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Genetic diversity of the endangered Chinese endemic herb *Primulina tabacum* (Gesneriaceae) revealed by amplified fragment length polymorphism (AFLP)

Xiaowei Ni, Yelin Huang, Lin Wu, Renchao Zhou, Shulin Deng, Darong Wu, Bosun Wang, Guohua Su, Tian Tang & Suhua Shi*
*State Key Laboratory of Biocontrol, Key Laboratory of Gene Engineering of the Ministry of Education, School of Life Sciences, Sun Yat-Sen University, 510275, Guangzhou, People's Republic of China *Author for Correspondenc: (E-mail: lssssh@zsu.edu.cn; Phone: +86-20-84113677; Fax: +86-20-34022356)*

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Abstract

Primulina tabacum Hance, is a critically endangered perennial endemic to limestone area in South China. Genetic variability within and among four extant populations of this species was assessed using AFLP markers. We expected a low genetic diversity level of this narrowly distributed species, but our results revealed that a high level of genetic diversity remains, both at population level (55.5% of markers polymorphic, $H_E = 0.220$, $I_S = 0.321$), and at species level ($P = 85.6\%$ of markers polymorphic, $H_E = 0.339$, $I_S = 0.495$), probably resulting from its refugial history and/or breeding system. High levels of genetic differentiation among populations was apparent based on Nei's genetic diversity analysis ($G_{st} = 0.350$). The restricted gene flow between populations is a potential reason for the high genetic differentiation. The population genetic diversity of *P. tabacum* revealed here has clear implications for conservation and management. To maintain present levels of genetic diversity, *in situ* conservation of all populations is necessary.

Introduction

Limestone vegetation is a unique geomorphologic formation, which is well known for its richness with endemic plant species (Rusea, Bibian & Julaihi, 2004). *Primulina tabacum* Hance (Gesneriaceae), one of such plants, is a calciphilous perennial herb endemic to China. It is narrowly distributed in the adjacent region between Guangdong and Hunan provinces and confined to limestone habitats (Li, 1996).

This species was firstly recorded by Hance (1883) in Lianxian (i.e. the present Lianzhou), North Guangdong. In 1936, several populations of this species were found in Yangshan, an adjacent county of Lianxian in Guangdong province. After then, no extant populations were found in several field surveys and this species had been speculated to be extinct. In 1991, Professor Ye Chuangxin (Sun Yat-sen University) found this species again at the boundary between Yangshan and Lianxian. In our following extensive survey, merely four populations were found and restricted to the abutting region between Hunan and Guangdong. As a species of cave plant, the density of *P. tabacum* increased along with the increase of distance away from the cave entrance (5–20 m, at most). Especially in one deep cave, it showed an aggregated distribution. Each population tends to a thousand individuals. Our 3 years of field observations showed this species was under threat of a serious decline in number in its original habitats.

Primulina tabacum grows around the entrance to limestone caves with other calciphilous and shade-tolerant plants, below 300 m in altitude (Ren et al., 2003). Limestone vegetation in southern China has been destroyed as much as other

vegetation types in recent years even though these limestone areas are more difficult to access and to farm. Limestone vegetation is more vulnerable than other vegetation types because it recovers slowly on usually thin soils (Zhu et al., 2003). Thereby, species such as *P. tabacum* that live on fragmented limestone habitat are expected to suffer loss of genetic variation (e.g. Brookes, 1992; Margules, Mikovitis & Smith, 1994; Van Dongen et al., 1998; Clarke & O'dwyer, 2000).

In the light of the endangered status and specific limestone habitat, *P. tabacum* are of high conservation interest. Understanding the population genetic structure of this rare species is a necessary prelude to conservation planning (Archibald et al., 2001). The amplified fragment length polymorphism (AFLP) technique has emerged as a powerful tool of genetic analysis (Vos et al., 1995). The AFLP technique is based on the PCR amplification of a fraction of restriction fragments generated by the digestion of total DNA. Its high multiples ratio, the ability to amplify several DNA markers in a single reaction, and the fact that no previous knowledge of DNA sequences is required for its application, makes this technique useful to first characterize variation, where little or no preliminary data exist (Lamote et al., 2002). In the present study, we evaluated genetic variation in four extant populations throughout the entire range of *P. tabacum*. Our aims were to (i) quantify genetic diversity of this narrowly distributed species, (ii) evaluate patterns of genetic differentiation among these fragmented populations of *P. tabacum*, and (iii) assess feasible approaches for its conservation.

Materials and methods

Sample collection and DNA extraction

A total of 96 individuals of *P. tabacum*, representing four extant populations, were sampled throughout the species' entire range (Figure 1). Twenty-four individuals were chosen randomly for

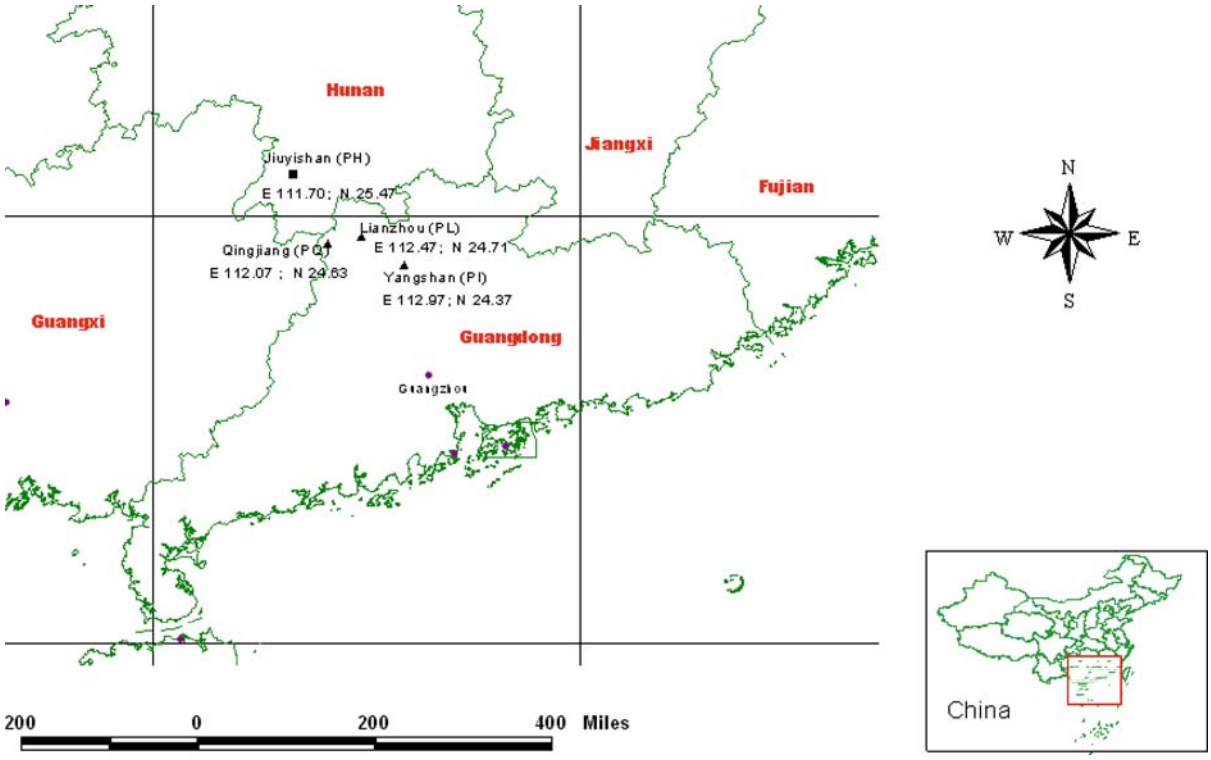

Figure 1. Map showing locations of *Primulina tabacum* populations sampled.

each population at intervals of at least 5 m. Leaves were dried directly with silica gel in zip-lock plastic bags.

Genomic DNA was extracted following the CTAB procedure (Doyle & Doyle, 1987). DNA concentrations were estimated and standardized on 1.0% (w/v) agarose gels. Aliquots from the DNA preparations were for AFLP analysis.

AFLP maker generation

The AFLP analysis was performed as described by Vos et al. (1995) with slight modification that *Eco*RI selective amplification primers were labeled with fluorescent 6-carboxy fluoresce in (6-FAM) on the 5′ nucleotide. Four AFLP primer combinations were chosen among 30 sets examined, with two samples randomly selected (Table 1). The amplified fragments were separated and detected with an ABI PRISM^{TM}-377 automated sequencer (Applied Biosystems, CA, USA).

Data analysis

Genotypes were scored for the presence (1) or absence (0) of all polymorphic bands. Statistical analysis was based on 250 polymorphic AFLP markers. The resulting binary data matrix was first analyzed using POPGENE v.1.31 (32-bit) (Yeh, Yang & Boyle, 1999), assuming Hardy–Weinberg equilibrium. The following parameters were used to estimate genetic diversity at a population level: the percentage of polymorphic fragment (P%), the genetic diversity (H_E), and Shannon's information index of diversity (I_s) (Lewinton, 1972). Genetic diversity parameters (P, H_E, and I_s) were also calculated at the species level. Nei's unbiased genetic identity (I) and genetic distance (D) (Nei, 1972) between populations were also computed using POPGENE v.1.31 (32-bit) (Yeh, Yang & Boyle, 1999).

Genetic differentiation among the populations was estimated by Nei's gene diversity statistics (Nei, 1973). The amount of gene flow among these populations was estimated as $N_m = (1/G_{ST} - 1)/4$ (Slatkin, 1987). In order to test for a correlation between genetic (D) and geographical distances (in km) among populations, a Mantel test was performed using NTSYSpc, v2.02j (Rohlf, 1998). (Computing 5000 permutations).

A matrix of genetic similarity using Dice coefficient was also calculated as $GS_{xy} = 2a/(2a + b + c)$, where a is the number of bands common for samples x and y, b is the number of bands present only in sample x, and c is the number of bands present only in sample y (Dice, 1945). The Dice coefficient is similar to the Jaccard similarity coefficient, but gives twice the weight to agreements assuming the

Table 1. DNA sequences of adaptors and primers used in the AFLP technique

Name		Sequence
*Eco*RI recognition sequence		5′-GAATTC-3′
*Mse*I recognition sequence		5′-TTAA-3′
*Eco*RI adaptor		5′-CTCGTAGACTGCGTAGG-3′
		3′-CATCTGACGCATGGTTAA-5′
*Mse*I adaptor		5′-GACGATGAGTCCTGAG-3′
		3′-TACTCAGGACTCAT-5′
*Eco*RI + 1 primer		5′-GACTGCGTACCAATTCA-3′
*Mse*I + 1 primer		5′-GATGAGTCCTGAGTAAC-3′
*Eco*RI + 3/*Mse*I + 3 primers	E4 + M1	5′-GACTGCGTACCAATTCACA-3′
		5′-GATGAGTCCTGAGTAACAG-3′
	E6 + M5	5′-GACTGCGTACCAATTCACC-3′
		5′-GATGAGTCCTGAGTAACAC-3′
	E1 + M2	5′-GACTGCGTACCAATTCAAG-3′
		5′-GATGAGTCCTGAGTAACTC-3′
	E1 + M5	5′-GACTGCGTACCAATTCAAG-3′
		5′-GATGAGTCCTGAGTAACAC-3′

one–one matches could reflect more similarity between samples than zero–zero matches (Kosman & Leonard, 2005). The mean similarity values (GS) within populations were calculated to quantify the degree of within-population diversity with the assistance of NTSYSpc v2.02j.

Results

Amplified fragment length polymorphism analysis of *P. tabacum* was optimized by determining which selective primer sets produce the clearest DNA fragments. Data from the 96 samples of four populations with four primer combinations revealed a total of 250 different AFLP fragments, corresponding to an average of 62.5 fragments per primer. Of these fragments, 214 (85.7%) were polymorphic at the species levels.

The percentages of polymorphic fragment (P) of a single population ranged from 47.2 to 60.8%, in which the highest is Lianzhou (PL) and the lowest is Qingjiang (PQ). Nei's gene diversity (H_E) was estimated to be 0.220 within populations (H_{pop}), and 0.339 at the species level (H_{sp}). The Shannon's information index (I_s) ranged from 0.279 to 0.344, with a total of 0.495 at the species level. The Dice coefficient of similarity varied considerably between populations, ranging from 0.781 for population Lianzhou (PL) to 0.863 for population Qingjiang (PQ), with an overall average of 0.700 (Table 2). Among the four populations, population Lianzhou (PL) and Yangshan (PI) exhibit the greatest level of variability, whereas the population from Qingjiang (PQ) exhibits the lowest level of variability (Table 2).

The coefficient of genetic differentiation among populations (G_{st}) was 0.350 as estimated by partitioning the total gene diversity, assuming Hardy–Weinberg Equilibrium. Genetic identities between populations varied from 0.719 to 0.923. Based on the G_{st} value, the level of gene flow (Nm) was estimated to be 0.47 among the four populations.

Genetic distance was calculated between all pairs of the four populations (Table 3). The least genetic distance (0.08) was between population Lianzhou (PL) and Yangshan (PI), and the greatest genetic distance (0.33) occurred between Qingjiang (PQ) and PH. A dendrogram based on the genetic distances showed that Jiuyishan (PH) population from Hunan was genetically distinct from three other populations from Guangdong (results not shown). None of the populations studied were very close to each other (Figure 1), and no significant correlation was found between genetic distance and geographic distance ($r = 0.183$, NS) based on a Mantel test.

Discussion

We predicted that the genetic diversity of *P. tabacum* would be low based on its strictly limited distribution. However, *P. tabacum* showed a high level of intrapopulation genetic diversity, rather than an expected low genetic diversity level: at a species level $P = 86\%$, $H_E = 0.34$, while *P averaged* $= 56\%$ and $H_E = 0.22$, among the four populations. Several studies also based on AFLP markers provided similar results: $H_E = 0.243$ for *Hibiscus tiliaceus* (Tang et al., 2003), $H_E = 0.158$–0.229 for *Trollius europaeus* (Despres

Table 2. Genetic diversity within populations of *Primulina tabacum* detected by AFLP analysis

Population	Sample size	N	P (%)	H_E	I_S	GS
Jiuyishan (PH)	24	134	53.6	0.220 (0.222)	0.318 (0.314)	0.861 (0.072)
Qingjiang (PQ)	24	118	47.2	0.192 (0.213)	0.279 (0.305)	0.862 (0.066)
Lianzhou (PL)	24	152	60.8	0.232 (0.212)	0.341 (0.299)	0.781 (0.098)
Yangshan (PI)	24	151	60.4	0.236 (0.216)	0.344 (0.304)	0.802 (0.098)
Mean	–	–	55.5	0.220 (0.017)	0.321 (0.026)	0.826 (0.358)
Total	96	214	85.7	0.339 (0.170)	0.495 (0.234)	0.700 (0.105)

N, number of polymorphic fragments; P, percentage of polymorphic fragments; H_E, Nei's (1973) gene diversity; I_S, Shannon's information index; GS, genetic similarity (Dice, 1945).
Standard deviations are given in parentheses.

Table 3. Nei's unbiased measures of genetic identity and genetic distance

Population	Qingjiang (PQ)	Jiuyishan (PH)	Lianshan (PL)	Yangshan (PI)
Qingjiang (PQ)	–	0.719	0.818	0.799
Jiuyishan (PH)	0.330	–	0.779	0.778
Lianzhou (PL)	0.201	0.250	–	0.923
Yangshan (PI)	0.224	0.252	0.080	–

Nei's genetic identity (above diagonal) and genetic distance (below diagonal).

et al., 2002) and $P = 56\%$ for *Populus nigra* subsp. *Betulifolia*, which was also in danger (Winfield et al., 1998). The many allozyme analyses available for plants suggest that even lower levels of genetic diversity are common: P ranging from 18.9–66.1% and H_E from 0.056–0.202 across 165 genera and 449 species (Hamrick & Godt, 1989).

According to Hamrick and Godt (1989), there are strong associations between geographical range and genetic diversity. Allozyme analyses concluded that endemic and geographically limited plant species generally possess less genetic variation within populations due to genetic drift and restricted gene flow (Karron, 1991; Hamrick & Godt, 1996). Nevertheless, our present result suggests that the size of geographic distribution is not necessarily a good predictor of genetic diversity for *P. tabacum*.

Historical events have been shown to be responsible for variation in genetic diversity (Karron, 1991). Numerous allozyme studies and an increasing number of cpDNA and mtDNA studies now provide substantial evidence that putative refugial plant populations harbor higher levels of genetic diversity relative to their likely descendant population (Lewis & Crawford, 1995; Comes & Kadereit, 1998). The high level of genetic diversity of *P. tabacum* may result from its refuge history. And, the high genetic diversity observed indicates that these populations might be able to adapt to environmental changes and thus can survive in rough habitat.

Despite the fact that populations remain variable, overall among-population differentiation was relatively high ($G_{st} = 0.350$). Therefore, little gene flow occurs among populations. This is not very surprising given the geographical and ecological properties. Geological or ecological barriers between areas of habitat patches frequently cause genetic differentiation (Clarke & O'Dwyer, 2000; Hudson et al., 2000; Medeiros et al., 2000). Among-population gene flow is limited by pollen and seed dispersal, especially as *P. tabacum* is an insect-pollinated plant whose corolla tube is cylindrical, probably restricting the pollinator to a small long-beak insect. Moreover, pollen dispersal is limited to the flying capacity of this pollinator, while the distances among the four populations is 50 km or more. *P. tabacum* relies on dense concentration of CO_2, high humidity and low light intensities in its habitat, deep in karst caves (Ren et al., 2003). In this kind of habitat, it is hard for seed dispersed by wind. Its seed is small ($\sim$0.5 mm), lacks hooks or pappi, are therefore the seeds of *P. tabacum* are not easily dispersed by animals. Thus, we predict no frequent long distance dispersal. In addition, the unfavorable acid soil conditions fragmentize the habitat for seed set have meant that even if the seed can be carried out of a cave, few seedlings would survive, which hampers prospects for short distance dispersal. The natural history of this species therefore corresponds well with our estimate of Nm, the effective gene flow per generation, at less than 0.5 per generation. This level of migration will not prevent continued divergence among populations (Wright, 1951; Slatkin, 1987).

Conservation considerations

The specific limestone habitat and strict ecological requirements make conservation of the endemic *P. tabacum* a major challenge for a number of reasons: (1) the four remaining wild populations are not sufficiently protected; (2) it is not known yet whether the recently discovered small populations at the boundary between Hunan and Guangdong are stable or declining; (3) the reasons for the decline are not sufficiently understood, but habitat loss is a potential problem; and (4) a 3-year *ex situ*

conservation experiment has not succeeded (Ren et al., 2003).

The relatively high genetic differentiation occurring in *P. tabacum* is most likely to be a result of obvious fragmented habitats, isolated by acidic soil around, and limited distribution. These isolated populations greatly diverged due to the restriction of gene flow. Therefore, all four populations should be protected. Thus, from a conservation perspective, we think that the current situation represents a 'window of opportunity' to preserve much of the variation present in the extant four populations. Conservation measures that offset the current decline and result in increases in population numbers are necessary to take advantage of this opportunity.

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