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Genetic diversity and population structure of *Salvia miltiorrhiza* Bge in China revealed by ISSR and SRAP

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Abstract *Salvia miltiorrhiza* Bge is a traditional Chinese medicinal herb used as an important drug to cure cardiovascular diseases. In this work, inter simple sequence repeats (ISSR) and sequence related amplified polymorphism (SRAP) markers, were applied to assess the level and pattern of genetic diversity in five important cultivated populations of *S. miltiorrhiza*. Among these populations, 120 bands were amplified by 5 ISSR primers, of which all were polymorphic, and 110 polymorphic bands (90.16%) were observed in 122 bands amplified by 6 SRAP primers. A high levels of genetic diversity at the species level was detected with $Hs = 0.1951$, 0.1927 respectively. Analysis of molecular variance revealed that a greater proportion of total genetic variation existed within populations (86.64 and 84.83% respectively) rather than among populations (13.36 and 15.17% respectively). Cluster analysis divided the five populations into two groups. The genetic relationships among populations have low correlation with their geographical distribution (Mantel test; $r = 0.4870$ and 0.5740 respectively). The study indicated that both ISSR and SRAP markers were effective and reliable for assessing the degree of genetic variation of *S. miltiorrhiza*. Our results suggested that random collecting, preserving and planting seeds without deliberate selection might be an efficient way to conserve genetic resources of medicinal plants. Their effective use was also discussed on the further breeding.

Keywords *Salvia miltiorrhiza* · Genetic diversity · ISSR · SRAP

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Introduction

Salvia miltiorrhiza Bge (also known as *Danshen* in Chinese) is a widely used medicinal herb in Asian countries (Yang 1990). Its root and rhizome officially listed in the Chinese Pharmacopoeia has been traditionally used for centuries to treat cardiovascular diseases such as angina pectoris, myocardial infarction, and stroke (China Pharmacopoeia Committee 2005). Some studies also indicated that the extract of *S. miltiorrhiza* could treat liver diseases (Liu et al. 2002), acquired immunodeficiency syndrome (AIDS) (Abd-Elazem et al. 2002), diabetic nephropathy (Jung et al. 2002), and other diseases. It is an important constituent of various medicinal preparations like Cardiotonic pills (Fufang Danshen Diwan), Fufang Danshen tablet (FDT) (Liu et al. 1997). The annual demand on raw materials is increased rapidly from 450×10^4 kg on 1998–1500×10^4 kg now.

S. miltiorrhiza (genus *Salvia*), belonging to Labiatae family, was a perennial plant with strong environmental adaptability and flourishes in warm and humid environments with abundant sunlight, it was widely distributed in the plain and hills of East China, Central China, and North China (Li et al. 2008). Floral biology of *S. miltiorrhiza* showed that the flowers were bisexual and never-ending. The breeding system was outcrossing with self-compatibility and the most frequent floral visitors were bees (Song et al. 2009).

As the wild resources decreased rapidly, *S. miltiorrhiza* was extensively cultivated since 1970s (Lin et al. 2008). At present commercial Danshen herb mainly depends on cultivated resource, its planting area concentrated in east and central China, such as Henan, Shandong, Shaanxi and Sichuan province (Lin et al. 2008). Some cities or counties of these provinces had become the main producing area of

S. miltiorrhiza in China, such as Tai'an city from Shandong province, Lushi county from Henan province, Zhongjiang city of Sichuan province. The species predominantly propagate sexually by seeds at most plant regions. At forest, farmers usually collected all mature seeds directly from local wild resources randomly, mixed them together, and planted them in the fields. In this way, farmers pass the germplasms on years after years within the same families. Meanwhile the species was propagated vegetatively by a short rhizome in few regions such as Zhongjiang, Sichuan province (Zhang et al. 2002).

As one of the most important herbs, most studies have focused on cultivation, effective ingredients, and pharmacology of *S. miltiorrhiza*, the reports about the genetic and breeding was little. Until now only few cultivars have been developed during a long history of horticulture in main planting populations, they are bred by individual selection.

Monitoring of the genetic diversity in cultivated populations is useful not only for gauging the impacts of breeding and production activities on resource diversity, but also for effective genetic conservation and utilization of existing natural populations (El-Kassaby and Ritland 1996). Could the cultivation practices like Danshen result in the homogeneity or decrease of genetic diversity after several decades of cultivation? This question becomes more important while wild gene pools of this species decreased rapidly and lack of good cultivars.

In recent years, studies about the genetic diversity of *S. miltiorrhiza* have been undertaken using different molecular markers. Wang et al. (2007) revealed a high genetic diversity of *S. miltiorrhiza* in wild populations by AFLP marker. Guo et al. (2002) studied the genetic diversity of cultivated and wild populations by RAPD marker, the populations mainly come from Shandong and Henan province, the sample number in each population ranged from 1 to 5. Wang et al. (2009) studied the genetic diversity and relationships of 16 plants from four cultivated populations applying a new marker CoRAP (conserved region amplification polymorphism), but which marker produced low polymorphism at species level (PPBs = 8.8%), this may lead to underestimate polymorphism analysis and genetic diversity level. Although previous researches has provided preliminary data regarding the genetic diversity at species level, more works need to be done about the genetic diversity and population structure within cultivated populations in main producing area of China.

The inter-simple sequence repeat (ISSR) marker involves polymerase chain reaction (PCR) amplifications of DNA using a primer composed of a microsatellite sequence anchored at 3′ or 5′ end by 2–4 arbitrary nucleotides (Zietkiewicz et al. 1994). It have proven to be a rapid, simple and inexpensive way to assess structure and genetic diversity (Culley et al. 2007; González et al. 2005; Nan et al. 2003; Jian et al. 2002), to analyze genetic relationships among cultivars (Martins et al. 2003), and to study evolutionary processes (Sun et al. 2005). Sequence-related amplified polymorphism (SRAP), is a newer molecular marker first introduced by Li and Quiros (2001). The SRAP technique consists of preferential amplification of open reading frames (ORFs) using PCR. SRAP had been applied extensively in genetic linkage map construction, genetic diversity analysis, and comparative genetics of different species and other fields (Li and Quiros 2001; Ferriol et al. 2003; Guo and Luo 2006; Ding et al. 2008).

Here we first report the comparison study on genetic diversity and genetic structure of 62 *S. miltiorrhiza* selected from five important geographical populations in China using both ISSR and SRAP markers. The objectives of the present work is: (1) to investigate genetic diversity of *S. miltiorrhiza* and distribution of genetic diversity in five main cultivated populations of China using ISSR and SSR markers; (2) to evaluate the impact of cultivation practices without deliberate selections on the genetic diversity within cultivated populations of *S. miltiorrhiza*; (3) to assess the levels of polymorphisms detected by the two different methods and compare the efficiency of ISSR and SRAP markers.

Materials and methods

Plant materials

In this study, a total of 62 individuals from five cultivated populations of *Salvia miltiorrhiza* Bge were used, details on sample collection are given in Table 1. These regions

Table 1 Location and sampling size of the five cultivation populations of *S. miltiorrhiza*

Populations	Location	Longitude (E)	Latitude (N)	No. of samples
Tai'an	Tai'an, Shandong province	117°13′33″	36°18′33″	14
Lushi	Lushi, Henan province	111°03′33″	34°06′67″	14
Neixiang	Neixiang, Henan province	111°83′33″	33°05′	14
Shangluo	Shangluo, Shaanxi province	109°90′	33°80′	14
Zhongjiang	Zhongjiang, Sichuan province	104°68′33″	31°06′67″	6

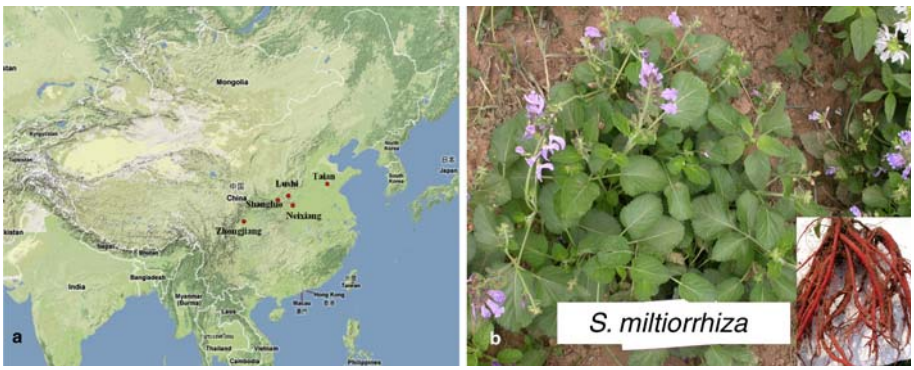

Fig. 1 Locations of different populations in China (**a**) and plant characters of *S. miltiorrhiza* (**b**)

for the sample collecting are the main producing area of China (Fig. 1), *S. miltiorrhiza* have been cultivated for decades and the planting area is larger than other regions. The distance between adjacent samples was at least 50 m. As 9 plants of 14 samples collected from Zhongjiang population amplified the same bands with all primers, and the species was propagated vegetatively in this region, only 6 distinct plants were chosen for further investigation.

DNA extraction

Total genomic DNA of fresh young leaves were extracted using a modified cetyltrimethylammonium bromide (CTAB) method (Guo and Lin 2002). The DNA was quantified by comparative analysis to diluted Lambda DNA in 1% agarose gel and stored at −20°C until use.

ISSR-PCR amplification

One hundred ISSR primers biosynthesised from the Shanghai Sangon Biological Engineering Technology and Service Company were screened initially. Five primers with good and clear amplified bands were selected for genetic diversity analysis (Table 2). Each 20 μl amplification reaction consisted of 1.5 mmol/l $MgCl_2$, 0.4 mmol/l of dNTP, 0.8 mmol/l primers, 10 ng template DNA, and 1 U *Taq* DNA polymerase (TaKaRa Biotechnology, Dalian, China). PCR amplification was performed under the following conditions: initial 5 min at 94°C, followed by 47 cycles of 30 s at 94°C, 45 s annealing at 51°C, and 90 s extension at 72°C, ending with a final extension of 5 min at 72°C. The PCR products were separated on 6% denaturing polyacrylamide gel (acrylamide:bisacrylamide = 29:1) and then silver stained.

SRAP-PCR amplification

Six different SRAP primers combinations were employed using five forward primers and four reverse primers (Table 3). Each 25 μl PCR reaction mixture consisted of 1.5 mmol/l of $MgCl_2$, 20 ng of genomic DNA, 0.2 mmol/l of dNTP, 0.5 mmol/l of primers, and 1 U of *Taq* polymerase (TaKaRa Biotechnology, Dalian, China). PCR amplification was performed under the following conditions: 5 min of denaturing at 94°C, 5 cycles of three steps: 1 min of denaturing at 94°C, 1 min of annealing at 35°C and 2 min of elongation at 72°C. In the following 30 cycles the annealing temperature was increased to 50°C, with a final elongation step of 5 min at 72°C. PCR products were separated and stained like ISSR analysis.

Data analysis

Amplified bands were scored 1/0 as presence/absence of homologous bands for all samples. The resulting presence/absence data matrix was analysed using POPGENE version 1.32 (Francis and Yang 2000) to estimate the level of genetic diversity. The following genetic diversity parameters including the percentage of polymorphic bands (PPB), Shannon's information index (*I*) (Shannon and Weaver

Table 2 Sequences of ISSR and SRAP primers used in the analysis

ISSR primers	Sequences	SRAP primer combinations	Forward primer sequences	Reverse primer sequences
UBC840	$(GA)_8YT$	M8E8	TGAGTCCAAACCGGACT	GACTGCGTACGAATTCAC
UBC846	$(CA)_8RT$	M6E8	TGAGTCCAAACCGGACA	GACTGCGTACGAATTCAC
UBC850	$GA(GT)_7YC$	M9E7	TGAGTCCAAACCGGAGG	GACTGCGTACGAATTCAA
UBC857	$(AC)_8YG$	M2E4	TGAGTCCAAACCGGAGC	GACTGCGTACGAATTTGA
UBC864	$(ATG)_6$	M8E1	TGAGTCCAAACCGGACT	GACTGCGTACGAATTAAT
		M1E4	TGAGTCCAAACCGGATA	GACTGCGTACGAATTTGA

Table 3 Polymorphism revealed by ISSR and SRAP

ISSR				SRAP			
Primer	Bands generated	*A*	PPB (%)	Primer combinations	Bands generated	*A*	PPB (%)
UBC840	27	27	100	M8E8	21	19	90.48
UBC846	24	24	100	M6E8	21	18	85.71
UBC850	25	25	100	M9E7	25	22	88.00
UBC857	17	17	100	M2E4	22	22	100
UBC864	27	27	100	M8E1	15	14	93.33
				M1E4	18	15	83.33
Total	120	120	100	Total	122	110	90.16
Average	24	24		Average	20.33	18.33	

A number of polymorphic loci, *PPB* percentage of polymorphic loci

1949) and Nei's gene diversity (*H*) (Nei 1973) were obtained at both species level and population level.

Gene differentiation between populations was estimated by the coefficient of gene differentiation (Gst) and gene flow (Nm) (Slatkin and Barton 1989) using POPGENE version 1.32 (Francis and Yang 2000). To examine the genetic relationship among populations, Nei's (1978) genetic distance was also generated by POPGENE and a dendrogram was constructed from Nei's (1978) genetic distance with the unweighted pair-group method of averages (UPGMA) with 1,000 permutations of bootstrapping using TFPGA software version 1.3 (Miller 1997). A Mantel test was performed to estimate a correlation between the matrices of Nei's (1978) genetic distances and of geographical distances using NTSYS-pc version 2.1 (1,000 permutations, Rohlf 2000). Correlation between similarity matrices generated by ISSR and SRAP dataset was estimated by means of the Mantel test (Mantel 1967).

Results

Polymorphism and genetic diversity of *S. miltiorrhiza* populations

Five ISSR primers amplified a total of 120 scorable bands (Table 3, Fig. 2), of which all were polymorphic, accounting for 100%. Number of bands varied from 17 (UBC 857) to 27 (UBC840, UBC 864), with an average of 24 polymorphic fragments per primer. Six SRAP primer combinations with high polymorphism and strong-signal bands were selected (Table 3, Fig. 3). The total bands amplified at species level were 122 ranging from 15 for M8E1 to 25 for M9E7, of which 110 were polymorphic, accounting for 90.16%. Average numbers of bands and polymorphic bands per primer were 20.33 and 18.33, respectively.

A summary of the ISSR and SRAP data from each population of *S. miltiorrhiza* are given in Table 4. In the

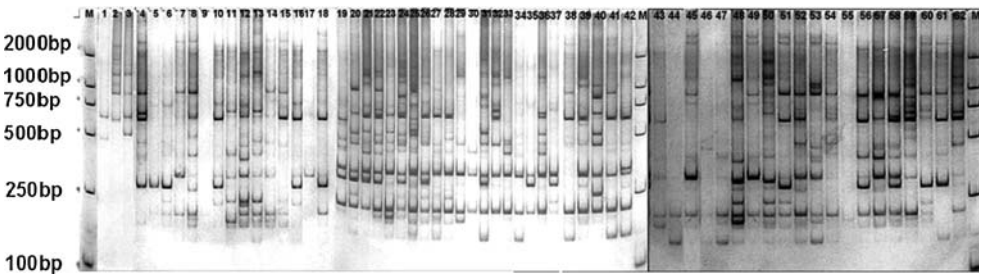

Fig. 2 ISSR amplified result of *Salvia miltiorrhiza* by primer UBC840. *Note*: The lanes from left to right were 1–6, Zhongjiang 1–6; 6–20, Shangluo 1–14; 21–34, Lushi 1–14; 34–48, Neixiang 1–14; 49–62, Tai'an 1–14; M was DL2000 DNA marker

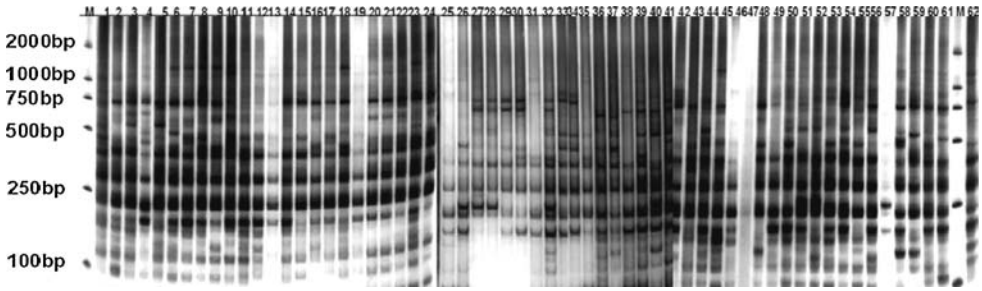

Fig. 3 SRAP amplified result of *S. miltiorrhiza* by primer M9E7. *Note*: The lanes from left to right were 1–6, Zhongjiang 1–6; 6–20, Shangluo 1–14; 21–34, Lushi 1–14; 34–48, Neixiang 1–14; 49–62, Tai'an 1–14; M was DL2000 DNA marker

Table 4 Genetic diversity of *S. miltiorrhiza* populations revealed by ISSR and SRAP

Populations	ISSR				SRAP			
	A	PPB (%)	*H*	*I*	*A*	PPB (%)	*H*	*I*
Tai'an	90	75.00	0.1809	0.2913	74	60.66	0.1548	0.2469
Lushi	101	84.17	0.1944	0.3198	96	78.69	0.1991	0.3147
Neixiang	88	73.33	0.1921	0.3054	91	74.59	0.1708	0.2806
Shangluo	75	62.50	0.1560	0.2515	68	55.74	0.1517	0.2374
Zhongjiang	36	30	0.0719	0.1188	45	36.89	0.1271	0.1915
Average	78	65	0.1591	0.2574	74.8	61.31	0.1607	0.2542
Species level	120	100	0.1951	0.3313	110	90.16	0.1927	0.3209

A number of polymorphic loci, *PPB* percentage of polymorphic loci, *H* Nei's gene diversity, *I* Shannon's information index

Table 5 Nei's unbiased measures of Nei's genetic distance revealed by ISSR and SRAP

Populations	Zhongjiang	Shangluo	Lushi	Neixiang	Tai'an
Zhongjiang	–	0.0092	0.0120	0.0267	0.0441
Shangluo	0.0222	–	0.0204	0.0349	0.0506
Lushi	0.0224	0.0310	–	0.0070	0.0384
Neixiang	0.0385	0.0479	0.0064	–	0.0421
Tai'an	0.0591	0.0477	0.0304	0.0423	–

Note: ISSR analysis (above diagonal) and SRAP analysis (below diagonal)

case of ISSR analysis, the value of Nei's gene diversity (*H*) was 0.1951 and Shannon's Information index (*I*) was 0.3313 at species level. Within each populations, the PPB varied from 30% for Zhongjiang population to 84.17% for Lushi population, and the mean value of *H* was 0.1591, ranging from 0.0719 for Zhongjiang population to 0.1944 for Lushi population. The value of *I* showed similar trends, ranging from 0.1188 to 0.3198 in populations Zhongjiang and Lushi respectively. In the case of SRAP analysis (Table 4), the value of *H* was 0.1927 and *I* was 0.3209 at species level. Within each of the populations, the PPB varied from 36.89% for Zhongjiang population to 78.69% for Lushi population. The value of *H* ranged from 0.1271 to 0.1991 (mean 0.1607) and *I* from 0.1915 to 0.3147. Results showed that the genetic diversity of *S. miltiorrhiza* from Lushi population was the richest among five populations and the Zhongjiang population was the lowest.

Genetic differentiation among populations

To assess the distribution of genetic diversity of *S. miltiorrhiza* in five cultivation bases, the POPGENE program was used to analysis genetic differentiation from both ISSR and SRAP data. For ISSR analysis, a relatively lower genetic differentiation among populations was revealed that the majority of genetic variation (86.64%, $G_{ST} = 0.1336$) existed within populations, whereas only a small amount of genetic variation (13.36%) was present among populations. Similarly, the analysis of SRAP bands indicated that most of the genetic differentiation (84.83%, $G_{ST} = 0.1517$) existed among populations, with minor variation (15.17%) found within populations.

Gene flow number (Nm) was 3.2362 and 2.7953 by ISSR and SRAP markers, which also indicated that there was higher gene exchange between populations.

Genetic identity and genetic distance among populations

To further elucidate the gene differentiation between populations, Nei's unbiased measure of genetic distance were evaluated (Table 5). Nei's genetic distance ranged from 0.0070 to 0.0506 based on the ISSR analysis (above the diagonal) with an average 0.02854. The largest genetic difference (0.0506) occurred between Shangluo and Tai'an populations and the least (0.0070) between Lushi and Neixiang populations from same province. The data from the SRAP analysis (below the diagonal) gave similar different results, with the largest genetic difference between Zhongjiang and Tai'an populations (0.0591). Although the average genetic distance (0.03479) derived from SRAP markers (below the diagonal in Table 4) showed higher genetic differences compared with the average genetic distance (0.02854) of ISSR markers, both indicated that there were high similarity and closer genetic distance between populations, and also confirmed low level of genetic differentiation among the populations indicated by G_{ST}.

Relationships between populations were further illustrated by a dendrogram using UPGMA algorithm based on Nei's genetic distance (Fig. 4a, b). A dendrogram (Fig. 4a) based on ISSR data grouped the five populations into two main clusters. Tai'an population formed a separate cluster showing less similarity with the other populations studied.

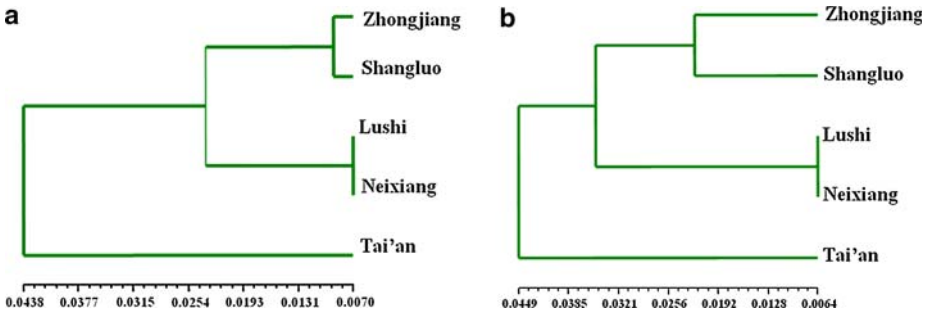

Fig. 4 Dendrogram obtained by UPGMA cluster based on data from ISSR and SRAP analysis. **a** ISSR. **b** SRAP

The other four populations grouped in another cluster can be further grouped into two subclusters. Shangluo and Zhongjiang populations constituted one sub-group. Lushi and Neixiang population clustered into the other sub-group which appeared to be closer to each other. A dendrogram (Fig. 4b) based on UPGMA analysis with SRAP data showed similar result with the ISSR data.

Mantel tests indicated a low correlation between geographical and genetic distances for both ISSR ($r = 0.4870$, $P = 0.1394$) and SRAP analyses ($r = 0.5740$, $P = 0.0742$). When the similarity matrices generated by ISSR and SRAP markers were compared, the values of $r = 0.8621$ at $P = 0.0012$ (Jaccard's coefficient) indicated a good correlation between the data generated by the two marker systems.

Discussion

Genetic diversity of *S. miltiorrhiza* revealed by ISSR and SRAP marker

Studies about the genetic diversity of *S. miltiorrhiza* have been undertaken by other researchers using different molecular markers such as RAPD, AFLP, CoRAP (Guo et al. 2002; Wang et al. 2007, 2009). However RAPD is simple and convenient, but its low stability and reproducibility have restricted its use. AFLP is now widely used for genomic fingerprinting due to its rapidity, reproducibility, and numerous polymorphisms. But two major disadvantages associated with this technique are prohibitive expenses and tedious procedures. And CoRAP produced fewer polymorphism bands in *S. miltiorrhiza*, this would lead to underestimate polymorphism analysis and genetic diversity level.

In this work, ISSR and SRAP markers were applied to assess the level and pattern of genetic diversity in five important cultivated populations of *S. miltiorrhiza*. Polymorphic bands ($A = 120$), its percentage (PPBs = 100%), the average Nei gene diversity ($Hs = 0.1951$) and Shannon's information index ($Is = 0.3313$) at species level generated by ISSR primer were higher than that of SRAP analysis ($A = 110$, PPBs = 90.16%, $Hs = 0.1927$, $Is = 0.3209$). The possible reason was the ISSR markers scattered throughout the genome which revealed the diversity of the entire genome, while SRAP markers only amplified target region of open reading frame (ORF), the functional regions. As dendrogram based on UPGMA analysis with SRAP data gave similar result with the ISSR data, the Mantel results ($r = 0.8621$, $P = 0.0012$) also showed a high correlation between the two molecular techniques which was proved by the studies of Chen et al. (2009). Our results indicated that both ISSR and SRAP markers were effective and reliable for accurately assessing the degree of genetic variation of *S. miltiorrhiza*.

Genetic diversity of cultivated populations of *S. miltiorrhiza* in China

Although the polymorphism detected by ISSR and SRAP markers were different, both markers revealed almost similar pattern of genetic diversity in cultivated populations of *S. miltiorrhiza* ($Hs = 0.1951$, 0.1927, respectively). Compared with the genetic diversity of *S. miltiorrhiza* in 24 wild populations ($Hs = 0.2612$) revealed by AFLP (Wang et al. 2007), the extent of genetic diversity of *S. miltiorrhiza* in cultivated populations at present research was lower. Comparing with these species from cultivation population in other most important herbs (Table 6) such as *Panax ginseng* C. A. Meyer, *Panax quinquefolius* L., *Platycodon grandiflorum, Rehmannia uralensis* Fisch and *Codonopsis pilosula, S. miltiorrhiza* in cultivated regions was also found to possess a moderate to high levels of genetic diversity at species or at the population level.

Although the results indicated that cultivation of peoples affected and decreased the genetic diversity of this species, the cultivated species still possess a relatively higher genetic diversity compared with other cultivated herbs. This would be good for the cultivation and breeding study of this species. Guo et al. (2002) studied the cultivated and wild populations of *S. miltiorrhiza* in Shandong and Henan province by RAPD and revealed that the genetic diversity in cultivated populations was similar with the result of wild

Table 6 Comparison of genetic diversity of *S. miltiorrhiza* with other important herbs in cultivation population

Species	Genetic diversity values	Markers	References
S. miltiorrhiza	PPBs = 100%; *Hs* = 0.1951; PPBp = 65%; *Hp* = 0.1591	ISSR	This paper
	PPBs = 90.16%; *Hs* = 0.1927; PPBp = 61.31%; *Hp* = 0.1607	SRAP	
Panax ginseng C. A. Meyer	PPBs = 98.9%; PPBp = 48.45%, *Is* = 0.2362	ISSR	Li et al. (2007)
Panax quinquefolius L.	PPBp = 28.2, *Hp* = 0.071	RAPD	Jennifer et al. (2005)
Platycodon grandiflorum	PPBs = 47.13%; *Hs* = 0.1612; PPBp = 27.58%; *Hp* = 0.1083	RAPD	Wei et al. (2006)
Glycyrrhiza uralensis Fisch	*Hs* = 0.25; *Hp* = 0.13-0.19	AFLP	Ge et al. (2009)
Codonopsis pilosula	PPBs = 87.23%, *Hs* = 0.2992 ± 0.1681	RAPD	Hong et al. (2007)

PPBs percentage of polymorphic loci at species level, *Hs* Nei's gene diversity at species level, *PPBp* percentage of polymorphic loci at population level, *Hp* Nei's gene diversity at population level, *Is*, Shannon's information index at species level

populations. The difference might be affected by different marker systems (Budak et al. 2004) and the sample number in population. Therefore, this aspect needs to be verified by a more extensive investigation.

Notably, the current high level of genetic diversity of *S. miltiorrhiza* may greatly attribute to the traditional, irregular and sparse agricultural practices of this plant. Traditionally, farmers collect and preserve seeds of *S. miltiorrhiza* randomly without deliberate selection, and mix them together before planting. And these would reserve more genetic diversity as the wild species. Breeding systems would be another one important factors that determine the genetic diversity in plant species (Hamrick 1982). Our observation based on pollination experiments of *S. miltiorrhiza* suggested that it is cross-pollinated plant with high cross-pollinated rate, which is believed important in the maintenance of the high levels of genetic variability of species (Hamrick and Godt 1996). In addition, the geographical distribution may affect the genetic diversity level of a species, widespread species often considered to be have higher level of genetic variability than narrowly distributed ones (Hamrick and Godt 1996). It was reported that *S. miltiorrhiza* have strong adaptability to environment (Li et al. 2008), and distributed widely in China and adapted to different survival environments, that would also result in higher level genetic diversity.

Population genetic structure of *S. miltiorrhiza*

Gene differentiation and gene flow are important index to evaluate the population genetic structure of a species. In the present study, the value of G_{ST} in *S. miltiorrhiza* were 0.1336 and 0.1517 based on the ISSR and SRAP markers, indicating that the major proportion of the total variation of *S. miltiorrhiza* existed within populations and the minor variations existed among populations. The result was consistent to the results revealed by RAPD (Guo et al. 2002) and CoRAP markers (Wang et al. 2009).

Gene flow, the movement of gene within and between populations, is negatively correlated with gene differentiation (Grant 1991), but is very important for population transfer and plant evolution, and is transferred by pollen and seed between populations for seed plant (Hamrick 1987). In the current study, gene flow (Nm) of *S. miltiorrhiza* was 3.2362 and 2.7953 detected by ISSR and SRAP markers, and this level of migration will prevent divergence between populations. The mean genetic distances of five populations in our study was 0.02854 and 0.03479 revealed by ISSR and SRAP respectively, which proved that the minor genetic difference of these populations. As to whether transferred by pollen or/and seed still need to further study. Moreover a low correlation between geographical and genetic distances for both ISSR ($r = 0.4870$, $P = 0.1394$) and SRAP analyses ($r = 0.5740$, $P = 0.0742$) were tested.

The *S. miltiorrhiza* in Zhongjiang populations from Sichuan province had the lowest genetic diversity than the other four populations. Although *S. miltiorrhiza* had been cultivated for a 100 years in Sichuan province, it had lower seed set and germination percentage (Sun and Wong 2001) probably due to its climate with high temperature and high humidity. So the production of *S. miltiorrhiza* in Sichuan province could not be cultivated in sexual reproduction but vegetatively by a short rhizome (Zhang et al. 2002). Therefore in the cultivated populations there only retained a low genetic diversity, which was also verified by RAPD markers (Guo et al. 2002). As the result of lower genetic diversity and long-term vegetative propagation of *S. miltiorrhiza* from Zhongjiang population, it would lead to the potential dangers in disease or decline in the quality and quantity. In order to improve its genetic diversity, new genetic resources should be introduced from its wild species or from other cultivated populations.

The other four cultivated populations of this paper possess higher genetic diversity, which would be benefit for the further genetic and breeding study. The results indicated that the primitive agricultural practices, i.e.,

collecting, preserving and planting seeds randomly without deliberate selection, seems still maintain rich genotypes of medicinal plants if the populations are sufficiently large. Such practices might be an effective way for the maintenance and conservation of gene pools of medicinal plants (Hong et al. 2007).

References

Abd-Elazem IS, Chen HS, Bates RB, Huang RCC (2002) Isolation of two highly potent and non-toxic inhibitors of human immunodeficiency virus type 1 (HIV-1) integrase from *Salvia miltiorrhiza*. Antiviral Res 55:91–106

Budak H, Shearman RC, Parmaksiz I, Dweikat I (2004) Comparative analysis of seeded and vegetative biotype buffalograsses based on phylogenetic relationship using ISSRs, SSRs, RAPDs, and SRAPs. Theor Appl Genet 109:280–288

Chen DX, Peng R, Li RY, Song NX, Zhong GY, Cai YF (2009) Study on genetic diversity of *Codonopsis tangshen* by SRAP and ISSR markers. Chin Tradit Herb Drugs 34(3):255–259

China Pharmacopoeia Committee (2005) Pharmacopoeia of P. R. China. Part I. People's Health Publishing House, Beijing, pp 52–53

Culley TM, Sbita SJ, Wick A (2007) Population genetic effects of urban habitat fragmentation in the perennial herb *Viola pubescens* (Violaceae) using ISSR markers. Ann Bot 100:91–100

Ding G, Zhang DZ, Ding XY, Zhou Q, Zhang WC, Li XX (2008) Genetic variation and conservation of the endangered Chinese endemic herb *Dendrobium officinale* based on SRAP analysis. Plant Syst Evol 276:149–156

El-Kassaby YA, Ritland K (1996) Genetic variation in low elevation Douglas-fir of British Columbia and its relevance to gene conservation. Biodivers Conserv 5:779–794

Ferriol M, Pico B, Nuez F (2003) Genetic diversity of a germplasm collection of *Cucurbita pepo* using SRAP and AFLP markers. Theor Appl Genet 107:271–282

Francis CY, Yang RC (2000) Popgene version 1.32. http// www.ualberta.ca/_fyeh/index.htm

Ge SJ, Li GM, Ma ZY, Wu XY, Meng YJ, Huo YQ (2009) Analysis on genetic diversity of wild populations of Licorice (*Glycyrrhiza uralensis* Fisch.) with AFLP markers. Scientia Agricultura Sinica 42:47–54

González A, Wong A, Delgado-Salinas A, Papa R, Gepts P (2005) Assessment of inter simple sequence repeat markers to differentiate sympatric wild and domesticated populations of common bean. Crop Sci 45:606–615

Grant V (1991) The evolutionary process: a critical study of evolutionary theory. Columbia University Press, New York

Guo BL, Lin S (2002) DNA extraction of dried leaves of *Salvia miltiorrhiza*. Chin Tradit Herb Drugs 33:418–420

Guo DL, Luo ZR (2006) Genetic relationships of some PCNA persimmons (Diospyros kaki Thunb.) from China and Japan revealed by SRAP analysis. Genet Resour Crop Evol 53:1597–1603

Guo BL, Lin S, Feng YX (2002) Primary research on genetic relationship among main populations of *Salvia miltiorrhiza* and genuineness of herb. Chin Tradit Herb Drugs 33:1113–1116

Hamrick JL (1982) Plant population genetics and evolution. Am J Bot 69:1685–1693

Hamrick JL (1987) Gene flow distribution of genetic variation in plant populations. In: Urbanska K (ed) Differentiation patterns in higher plants. Academic Press, New York, pp 53–57

Hamrick JL, Godt MJW (1996) Effects of life history traits on genetic diversity in plant species. Philos Trans R Soc Lond B Biol Sci 351:1291–1298

Hong BG, Bao RL, Qian Ho W, Jia KC, Tong SZ (2007) Abundant genetic diversity in cultivated *Codonopsis pilosula* populations revealed by RAPD polymorphisms. Genet Resour Crop Evol 54:917–924

Jian SG, Shi SH, Zhong Y (2002) Genetic diversify among south China *Heritiera littoralis* detected by inter-simple sequence repeats (ISSR) analysis. J Genet Mol Biol 13(4):272–276

Jung M, Lee HC, Ahn CW, Park W, Choi S, Kim H (2002) Effective isolation of magnesium lithospermate B and its inhibition of aldose reductase and fibronectin on mesangial cell line. Chem Pharm Bull 50:1135–1136

Li G, Quiros CF (2001) Sequence-related amplified polymorphism (SRAP), a new marker system based on a simple PCR reaction: its application to mapping and gene tagging in Brassica. Theor Appl Genet 103:455–461

Li J, Chen Z, Yang XL, Li S, Gu RQ, Wan SW, Zhang WJ (2007) ISSR analysis on genetic diversity of *Panax ginseng* landraces. Chin Tradit Herb Drugs 38(9):1392–1395

Li MH, Chen JM, Peng Y, Wu QL, Xiao PG (2008) Investigation of Danshen and related medicinal plants in China. J Ethnopharmacol. doi:10.1016/j.jep.2008.09.013

Lin WL, Deng JH, Lu M, Ou XH, Lin N, Yang GS, Liang HY (2008) The research in production of Danshen in three main cultivations in China. J Chin Med Mater 31:338–340

Liu ZL, Gao SY, Deng J, Li H (1997) The effect of cardiotonic pill on coronary heart disease with symptom of angina. Chin Tradit Pat Med 19:20–21

Liu P, Liu CH, Wang HN, Hu YY, Liu C (2002) Effect of salvianolic acid B on collagen production and mitogen-activated protein kinase activity in rat hepatic stellate cells. Acta Pharmacol Sin 23:733–738

Mantel NA (1967) The detection of disease clustering and a generalized regression approach. Cancer Res 27:209–220

Martins M, Tenreiro R, Oliveira MM (2003) Genetic relatedness of Portuguese almond cultivars assessed by RAPD and ISSR markers. Plant Cell Rep 22:71–78

Miller MP (1997) Tools for population genetics analysis (TFPGA), version 1·3. Department of Biological Sciences, Northern Arizona University, Flagstaff

Nan P, Shi SH, Peng SL (2003) Genetic diversity in *Primula obconica* (Primulaceae) from central and south-west China as revealed by ISSR markers. Annal Bot 91(3):329–333

Nei M (1973) Analysis of gene diversity in subdivided populations. Proc Natl Acad Sci USA 70:3321–3323

Nei M (1978) Estimation of average heterozygosity and genetic distance from a small number of individuals. Genetics 89:583–590

Rohlf FJ (2000) NTSYS-pc: numerical taxonomy and multivariate analysis system, version 2.1., New York, USA

Shannon CE, Weaver W (1949) The mathematical theory of communication. University of Illinois Press, Urbana

Slatkin M, Barton NH (1989) A comparison of three indirect methods for estimating average levels of gene flow. Evolution 43:1349–1368

Song ZQ, Wang JH, Wang HG, Zhao FJ, Hao LW (2009) Studied of the floral biology, breeding characters of *Salvia miltiorrhia*. Acta Horticulturae Sinica 36:905–910

Sun M, Wong KC (2001) Genetic structure of three orchid species with contrasting breeding systems using RAPD and allozyme markers. Am J Bot 88:2180–2188

Sun JH, Li ZC, Jewett DK (2005) Genetic diversity of *Pueraria lobata* (Kudzu) and closely related taxa as revealed by inter-simple sequence repeat analysis. Weed Res 45(4):255–260

Wang B, Zhang Y, Cheng CB, Li XL, Chen RY, Chen L (2007) Analysis on genetic diversity of different *Salvia miltiorrhiza* geographical populations in China. Chin J Chin Mater Medica 32:1988–1991

Wang QH, Zhang BL, Lu QS (2009) Conserved region amplification polymorphism (CoRAP), a novel marker technique for plant genotyping in *Salvia miltiorrhiza*. Plant Mol Biol Rep 27:139–143

Wei JH, Yang CM, Chen SL, Cheng HZ, Huang LQ (2006) Analysis of genetic diversity of the cultivation and wild germplasms of *Platycodon grandiflorum* based RAPD markers. World Sci Technol/Mod Tradit Chin Med Mater Medica 8(3):37–41

Yang B (1990) Phytochemistry of *Salvia miltiorrhiza*. In: Xu R (ed) Danshen, biology and application. Science Press, Beijing, pp 81–157

Zhang XG, Wang YM, Luo GA, Cheng FX (2002) Studies on resource characteristics of *Salvia miltiorrhiza* varietie. Chin Tradit Herb Drugs 33:742–747

Zietkiewicz E, Rafalski A, Labuda D (1994) Genome fingerprinting by simple sequence repeat (SSR) anchored polymerase chain reaction amplification. Genomics 20:176–183