.25	0.38	79.56	0.33	–	–

N, sample size; A_o, observed number of alleles per locus; A_e, the effective number of alleles per locus; H_e, expected heterozygosity; H_o, Shannon's information index; *PPB*, percentage of polymorphic loci; H_B, expected Bayesian heterozybosity; DW, frequency-down-weighted values; π, mean number of pairwise differences between ISSR haplotypes. Standard deviations are shown in parentheses.

pairwise estimates of Nei's D were calculated from allele frequencies, and subjected to cluster analyses by an unweighted pair-group method with arithmetic means (UPGMA). Bootstrap values were obtained by re-sampling with replacement over loci (1000 replicates). In addition, to visualize the genetic relationships among all ISSR phenotypes, their Euclidean distance matrix was subjected to a principal coordinates analysis (PCoA), using the program MVSP version 1.3 (Kovach, 1999). The correlation between genetic distance (*D*) and log 10 geographical distances (Mantel's r_M) was tested for all populations by using Mantel's test with 3000 permutations (Mantel, 1967).

3. Results

3.1. ISSR polymorphism

The 10 selected primers generated 137 unambiguous and reproducible bands, of which 109 (*PPB* = 79.56%) were polymorphic, the size ranging from 250 to 2500 bp. The number of bands varied from 10 (UBC895) to 19 (UBC814 and UBC835), with an average of 13.7 bands per primer (Table 2). The percentage of polymorphic bands (*PPB*) for a single population ranged from 35.77% to 54.74% with an average of 44.28%, and the effective number of alleles per locus ranged from 1.15 to 1.27 with an average being 1.19. ISSR polymorphism within populations (in terms of A_o, A_e, H_e, H_o, H_B or *PPB*) varied across populations (Table 3). The highest genetic diversity was observed in population of QYS, with $A_o = 1.55$, $A_e = 1.24$, $H_e = 0.15$, $H_o = 0.24$, $H_B = 0.22$ and *PPB* = 54.74%. The genetic diversity was lowest in population of GC, with $A_o = 1.36$, $A_e = 1.15$, $H_e = 0.09$, $H_o = 0.14$, $H_B = 0.14$ and *PPB* = 35.77%. The population genetic diversity was not positively correlated to population size (i.e. in terms of *PPB*, $r = 0.360$, $n = 6$, $P = 0.481$), but was correlated to habitat type. PLD and QYS populations, located at the edge of deciduous and latifolious forests, conserved higher levels of diversity (e.g., *PPB* > 50%, $H_B > 0.20$) when compared to those populations under the deciduous and latifolious forest. Likewise, the former populations (PLD and QYS) exhibited higher proportions of rare ISSR markers (higher DW values), as well as higher levels of divergence (in terms of π), than the latter populations (THF, TJQ, LD and GC).

3.2. Genetic structure of populations

The total gene diversity (H_T) and gene diversity within populations (H_S) were 0.25 and 0.12, respectively. The coefficient of genetic differentiation between populations (G_{ST}), equaling 0.5293, indicated that 52.93% of the total gene diversity was among populations. The Shannon's diversity index analysis also showed that 50.13% of the total variation was among populations. The AMOVA analysis provided corroborating evidence for the genetic structure obtained from Nei's genetic diversity statistics. There were highly significant ($P < 0.001$) genetic differences among the populations of *P. merrilliana*. Of the total genetic diversity, 49.41% was attributable to among-populations diversity and the rest (50.49%) to differences within populations (Table 4). The above analysis of the population genetic structure revealed that a high level of genetic differentiation was among the six populations of *P. merrilliana* investigated. The level of gene flow (N_m, the number of migrating individuals among populations per generation) was estimated to be only 0.4447.

Table 4
Analysis of molecular variance (AMOVA) within/among populations of *P. merrilliana*.

Source of variation	d. f.	SSD	MSD	Variance component	Total variance	*P*[a]
Among populations	6	1275.286	212.548	9.976	49.41%	<0.001
Within populations	136	1389.231	10.215	10.215	50.59%	<0.001

[a] *P* values are the probabilities of having a more extreme variance component than the observed values by chance alone. Probabilities calculated by 3000 random permutations of individuals across populations.

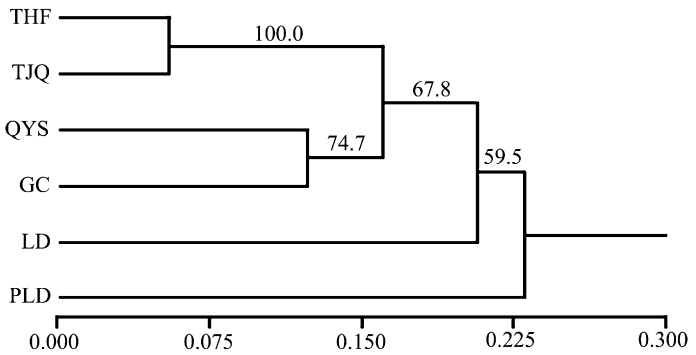

Fig. 2. UPGMA dendrogram showing genetic relationships among six populations of *Primula merrilliana*, based on Nei's (1972) genetic distance measure calculated from 137 ISSR markers. Numbers on branches indicate bootstrap value from 1000 replicates. Abbreviations as in Table 1.

3.3. Genetic relationships

Estimates of genetic distance, in terms of Nei's (1972) D, between all pairs of populations ranged from 0.0471(between THF and TJQ) to 0.2429 (between PLD and THF). Subjecting the genetic distance matrix to UPGMA clustering was shown in Fig. 2. The plot of the first and second principal coordinates from a principal coordinates analysis (PCoA) (accounting for 16.73% and 13.92% of variation, respectively) is depicted in Fig. 3. Both PCoA and UPGMA clustering revealed that four clades existed in *P. merrilliana* investigated populations. Mantel's test showed that there was no significant correlation between genetic and geographical distances ($r = 0.194$, $P = 0.489$).

4. Discussion

4.1. Partitioning of genetic variation

Previous researches have shown that the genetic differentiation is particularly weak in populations of the plant in the genus of *Primula*, e.g, *Primula farinosa* (Φ_{ST}: 0.206) (Reisch et al., 2005), *Primula interjacens* (G_{ST}: 0.261) (Xue et al., 2004), *Primula vulgaris* (F_{ST}: 0.165) (Van Rossum and Triest, 2003), *Primula elatior* (G_{ST}: 0.036) (Jacquemyn et al., 2004), and *Primula veris* (F_{ST}: 0.150)(Van Rossum et al., 2004). However, in this study, the Φ_{ST} value of *P. merrilliana* was 0.494, suggesting that 49.41% of total genetic diversity was among populations (Table 4), which is much higher than those of its congenerous species, and the mean Φ_{ST} value of 0.35 for 78 taxa (Nybom and Bartish, 2000). Strong genetic differentiation has also been found in some other species of Primrose, such as *Primula obconica* ($G_{ST} = 0.52$) (Nan et al., 2003), *Primula sikkimensis* ($\Phi_{ST} = 0.50$) (Wang et al., 2008), and *Primula maguirei* ($\Phi_{ST} = 0.41$) (Wolf and Sinclair, 1997).

The genetic structure of plant populations reflects the interactions of many different processes such as the long-term evolutionary history of the species (e.g., shifts in distribution, habitat fragmentation, and/or population isolation), mutation, genetic drift, mating system, gene flow, selection and life form (Slatkin, 1987; Schaal et al., 1998; Nybom and Bartish, 2000).

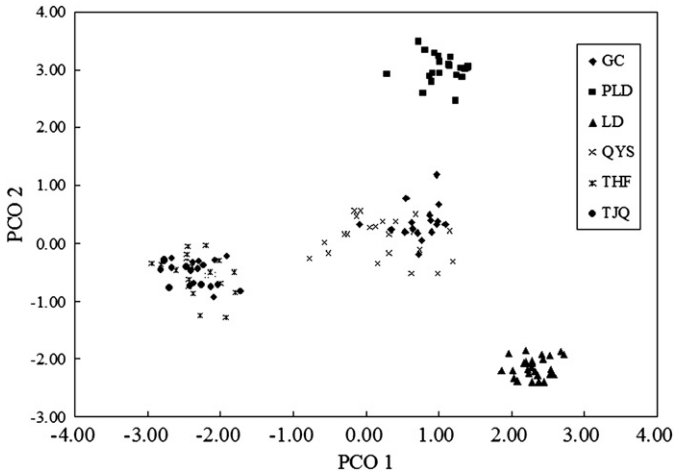

Fig. 3. Principal coordinates (PCO) analysis. The first and second axis extracted 16.73% and 13.92% of the total genetic variance, respectively. Population codes are identified in Table 1.

All of these factors may be associated with the amount of total genetic variation and its partitioning among and within populations.

Among these factors, limited gene flow is one of the important factors that lead to high differentiation between populations of *P. merrilliana*. Our field observations indicated that *P. merrilliana* preferred shady and moist habitat at the vegetative stage (from June to the next January); whereas in flowering period (February–April), in contrast, it favored relatively well-illuminated habitat, by which sufficient pollinators can be attracted. Therefore, the normal growth and successful reproduction of *P. merrilliana* is bound up with a peculiar habitat, which often occurs at the waterside under or at the edge of deciduous and latifolious forests. However, during the last decades, human activities have caused a considerable decrease of this once common deciduous and latifolious forests, and consequently *P. merrilliana* is forced to survive in small and isolated patches, such as road verges, ditch banks and tilth-side (Shao et al., 2008a). In addition, the level of gene flow in the species has also been affected by the biological characteristics of the species itself and the habit of the pollinators. *P. merrilliana* lacks specialized seed dispersal mechanisms, and most seeds directly disperse on the ground near the mother plant. The two effective pollinators of *P. merrilliana*, i.e. *Bombylius major* L. and *Anastoechus chinensis* Paramonow, not only carry pollens of *P. merrilliana* by long proboscis, but also often visited other plant species (e.g., *Corydalis edulis*, *Viola alata*, *Cardamine hirsute*) in early spring. Such pollinator behaviour would reduce the pollen-mediated gene flow among *P. merrilliana* populations (Shao et al., 2008b). Thus, these limitations to seed and pollen dispersal, together with habitat fragmentation, may explain the currently limited gene flow among populations (based on indirect estimates of gene flow in the present study, $N_m = 0.45$), which resulted in genetic erosion and increase of genetic divergence among populations through randomly genetic drift.

Breeding system is often the most important determinant of population genetic structure, affecting both genetic diversity within populations and genetic differentiation among them (Hamrick and Godt, 1989). Typically inbreeding species maintain relatively more of their genetic diversity among populations rather than within populations than do outcrossing species (Nybom and Bartish, 2000). The controlled pollination experiments conducted on *P. merrilliana* have shown that both cross-pollination and self-pollination can lead to successful set seed (Shao, unpubl. data). Therefore, unlike most other species of this genus are strictly self-incompatible, the breeding system of *P. merrilliana* is self-compatible, which could also contribute to the observed high genetic differentiation among populations of *P. merrilliana.*

Both allozyme- and RAPD-based data indicated that short-lived perennial species presented a significantly higher level of population differentiation than long-lived perennial plant (Hamrick and Godt, 1989; Nybom and Bartish, 2000). Although most species of *Primula* are long-lived herbaceous perennial, *P. merrilliana* is herbaceous biennial. The biennial life form of *P. merrilliana* may have played an important role in promoting differentiation among these populations.

4.2. Genetic variability within populations

The life form and breeding system attributes significantly affected the genetic diversity within populations. Long-lived and outcrossing species commonly have higher levels of genetic diversity within populations than short-lived and selfing plants (Hamrick and Godt, 1989; Nybom and Bartish, 2000). Most species of *Primula* are long-lived perennial herbs with self-incompatible breeding system, and accordingly, the reported levels of genetic variations (H_e: expected heterozygosity) within population are high, e.g., 0.26 in *P. sikkimensis* (Wang et al., 2008), 0.21 in *P. farinosa* (Reisch et al., 2005), 0.28 in *P. elatior* (Jacquemyn et al., 2004), 0.24 in *P. interjacens* (Xue et al., 2004), and 0.17 in *P. obconica* (Nan et al., 2003). The levels of within-population genetic diversity in *P. merrilliana* ($H_e = 0.12$; Table 3) are conspicuously low when compared to these self-incompatible long-lived perennial species of *Primula*. This may indicate that the self-compatible breeding system and biennial life form might contribute to the low levels of within-population diversity in *P. merrilliana*.

The level of genetic diversity in the *P. merrilliana* populations was not positively correlated to population size. For example, population TJQ with the biggest population size but possessed a comparatively low genetic diversity ($PPB = 39.42\%$), while population QYS with relatively small population size contained highest genetic diversity ($PPB = 54.74\%$) among the six populations. In contrast, the uneven distribution of genetic diversity was apparently related to the habitat. The habitat of populations PLD and QYS were similar, both occurring at steep slope on the edge of deciduous and latifolious forest, and their genetic diversity were both high (e.g., $PPB > 50\%$). The other four populations grew under deciduous and latifolious forest, possessing comparatively lower gene diversity (e.g., $PPB \approx 40\%$). Previous studies have shown that extinction and recolonization dynamics may lead to either increased or decreased gene diversity and differentiation among populations, depending on the number of founding events, the origin of colonists, and the degree of migration after colonization (McCauley, 1991; Pannell and Charlesworth, 2000). Jacquemyn et al. (2004) also demonstrated that the within-population diversity levels tended to be higher for populations located in older forests compared with those for populations located in young forests. *P. merrillana* is comparatively sensitive to habitat change because of its growth form (a small biennial rosette herb) and distylous flowers. The four populations (LD, GC, THF and TJQ) grow under the young secondary deciduous and latifolious forests to get shady habitats for surviving, while the two populations (PLD and QYS) occur on the cliffy and northern slope where the plants of species can get a fairly constant moist habitat condition to meet their normal growth requirement. The present ISSR diversity and divergence data (Table 3) also reveal that habitat change had contrasting impacts on population groups from two types of habitat. For PLD and QYS, the combination of relatively high levels of genetic diversity (H_e and H_B), rare fragments (DW) and divergence (π) suggests that time was sufficient for the accumulation of high levels of ISSR variation via *de novo* mutations and/or effects of lineage sorting. By contrast, markedly lower values of genetic diversity

(H_e and H_B), rarity (DW) and divergence (π) were observed in four populations (LD, GC, THF and TJQ) (Table 3). This pattern may be due to long-term population bottlenecks *in situ*, possibly as a result of habitat fragmentation.

4.3. Implication for conservation

A major goal of conservation is the maintenance of genetic diversity and evolutionary processes in viable populations in order to prevent potential extinction (Godt and Hamrick, 1998). Loss of genetic diversity could lead to a decline in a species' ability to cope with changing environment and demographic fluctuations both in the short and long term (Ellstrand and Elam, 1993; Qiu et al., 2006). Considering that genetic diversity of *P. merrilliana* was not positively correlated to population size, genetic variation within populations was low and differentiation among populations was high, preservation of some populations would be insufficient to conserve all the genetic variation in this species. Therefore, priority must be given to all the extant populations *in situ*, in particular, attention should be paid to those populations small in size but conserved fairly high genetic diversity and high level of rare fragments like QYS and THF. In addition, *P. merrilliana* is a small biennial herbs with distylous flowers, normal growth and reproduction rely on peculiar habitats and the genetic diversity is related to the habitat. So it is essential to protect local deciduous and latifolious forests which can provide their suitable habitats. When a program of *ex situ* conservation is implemented, in order to capturing most of the detected genetic variability, samples should be collected from as many populations as possible rather than from one or two populations. Pollination by hand between different morphs should be performed when pollinators are not enough for effective pollination under cultivated conditions.

Acknowledgements

The authors are grateful to two anonymous referees who provided very useful comments on an earlier version of the manuscript. This work was supported by Anhui Provincial Natural Science Foundation (No. 090413078), the National Science Foundation of China (No. 30840020) and the foundation of the Key Laboratory of Biotic Environment and Ecological Safety in Anhui Province.

References

Barrett, S.C.H., Kohn, J.R., 1991. Genetic and evolutionary consequences of small population size in plants: implication for conservation. In: Falk, D.A., Holsinger, K.E. (Eds.), Genetics and Conservation of Rare Plants. Oxford University Press, New York, pp. 3–30.
Boyd, M., Silvertown, J., Tucker, C., 1990. Population ecology of heterostyled and homostyle *Primula vulgaris*: growth, survival and reproduction in field populations. J. Ecol. 78, 799–813.
Chen, F.H., Hu, C.M., 1990. Primulaceae. In: Flora Reipublicae Popularis Sinicae, Tomus, vol. 59 (2). Science Press, Beijing, p. 57.
Doyle, J.J., 1991. DNA protocols for plants–CTAB total DNA isolation. In: Hewitt, G.M., Johnston, A. (Eds.), Molecular Techniques in Taxonomy. Springer-Verlag, Berlin, Germany, pp. 283–293.
Ellstrand, N.C., Elam, D.R., 1993. Population genetic consequences of small population size: implications for plant conservation. Annu. Rev. Ecol. Syst. 24, 217–242.
Esselman, E.J., Jianqiang, L., Crawford, D.J., Winduss, J.L., Wolfe, A.D., 1999. Clonal diversity in the rare *Calamagrostis porteri* ssp. *insperata* (Poaceae): comparative results for allozymes and random amplified polymorphic DNA (RAPD) and inter simple sequence repeat (ISSR) markers. Mol. Ecol. 8, 443–451.
Excoffier, L., 1993. Analysis of Molecular Variance (AMOVA) Ver 1.55. Genetics and Biometry Laboratory, University of Geneva, Switaerland.
Excoffier, L., Smouse, P.E., Quattro, J.M., 1992. Analysis of molecular variance inferred from metricdistances among DNA haplotypes: application to human mitochondrial DNA restriction data. Genetics 131, 479–491.
Godt, M.J.W., Hamrick, J.L., 1998. Allozyme diversity in the endangered pitcher plant *Sarracenia rubra* ssp. *alabamensis* (Sarraceniaceae) and its close relative *S.rubra* ssp. *rubra*. Am. J. Bot. 85, 802–810.
Hamrick, J.L., Godt, M.J.W., 1989. Allozyme diversity in plant species. In: Brown, A.H.D., Clegg, M.T., Kahler, A.L., Weir, B.S. (Eds.), Plant Population Genetics, Breeding and Genetic Resources. Sinauer, Sunderland, Massachusetts, USA, pp. 43–63.
Hao, G., Hu, C.M., Lee, N.S., 2002. Circumscriptions and phylogenetic relationships of *Primula* Sects. *Auganthus* and *Ranunculoides*: evidence from nrDNA ITS sequences. Acta Bot. Sin. 44, 72–75.
Holsinger, K.E., Lewis, P.O., 2003. Hickory: a Package for the Analysis of Population Genetic Data, Version 0.8. Distributed by the authors. Department of Ecology and Evolutionary Biology, University of Connecticut, Storrs, Connecticut, USA.
Hu, C.M., Kelso, S., 1996. Primulaceae. In: Wu, Z.Y., Raven, P.H. (Eds.), Flora of China, vol. 15. Science Press, Beijing, pp. 39–189.
Ishihama, F., Ueno, S., Tsumura, Y., Washitani, I., 2005. Gene flow and inbreeding depression inferred from fine-scale genetic structure in an endangered heterostylous perennial, *Primula sieboldii*. Mol. Ecol. 14, 983–990.
Jacquemyn, H., Olivier, H., Peter, G., Isabel, R.U., 2004. Genetic structure of the forest herb *Primula elatior* in a changina landscape. Mol. Ecol. 13, 211–219.
Kovach, W.L., 1999. MVSP – A Multivariate Statistical Package for Windows, Version 3.1. Kovach Computing Services, Pentraeth, Wales.
Lewinton, R.C., 1972. The apportionment of human diversity. Evol. Biol. 6, 381–398.
Mantel, N., 1967. The detection of disease clustering and a generalized regression approach. Cancer Res. 27, 209–220.
McCauley, D.E., 1991. The effect of host plant patch size variation on the population structure of a specialist herbivore insect, *Tetraopes tetraophthalmus*. Evolution 45, 1675–1684.
Miller, M.P., 1997. Tools for Population Genetic Analysis (TFPGA), Version 1.3. Department of Biological Sciences, Northern Arizona University, AZ, USA.
Miller, M.P., 1998. AMOVA-PREP 1.01: a program for the preparation of AMOVA input files from dominant-markers raw data. Computer software distributed by author.
Nan, P., Peng, S., Ren, H., Shi, S., Tian, C., Zhong, Y., 2002. Genetic diversity of *Primula ovalifolia* from central and southwest China based on ISSR markers. J. Genet. Mol. Biol. 13, 119–123.
Nan, P., Shi, S.H., Peng, S.L., Tian, C.J., Zhong, Y., 2003. Genetic diversity of *Primula obconica* from central and southwest China as revealed by ISSR markers. Ann. Bot. 91, 329–333.
Nei, M., 1972. Genetic distance between populations. Am. Nat. 106, 283–292.
Nei, M., 1973. Analysis of gene diversity in subdivided population. P. Natl. Acad. Sci. U.S.A 70, 3321–3323.
Nybom, H., Bartish, I.V., 2000. Effects of life history traits and sampling strategies on genetic diversity estimates obtained with RAPD markers in plants. Perspect. Plant Ecol. Evol. Syst. 3, 93–114.

Pannell, J.R., Charlesworth, B., 2000. Effects of metapopulation processes on measures of genetic diversity. Philos. T. Roy. Soc. B 355, 1851–1864.
Qian, W., Ge, S., Hong, D.Y., 2001. Genetic variation within and among populations of a wild rice *Oryza granulata* from China detected by RAPD and ISSR markers. Theor. Appl. Genet. 102, 440–449.
Qiu, Y.X., Li, J.H., Liu, H.L., Chen, Y.Y., Fu, C.X., 2006. Population structure and genetic diversity of *Dysosma versipellis* (Berberidaceae), a rare endemic from China. Biochem. Syst. Ecol. 34, 745–752.
Reisch, C., Anke, A., Röhl, M., 2005. Molecular variation within and between ten populations of *Primula farinosa* (Primulaceae) along an altitudinal gradient in the northern Alps. Basic Appl. Ecol. 6, 35–45.
Schaal, B.A., Hayworth, D.A., Olsen, K.M., Rauscher, J.T., Smith, W.A., 1998. Phylogeographic studies in plants: problems and prospects. Mol. Ecol. 7, 465–474.
Schemske, D.W., Husband, B.C., Ruckelhaus, M.H., Goodwillie, C., Parker, I.M., Bishop, J.G., 1994. Evaluating approaches to the conservation of rare and endangered plants. Ecology 75, 584–606.
Schneider, S., Roessli, D., Excoffier, L., 2000. ARLEQUIN: a Software for Population Genetics Data Analysis, Version 2.000. Genetics and Biometry Laboratory, University of Geneva, Geneva, Switzerland. Available at website. http://lgb.unige.ch/arlequin/.
Schönswetter, P., Tribsch, A., 2005. Vicariance and dispersal in the alpine perennial *Bupleurum stellatum* L. (Apiaceae). Taxon 54, 725–732.
Shao, J.W., Zhang, X.P., Zhang, Z.X., Zhu, G.P., 2008a. Effects of population size on reproductive success of the endangered and endemic species *Primula merrilliana*. J. Integr. Plant Biol. 50, 1151–1160.
Shao, J.W., Zhang, X.P., Zhang, Z.X., Zhu, G.P., 2008b. Identification of effective pollinators of *Primula merrilliana* and effects of flower density and population size on pollination efficiency. J. Syst. Evol. 46 (4), 537–544.
Slatkin, M., 1987. Gene flow and the geographic structure of populations. Science 236, 787–792.
Tajima, F., 1983. Evolutionary relationship of DNA sequences in finite populations. Genetics 105, 437–460.
Van Rossum, F., 2008. Conservation of long-lived perennial forest herbs in an urban context: *Primula elatior* as study case. Conserv. Genet. 9, 119–128.
Van Rossum, F., De Sousa, S.C., Triest, L., 2004. Genetic consequences of habitat fragmentation in an agricultural landscape on the common *Primula veris*, and comparison with its rare congener, *P. vulgaris*. Conserv. Genet. 5, 231–245.
Van Rossum, F., Echchgadda, G., Szqbadi, I., Triest, L., 2002. Commonness and long-term survival in fragmented habitats: *Primula elatior* as study case. Conserv. Biol. 16, 1286–1295.
Van Rossum, F., Triest, L., 2003. Spatial genetic structure and reproductive success in fragmented and continuous populations of *Primula vulgaris*. Folia Geobot 38, 239–254.
Wang, F.Y., Ge, X.J., Gong, X., Hu, C.M., Hao, G., 2008. Strong genetic differentiation of *Primula sikkimensis* in the East Himalaya–Hengduan mountains. Biochem. Genet. 46, 75–87.
Washitani, I., Kato, M., Nishihiro, J., Suzuki, K., 1995. Importance of queen bumble bees as pollinators facilitating inter-morph crossing in *Primula sieboldii*. Plant Species Boil 9, 169–176.
Wolf, P.G., Sinclair, R.B., 1997. Highly differentiated populations of the narrow endemic plant Maguire primrose (*Primula maguirei*). Conserv. Biol. 11, 375–381.
Woodell, S.R.J., 1960. What pollinates *Primulas*? New Sci. 8, 568–571.
Xue, D.W., Ge, X.J., Hao, G., Zhang, C.Q., 2004. High genetic diversity in a rare, narrowly endemic primrose species: *Primula interjacens* by ISSR analysis. Acta Bot. Sin. 46, 1163–1169.
Yeh, F.C., Yang, R.C., 1999. POPGENE VERSION 1.31, Microsoft Window-based Freeware for Population Genetic Analysis. University of Alberta And Tim Boyle, Centre for International Forestry Research. fttp://ftp.microsoft.com/softlib/slfiles/hpgl. exe.
Zhivotovsky, L.A., 1999. Estimating population structure in diploids with multilocus dominant DNA markers. Mol. Ecol. 8, 907–913.
Zietkiewicz, E., Rafalski, A., Labuda, D., 1994. Genome fingerprinting by simple sequence repeat (SSR)-anchored polymerase chain reaction amplification. Genomics 20, 176–183.