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Full Length Research Paper

Genetic diversity analysis and conservation of the Chinese herb *Salvia miltiorrhiza* collected from different geographic origins in China

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Salvia miltiorrhiza **is an economically important floral herb. However, little work has been conducted to further our understanding of the genetics of this herb. In this study, a representative set of germplasm of *S. miltiorrhiza* populations was used to analyze genetic diversity using amplified fragment length polymorphism (AFLP) methodology. Twenty seven *S. miltiorrhiza* geographical populations from ten provinces in China were selected based on morphological diversity and geographic origin. A total of 528 unambiguous bands were identified by ten primer combinations of *Eco*RI +3 and *Mse*I +3. Of those, 476 showed a clear polymorphism, representing 90% of the total bands. The samples showed different levels of similarity ranging between 0.504 and 0.789. The unweighted pair-group method with arithmetic averaging (UPGMA) cluster analysis conducted on polymorphic AFLP markers revealed that all these *S. miltiorrhiza* populations could be clearly distinguished into eight distinct groups as well as an intermediate. The population genetic diversity of *S. miltiorrhiza* revealed here had clear implications for conservation, management and use of the *S. miltiorrhiza* germplasm.**

Key words: Amplified fragment length polymorphism, genetic diversity, *Salvia miltiorrhiza*, population.

INTRODUCTION

Salvia miltiorrhiza was a perennial herb belonging to the Lamiaceae family and is mainly distributed in China (Guo et al., 2002). *S. miltiorrhiza* was used in folk medicine by the local people and recognized for potential value of phytopharmaceuticals (Zhang et al., 2002). Dan-shen, the dried roots of *S. miltiorrhiza*, was officially listed in the Chinese Pharmacopoeia and has been used for the treatment of disorders caused by poor blood supply such as coronary artery disease and angina pectoris (Chen et al., 1999).

The life cycle of *S. miltiorrhiza* is 5 or 6 years. Flowers blossom before leaves grow out. The plant has a morphological and biological character adapted to cross-pollination and good sexual reproduction (Zhang et al., 2002). In natural conditions, the seed dispersal distance of *S. miltiorrhiza* is short. *S. miltiorrhiza* has a wide region of distribution; however, within that region, it mostly appears in valleys with secondary vegetation. In any population, the plant shows a kind of clumping distribution pattern, and individual plants were mostly observed growing under shrubs or around tree trunks (Zhang et al., 2002). Earlier, *S. miltiorrhiza* was used in folk medicine by the local people in small quantities, but commercialization of this plant in recent years has increased demand and consequent exploitation. Recently, the size of the wild population has been declining rapidly in China, owing to habitat fragmentation, overexploitation, long dormancy, low rate of natural regeneration and human disturbance (Guo et al., 2002). Thus, there is a need to conserve genetic diversity of this prized medicinal plant, which may become extinct if its reckless exploitation continues. Estimation of the level and distribution of genetic variation in endangered species is a primary objective of conservation genetics (Fritsch and Rieseberg,

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Table 1. List of sites where *Salvia miltiorrhiza* used in this study was collected.

No.	Population	Origin	Attitude (m)	Latitude (N), Longitude (E) (N), Longitude (E)	Sample (N) size (*N*)
1	YC-1	Yuncheng city, Sanxi province	1126	35°36', 111°29'	20
2	YC-2	Yuncheng city, Sanxi province	825	35°71', 111°38'	20
3	YC-3	Yuncheng city, Sanxi province	1679	35°52', 111°55'	20
4	LN-1	Luonan county, Shanxi province	532	34°35', 110°16'	20
5	LN-2	Luonan county, Shanxi province	379	34°28', 110°28'	20
6	SL	Shangluo county, Shanxi province	238	33°77', 110°03'	20
7	TSL-4X	Tianshili 4x, Shanxi province	215	33°50', 110°18'	20
8	SHY	Shanyang county, Shanxi province	326	33°46', 110°20'	20
9	NX	Neixiang county, Henan province	1325	33°06', 112°09'	20
10	FC	Fangcheng county, Henan province	680	33°29', 113°02'	20
11	JN	Jinan city, Sahndong province	186	36°43', 117°03'	20
12	YT	Yantai city, Sahndong province	157	37°31', 121°53'	20
13	SX-1	Sjouxi county, Zhejiang province	657	29°93', 121°59 '	20
14	SX-2	Sjouxi county, Zhejiang province	712	29°91', 121°61 '	20
15	CM-1	Chunming county, Shanghai city	18	31°52', 122°05 '	20
16	CM-2	Chunming county, Shanghai city	23	31°50', 122°08 '	20
17	SY-1	Sheyang county, Jiangshu province	3	33°30', 120°48 '	20
18	SY-2	Sheyang county, Jiangshu province	7	34°12', 119°83 '	20
19	SY-3	Sheyang county, Jiangshu province	8	34°07', 119°78 '	20
20	SY-4	Sheyang county, Jiangshu province	5	33°27', 120°36 '	20
21	SY-5	Sheyang county, Jiangshu province	5	33°29', 120°43 '	20
22	BZ	Bozhou county, Anhui province	517	33°11', 116°29 '	20
23	JC	Jichun county, Hubei province	756	30°25', 116°23 '	20
24	SZ	Suizhou county, Hubei province	813	31°75', 113°38 '	20
25	ZJ	Zhongjiang county, Sichuan province	378	31°07', 104°63 '	20
26	JT	Jingtang county, Sichuan province	451	30°78', 104°51 '	20
27	LW	Laiwu county, Shandong province	753	36°27', 117°45 '	20

1996).

In the present study, we evaluated genetic variation in 27 *S. miltiorrhiza* geographical populations. The aims were to (i) examine levels and distribution of genetic variability within and among populations of *S. miltiorrhiza*; and (ii) assess the possible factors contributing to the observed patterns. Such information could be used to guide management strategies for the conservation of *S. miltiorrhiza*.

MATERIALS AND METHODS

Plant material

Ten individuals of *S. miltiorrhiza* were randomly chosen for sampling from each of 27 wild populations (Table 1, Figure 1). All populations were separated geographically by at least 30 km and the sampled plants within population were at least 10 m interval. Young leaves were collected and dried in silica gel.

DNA extraction

The genomic DNA was isolated from 200 mg leaf tissue using the CTAB method according to Murray and Thompson (1980). Finally, the high molecular weight DNA was checked for quality and quantity using agarose gel (0.8%) electrophoresis and fluorimetry (ND-1000, NanoDrop, America). Template DNA was performed by combining genomic DNA in equal proportions from ten individual genotypes from each of these 27 populations. Total DNA was diluted to a working concentration of 200 ng/µL and stored at -20°C.

AFLP analysis

AFLP analysis was performed essentially as described by Vos et al. (1995) using the adapter and PCR primer sequences and PCR cycles with minor modifications as described previously (Zhang et al., 2008). The resulting banding pattern was analyzed manually. Reproducibility of each primer pair was checked by carrying out two times the whole AFLP protocol for three individuals chosen randomly. Only reproducible bands across two PCR amplification replicates were used in the subsequent analysis.

Data analysis

Amplified fragments were scored for presence (1) or absence (0) of homologous bands and entered into a binary matrix representing the AFLP profile of each sample. The resulting binary data matrix was

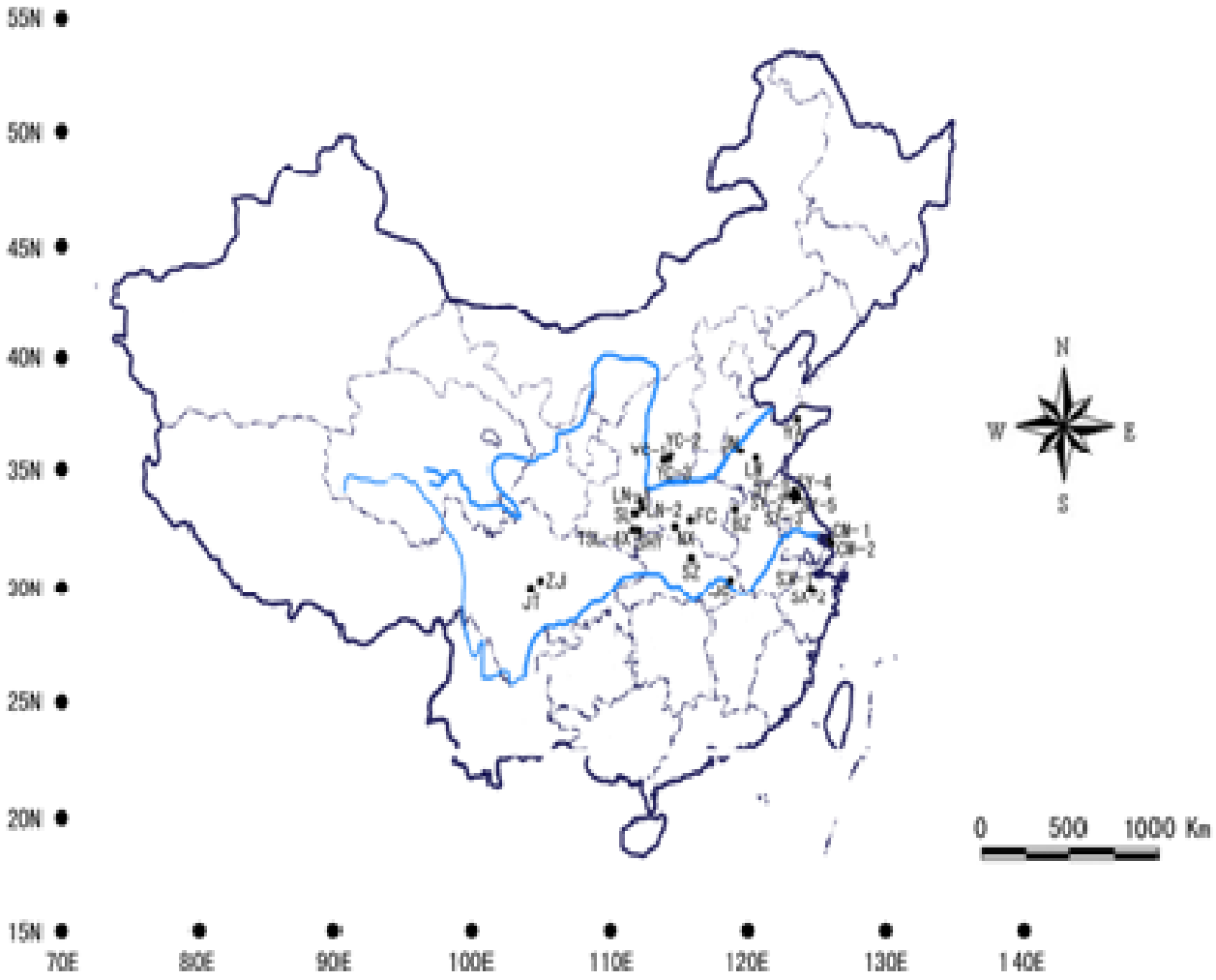

Figure 1. Geographic distribution of populations included in *Salvia miltiorrhiza* AFLP analysis.

first analyzed using Popgene software package version 1.31 (Yeh et al., 1997), assuming Hardy-Weinberg equilibrium. The following parameters were used to estimate genetic diversity between populations: (i) the percentage of polymorphic loci out of all polymorphic loci (P%), (ii) Nei's (1978) unbiased expected heterozygosity (H_E) and (iii) Shannon's index of phenotypic diversity (I_S) (Lewontin, 1972). Estimates of H_E and I_S were obtained by averaging across loci.

The genetic relationships among the populations were determined by calculating the simple matching coefficient (SM). The resultant pairwise similarity matrix was employed to construct cluster plots by the unweighted pair group method with arithmetic mean (UPGMA) using NTSYS-PC software package version 2.1 (Rohlf, 2000). Bootstrap analysis, with 1000 re-samples, was computed using WinBoot software (Yap and Nelson, 1996) to determine the confidence limits of the UPGMA dendrogram.

RESULTS

Genetic diversity between *S. miltiorrhiza* populations

Although large numbers of amplified fragments were generated by each primer combination, only fragments that were unambiguous and polymorphic within at least one of the populations were scored (Figure 2). Wide diversity screen AFLP fingerprinting of the 27 *S. miltiorrhiza* samples (Table 1, Figure 1) using 10 primer combinations generated a total of 528 bands, ranging in length from 150 -500 bp. Of these fragments, 476 (P = 90.15%) were polymorphic at the population level (Table 2). Genetic diversity analysis showed that Nei's gene diversity (H_E) was 0.2612 and Shannon's genetic diversity index (I_S) was 0.4033.

The genetic similarity coefficients (SM) based on the AFLP data between the pairs of populations ranged from 0.5909 to 0.8295. Populations TSL-4X collected from Shanxi province and YC-1 collected from Sanxi province had the lowest SM (0.5909) and SX-1 and SX-2 both collected from Zhejiang province had the highest SM (0.8295). Cluster analyses were performed with each primer combination separately and compared. Mantel tests gave very strong evidence that the results from each primer combination are closely associated ($P < 0.001$). For each Mantel randomization test performed (5000

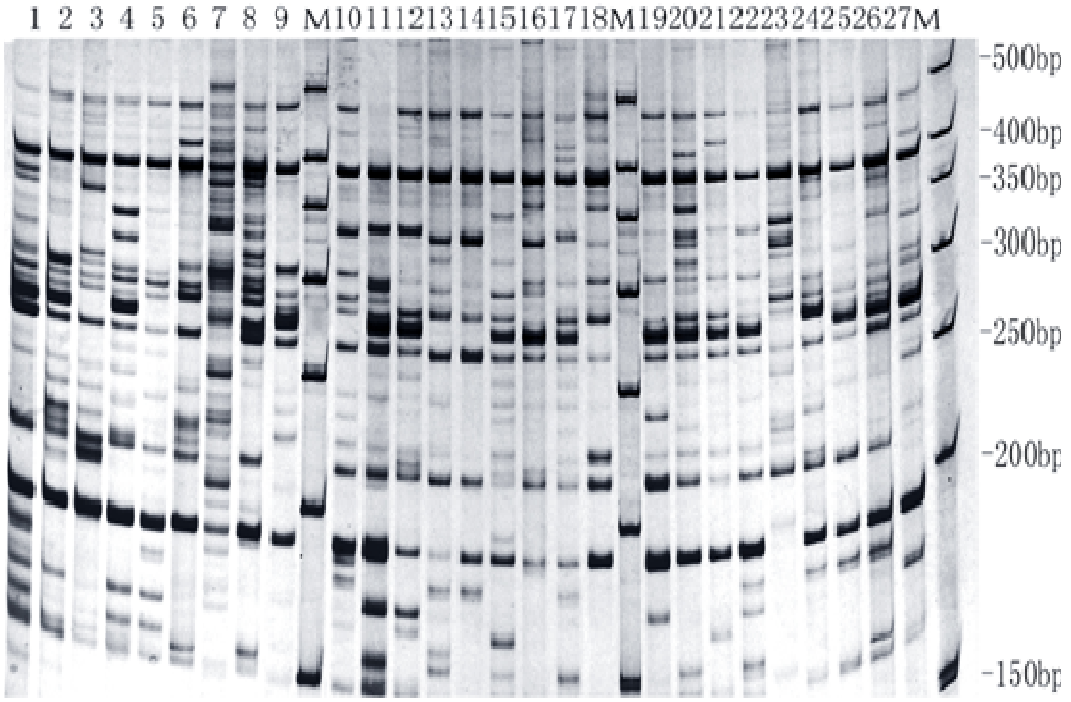

Figure 2. AFLP profile of 27 *Salvia miltiorrhiza* populations created using E-ACG and M-CAA primer combination. M: the DNA molecular-weight mark; 1 - 27: correspond to populations (code 1 - 27) listed in Table 1.

Table 2. The numbers of DNA fragments generated by the selected primer combinations.

Primer combinations	Total fragments	Monomorphic fragments	Polymorphic fragments	Percent polymorphic fragments (%)
E-ACC / M-CAT	44	4	40	90.91
E-ACC / M-CAA	41	5	36	87.80
E-ACT / M-CTA	60	6	54	90.00
E-ACC / M-CTA	43	7	36	83.72
E-ACG / M-CAA	49	4	45	91.84
E-ACG / M-CAT	51	2	49	96.08
E-ACT / M-CAT	67	8	59	88.06
E-ACC / M-CTT	85	8	77	90.59
E-ACT / M-CTG	46	3	43	93.48
E-ACT / M-CTG	42	5	37	88.10
Total or average	528	5.2	47.6	90.15

Genetic relationships between *S. miltiorrhiza* populations.

randomizations was carried out in each case), the value obtained when data matrices from the different primer combinations were compared was greater than obtained from any of the 5000 randomizations. These results indicated that the data from the 10 primer combinations could be combined into a single set for analysis.

The dendrogram was constructed with the UPGMA method using the SM based on the AFLP data (Figure 3). Eight clusters were defined among 27 populations according to their geographical distribution at the 72% similarity level approximately. The cluster A consisted of 4 populations collected from Zhejiang province and Jiangsu province (SX-1, SX-2, SY-2 and SY-3), with an average SM of 0.7843. The populations BZ and FC were clustered into the cluster B (SM = 0.7652). The cluster C possessed populations ZJ and JT both collected from Sichuan province (SM = 0.8106). The cluster D, with a little complex, contained four populations, including SL and

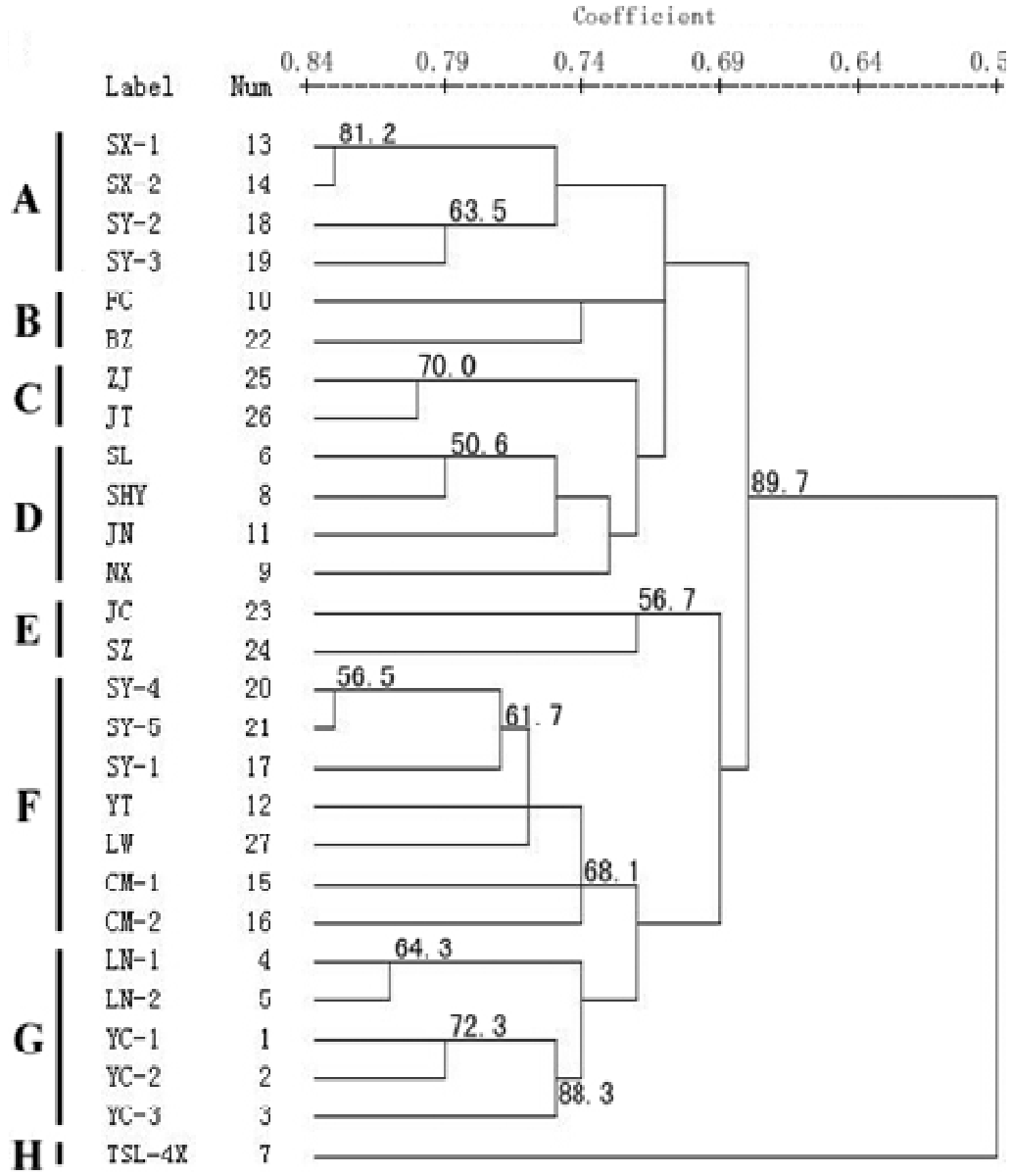

Figure 3. UPGMA cluster analysis of AFLP data generated by ten primer combinations for 27 *Salvia miltiorrhiza* populations based on SM similarity coefficient. Numbers represent the percentage of 5000 bootstrap replicates and only bootstrap values greater than 50% are reported.

SHY both collected from Shanxi province, JN collected from Shandong province and NX collected from Henan province, with an average SM of 0.7683. The two populations from Hubei province (JC and SZ) formed the cluster E (SM = 0.7500). The cluster F possessed populations CM-1, CM-2, LW, SY-1, SY-4, SY-5 and YT collected from the region in east China, with an average SM of 0.7722. The 2 populations from Shanxi province (LN-1 and LN-2) and 3 populations from Sanxi province (YC-1, YC-2 and YC-3) formed the cluster G, with an average SM of 0.7746. The cluster H contained population TSL-4X only, which is located in the south part of the Qinling region of Shanxi province.

DISCUSSION

Identification of genetically related genotypes and (or) populations within *S. miltiorrhiza* based on a limited number of morphological and ampeolographic characteristics has proven difficult in this plant species (Zhang et al., 2002). In this paper, we have optimized the AFLP protocol for reproducible genomic fingerprinting of *S. miltiorrhiza* population and demonstrated the usefulness of the approach by surveying genetic diversity and relatedness of this plant in selected different geographic origins in China.

The AFLP analysis of 10 primer combinations on 27 *S.*

miltiorrhiza populations yielded a total of 476 polymorphic bands. On average, each primer combination gave rise to 47.6 polymorphic amplified fragments that could be detected via silver staining. This finding indicated that the amount of genetic variability present in *S. miltiorrhiza* was relatively high. Currently, there was little knowledge about the genome of *S. miltiorrhiza*, as it has not been adequately investigated. The AFLP technique was well suited for the analysis of unknown genomes as it was more reproducible than other molecular marker systems, and AFLP profiles did not alter with minor variations in experimental conditions (Vos et al., 1995; Tomkins et al., 2001). In this study, the AFLP technique has been proven to be useful in elucidating the genetic variation present in the *S. miltiorrhiza* genome. The AFLP profiles obtained could be used to distinguish between the different populations by their unique banding patterns (Figure 2). These results compared favorably to those of Guo et al. (2002), whose RAPD studies with 11 primers yielded 129 polymorphic bands in 9 populations. As AFLP generated a much greater number of markers per reaction (high multiplex ratio) compared to RAPD (Breyne et al., 1997), the technique had the ability to provide substantial information for genetic studies of *S. miltiorrhiza* populations, as demonstrated by this study.

We predicted that the genetic diversity of *S. miltiorrhiza* wild populations would be low based on its limited distribution. However, *S. miltiorrhiza* showed a high level genetic diversity between wide distributing populations, rather than an expected low genetic diversity level, while P = 90.15%, H_E = 0.2612 and I_S = 0.4033, among the 27 populations. Several studies also based on AFLP markers provided similar results: H_E = 0.158-0.229 for *Trollius europaeus* (Despres et al., 2002), H_E = 0.243 for *Hibiscus tiliaceus* (Tang and Kanpp, 2003) and H_E = 0.220-0.339 for *Primulina tabacum* (Ni et al., 2006). The many allozyme analyses available for plants suggested that even lower levels of genetic diversity were common: P ranging from 18.9-66.1% and H_E from 0.056-0.202 across 165 genera and 449 species (Hamrick, 1990).

Genetic diversity maintained in a plant species was influenced by specific characteristics of the species (Hamrick, 1990). The high level of genetic variation in *S. miltiorrhiza* may be attributed both to its breeding reproductive behavior and to its ecological distribution type. Cross-pollination could lead to increase of genetic diversity. Habitat fragmentation and deterioration under human disturbance was another key factor leading to high genetic diversity between populations (Hunter, 1996). The result of our field survey agrees with previous studies on its ecological distribution (Guo et al., 2002). Because of habitat fragmentation and deterioration under human disturbance, *S. miltiorrhiza* appeared mostly in valleys with secondary vegetation in its distribution region, and individual plants were mostly observed growing under shrubs or around tree trunks. In China, the size of most wild populations was very low and was declining each year. The rapid decrease in the number of individuals in the *S. miltiorrhiza* population brought out more directly the loss of genetic diversity (data not shown).

Despite the fact that populations remain variable, overall among-population differentiation was relatively high (P = 90.15%, H_E = 0.2612 and I_S = 0.4033). Therefore, little gene flow occurred among populations. This was not very surprising given the geographical and ecological properties. Geographical and ecological barriers between areas of habitat patches frequently caused the distribution of genetic diversity of plant populations (Clarke and O'Dwyer, 2000; Hudson et al., 2000; Medeiros et al., 2000). In this paper, the UPGMA cluster analyses revealed a similar result in that most *S. miltiorrhiza* populations collected from the near or similar habitat in China were clustered together, except for populations JN and NX. This demonstrated that there was a certain association between genetic diversity among populations and geographical locations. Preliminary studies indicated that the distribution pattern and morphological polymorphism of *S. miltiorrhiza* should be considered, the result of its adaptation to various environmental factors, such as illumination, moisture, soil, temperature, and elevation (Guo et al., 2002). These analyses demonstrated that the genetic diversity and genetic structure of *S. miltiorrhiza* were determined to a certain extent by the ecological factors in distinct population distributive regions.

A low level or absence of gene flow among populations was characteristic of many rare species (Slatkin, 1985). Some studies have found that seed dispersal was a primary factor influencing variation in gene flow and population structure (Kalisz et al., 1999), especially as *S. miltiorrhiza* was an insect-pollinated plant whose corolla tube was cylindrical, probably restricting the pollinator to a small long-beak insect. Moreover, pollen dispersal was limited to the flying capacity of this pollinator, while the distances among the four populations were 50 km or more. In natural conditions, the short seed dispersal distance of *S. miltiorrhiza* probably resulted in limited seed-mediated gene flow among populations.

The relatively high dissimilarity coefficients discovered in the *S. miltiorrhiza* plant material under investigation indicated that a fairly high amount of diversity was present in the gene pool from which these populations were derived. The diversity of germplasm available for breeding efforts indicated that there was a good potential for genetic improvement of this species. Since the genetic background of the groups of *S. miltiorrhiza* populations included in this study was unavailable, the results of the genetic analysis obtained in this study could be very useful. Determining the level of genetic variation present in *S. miltiorrhiza* provided opportunities for genetic improvement of this plant.

Low genetic diversity could reduce the potential of species or populations to survive in a changing environment (Ellstrand and Elam, 1993). Although wild *S. miltiorrhiza* germplasm has been considered a rare and threatened

species (Guo et al., 2002), because of higher economic returns and commercial demand, the removal of its underground parts (generally during the middle of the growing season and well before seed set in the wild) continues at rates well over natural regeneration. On the basis of our field survey of the natural populations of *S. miltiorrhiza* in China, we found that the habitats of most wild populations have been destroyed under human disturbance. These factors have led to a decrease in population size and probably a subsequent increase in inbreeding, resulting in a loss of part of its genetic diversity. Therefore, keeping a stable environment suitable for population growth and breeding was a very emergent task. The wild populations with a relatively high level of genetic diversity should be conserved as key populations *in situ*. This would guarantee the maintenance of most of the species' genetic variation. There was an urgent need to take effective measures to protect this species against further loss of genetic diversity. Considering the high genetic differentiation of *S. miltiorrhiza*, preservation of any one population would be insufficient to conserve all the variation in the species. Thus, the priority must be to protect all the existing populations *in situ* and prevent anthropogenic destruction, allowing them to propagate and increase in size through natural regeneration.

For future use of available genetic resources included in wild species, it must be considered how we should conserve such genetic variations in natural populations. From this point, the best way was *in situ* conservation, that was, maintenance of the accessions in the original habitat. However, because of difficulty of management and limitation of facilities, it was impossible to conserve all accessions in *in situ* condition. Therefore, we must consider *ex situ* conservation instead of *in situ*. Because most genetic variation was among populations, we should intensively sample and preserve more populations with fewer individuals from each population. For the purpose of capturing most of the genetic variability, germplasm resources should be established with seeds from multiple sources (Montalvo et al., 1997). It might be possible to preserve the majority of local genotypes by collecting seeds from representative ecotypes. However, it was possible that some rare alleles could still potentially be lost with this practice, and this may ultimately affect the long-term survival of this species.

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