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Genetic diversity among *Salvia miltiorrhiza* Bunge and related species inferred from nrDNA ITS sequences

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Abstract: To investigate the genetic diversity and phylogenetic relationships of *Salvia miltiorrhiza* and related species, we analyzed the nuclear ribosomal DNA internal transcribed spacer (ITS) region for 7 accessions of *Salvia miltiorrhiza* and another 23 samples from other taxa within the genus *Salvia* by maximum parsimony and Bayesian inference analyses. There were 257 variation sites amounting to 40.8% of the total base pairs. All of the data revealed abundant genetic diversity in the genus *Salvia*. The results showed that the tested materials could be grouped into 3 clusters, and the 3 different occasions in *Salvia* were clustered into 1 clade, which suggests that the species from different occasions form independent lineages. We can easily distinguish *S. miltiorrhiza* from a variety of *Salvia* species in this manner. Moreover, according to cluster analysis, *S. bowleyana*, *S. yunnanensis*, and *S. cavaleriei* var. *simplelefolia* are good or potential germplasm resources for *S. miltiorrhiza*.

Key words: ITS, genetic diversity, *Salvia miltiorrhiza*

Introduction

The genus *Salvia* L. (Lamiaceae) represents an enormous and cosmopolitan assemblage of nearly 1000 species, exhibiting a remarkable range of variation. It is distributed throughout the Old and the New World, in subtropical and temperate areas (1). In China, there are 78 species, 24 varieties, and 8 forma of *Salvia*; about 70 of these species are endemic to China. Most taxa are distributed in southwestern regions such as Sichuan, Yunnan, and Guizhou. *Salvia* is distinguished from the other genera in Lamiaceae by its 2 lever-like modified stamens. In *Flora of China*, according to the morphological characters of stamen, the *Salvia* species are classified into 4 subgenera: subgen. *Salvia*, subgen. *Sclarea*, subgen. *Jungia*, and subgen. *Allagospadonopsis* (2).

Since ancient times, species of *Salvia* have been used in folk medicine for the treatment of diabetes and skin diseases such as psoriasis and eczema (3). The majority of the Chinese *Salvia* species are known for their characteristic fragrant oils, and use of the family Lamiaceae is well recorded in ancient and contemporary pharmacopoeias and literature documenting folk uses of plants. Some *Salvia* species in China, for example *S. miltiorrhiza* Bunge, are important from medicinal and economic perspectives

and are cultivated for pharmaceutical use. The dried root of *S. miltiorrhiza*, which is called Danshen in Chinese, has been used since ancient times as a medicinal plant. In *Pharmacopoeia of China*, it is a very important traditional Chinese medicine that promotes blood flow to overcome blood stasis and cools the blood to resolve abscesses (4). Modern pharmacological and clinical studies show that Danshen has curative effects in cardiovascular disease (5). In China, Danshen is widely used in many finished Chinese medicines. Every year about 80,000,000 kg of this crude medicine is consumed in China; as a result, the supply of Danshen is inadequate (6). Therefore, it is very important to find new medicinal plant resources, as well as a gene germplasm resource for *S. miltiorrhiza*.

The root and rhizome of more than 36 species (including varieties and forma) in subgen. *Salvia* or subgen. *Sclarea* can be used to serve the function of *S. miltiorrhiza* (7). However, this usage is largely traditional, and its scientific basis needs further research. The traditional methods are mainly based on slight differences in morphological characters and analysis of compounds by high performance liquid chromatography (HPLC) fingerprints to distinguish *S. miltiorrhiza* from adulterants (6,8). The accuracy of authentication has limitations because of the sample amounts, stability of chemical constituents, variable sources, and chemical complexity. However, DNA can be extracted from fresh or dried organic tissue of the plant materials and is not restricted by the form of the samples. DNA markers are reliable for informative polymorphisms because genetic composition is unique for each species (9). The species that have close relationships may have similar functions. However, few studies on the relationships of *Salvia* species have been carried out, and the status of each herbal medicine has not been established. In order to utilize these species accurately, more basic studies, such as genetic diversity and relationship studies, should be pursued in order to obtain a standard plant base for high quality herbal medicine and new germplasm resource selection.

Molecular studies have successfully revealed the origin and evolutionary history of polyploids in plants and clarified the nature of different polyploids and the hybridization events involved in their formation (10). The nuclear ribosomal DNA internal transcribed spacer (nrDNA ITS) region has been established as a useful marker to decipher phylogenetic relationships and genomic relationships of plants at lower taxonomic levels (11-13). Utilization of ITS phylogeny in Lamiaceae has also been well established (14-17). Furthermore, while the plastid genome has been the target of most DNA barcode proposals, the nuclear ribosomal ITS (ITS1 and ITS2) sequences have also been identified as potential barcode regions, if used in combination with 1 or 2 plastid genome sequences (18). Some species in *Salvia* have been investigated for phylogenetic relationship analysis based on the nuclear ITS region (19-23). However, the nuclear ITS regions of most *Salvia* species in China have not been studied.

In this study, we sequenced and analyzed the nuclear ribosomal ITS regions for 21 species, 1 variety, and 1 form of *Salvia*, in addition to another 7 accessions of *S. miltiorrhiza* from different regions of China in subgen. *Salvia* and subgen. *Sclarea* that are used mainly as medicinal plants. The aims of this study were to estimate the relationships of *Salvia* species in different distributions, to elucidate the relationships of *Salvia* species in China between subgen. *Salvia* and subgen. *Sclarea*, and to search for new Danshen germplasm resources in *Salvia* and obtain scientific data for further use.

Materials and methods

Materials sampling

A total of 32 nrDNA ITS sequences are used in this study; 20 new nrDNA ITS sequences were employed and 12 ITS sequences from earlier investigations in the Lamiaceae were obtained from GenBank. Taxa, subgenera, vouchers, localities, and GenBank numbers of these sequences are listed in the Table. The data matrix for the phylogenetic analysis comprised 30 accessions of *Salvia*. *Dorystaechas hastata* and *Meriandra bengalensis* were both in the tribe Mentheae of Lamiaceae and were selected as outgroups for the *Salvia* phylogenetic analysis. The voucher specimens for the newly sequenced taxa were deposited in the Triticeae Research Institute of Sichuan Agricultural University, Sichuan, China.

Table. Species of *Salvia* used in this study and related general information.

Taxon	Subgen.us	Location	Voucher	GenBank NO.
Salvia aerea Lévl.	*Salvia*	Asia: Southwest China	S009	EU169469*
Salvia brevilabra Franch.	*Salvia*	Asia: Southwest China	Y. H. Zhou 06011	EF373636
Salvia castanea Diels	*Salvia*	Asia: Southwest China	S017	EU169463*
Salvia cyclostegia Stib.	*Salvia*	Asia: Southwest China	S011	EU169475*
Salvia cynica Dunn	*Salvia*	Asia: Southwest China	Z. J. Yang 05005	EF373639
Salvia digitaloides Diels	*Salvia*	Asia: Southwest China	S005	EU169473*
Salvia evansiana Hand.-Mazz.	*Salvia*	Asia: Southwest China	X. Fan 04002	EF373621
Salvia flava Forrest ex Diels	*Salvia*	Asia: Southwest China	X. Fan 04001	EF373626
Salvia omeiana Stib.	*Salvia*	Asia: Southeast China	Z. J. Yang 05001	EF373643
Salvia pauciflora Stib.	*Salvia*	Asia: Southwest China	S015	EU169476*
Salvia przewalskii Maxim.	*Salvia*	Asia: Southwest China	Z. J. Yang 06005	EF373628
Salvia roborowskii Maxim.	*Salvia*	Asia: Southwest China	Z. J. Yang 06009	EF373630
Salvia tricuspis Franch.	*Salvia*	Asia: Southeast China	Z. J. Yang 06008	EF373635
Salvia bowleyana Dunn	*Sclarea*	Asia: Southwest China	L. Zhang 05008	EF373645
Salvia cavaleriei var. *simplicifolia* Stib.	*Sclarea*	Asia: Southwest China	Z. J. Yang 06006	EF373619
Salvia miltiorrhiza Bunge 1	*Sclarea*	Asia: East China	L. Zhang 05001	EF373590
Salvia miltiorrhiza Bunge 2	*Sclarea*	Asia: North China	L. Zhang 05002	EF373595
Salvia miltiorrhiza Bunge 3	*Sclarea*	Asia: Southeast China	L. Zhang 05003	EF373597
Salvia miltiorrhiza Bunge 4	*Sclarea*	Asia: South China	L. Zhang 05004	EF373602
Salvia miltiorrhiza Bunge 5	*Sclarea*	Asia: Northeast China	L. Zhang 05005	EF373604
Salvia miltiorrhiza Bunge 6	*Sclarea*	Asia: Southwest China	L. Zhang 05006	EF373606
Salvia miltiorrhiza Bunge 7	*Sclarea*	Asia: North China	L. Zhang 05007	EF373609
Salvia miltiorrhiza f. *alba* C.Y.Wu et H. W. Li	*Sclarea*	Asia: Northeast China	H. Q. Yu 05001	EF373612
Salvia plebeia R.Br.	*Sclarea*	Asia: Southwest China	L. Zhang 05010	EF373648
Salvia yunnanensis C.H.Wright	*Sclarea*	Asia: Southeast China	X. Fan 04003	EF373616
Salvia aegyptiaca L.	–	Mediterranean: North Africa	McLeish 3728	DQ667285*
Salvia aristata Aucher	–	Mediterranean: West Iran	Wedelbo&Assadi s.n.	DQ667280*
Salvia apiana Jepson	–	North America: California	JBW 2509	DQ667214*
Salvia splendens Sello ex Roem et Schulf.	–	South America: Bolivia	–	AF477788*
Salvia patens Cav.	–	Latin America: Mexico	1973-9197	DQ667253*
Dorystaechas hastata Boiss. & Heldr. ex Benth.	–	–	1972-0177D	DQ667252*
Meriandra bengalensis (Roxb.) Benth.	–	–	Lavranus & Newton 15796	DQ667329*

*Data from published sequences in GenBank (http://www.ncbi.nlm.nih.gov).

DNA extraction, amplification, and sequencing

Total genomic DNA was extracted from young fresh leaves using the CTAB method (24). All nrDNA ITS regions were amplified by polymerase chain reaction (PCR) with universal rice primers P1: 18S 5'-CGTAACAAGGTTTCCGTAGGTGAA -3' and P2: 26S 5'- TTATTGATATGCTTAAACTCAGCGGG -3' (21). The PCR amplification of ITS DNA was performed in a total reaction volume of 30.0 µL containing 10× Ex Taq reaction buffer, 1.5 mM of $MgCl_2$, 0.8 mM of dNTP mixture, 10.0 µM of each primer, 1.25 U of Ex Taq polymerase (TaKaRa Inc., Dalian, China), 20 ng of template DNA, and double distilled H_2O to reach the final volume. The thermal cycling program for PCR consisted of an initial denaturation step at 95 °C for 4 min, followed by 30 cycles of 1 min at 95 °C for denaturation, 45 s at 60 °C for annealing, 1.5 min at 75 °C for extension, and a final extension step of 10 min at 72 °C. The PCR products were purified with the ENZA™ gel extraction kit (Omega Bio-Tek, Norcross, GA, USA) and linked to a pMD18-T vector (Promega, Madison, WI, USA) according to the manufacturer's instructions. Transformation, plating, and isolation of plasmids were performed as described in Liu et al. (25). Purified plasmid DNAs were digested with *EcoR*I and *Hind*III. For each of the accessions, 3-5 cloned PCR products were sequenced by TaKaRa.

Phylogenetic analyses

Multiple sequences were aligned using the ClustalW algorithm followed by manual adjustment implemented by MegAlign software (DNAStar, Inc., Madison, WI, USA) (26). Sequence statistics, including nucleotide substitutions, transition/transversion ratio, and variability in different regions of the sequences, were calculated with MEGA 4 (27).

Analyses were conducted using maximum parsimony (MP) and Bayesian inference (BI). MP analysis was performed in PAUP*4.0b10 (28). MP heuristic searches were carried out with the following options implemented: heuristic search mode, 100 random-addition-sequence replicates, tree bisection-reconnection (TBR) branch swapping, and MUL Trees. The MP trees from each data analysis were used to generate the 50% majority-rule consensus trees. Topological robustness MP analysis was assessed by bootstrap analysis with 1000 replicates using simple taxon addition.

The BI analysis was conducted with MrBayes v3.1.2 (29). ModelTest 3.06 was used to determine the appropriate DNA substitution model, and gamma rate heterogeneity was determined using the Akaike information criterion (30). The best-fit model identified was GTR+G+I for ITS data. Applying MrBayes default heating values (t = 0.2), 4 Markov chain Monte Carlo chains (1 cold and 3 heated) were run for 1,836,000 generations, each sampling every 100 generations. The first 4950 trees were discarded as "burn-in." The remaining trees were used to construct the 50% majority-rule consensus trees. Two independent runs were performed to check whether convergence on the same posterior distribution was reached. The statistical confidence in nodes was evaluated by posterior probabilities.

Results

The ITS sequences in this study included 2 regions: 21 nucleotides of the 18S rRNA gene, and the complete sequences of ITS1, 5.8S rRNA gene, and ITS2. Sequences of the 18S rRNA genes showed no variations for all accessions included in this study. The length of sequences ranged from 204 to 216 bp in the ITS1 region and from 192 to 211 bp in the ITS2 region. The 5.8S rRNA gene was 161 bp long and completely identical for all cloned sequences of the 32 accessions.

The aligned ITS sequences yielded a total of 630 characters; 257 of these were variable characters and 85 were informative. The parsimony analysis for ITS sequences resulted in 10,000 most parsimonious trees (tree length = 538; consistency index = 0.6636; retention index = 0.8697). The 50% MP majority-rule consensus tree was identical to the tree obtained from BI with the exception of some nodes presenting different statistical support. The tree illustrated in the Figure is a BI tree of posterior probabilities (PP) above and bootstrap support (BS) below the branches.

In the Figure, all of the *Salvia* accessions formed 3 major clades with high support corresponding to the various distributions (100%, 99%, and 94% PP, respectively). Clade I consisted of 16 species, 1 variety, 1 form, and 7 accessions of *S. miltiorrhiza* in subgen. *Salvia* and subgen. *Sclarea*, and all of these are from China, mainly from southwestern China (Old World).

Figure. The strict consensus tree of 2 most parsimonious (MP) trees inferred from the ITS sequences of 32 taxa. Numbers with bold above nodes are Bayesian posterior probability values (PP) ≥90%; numbers below nodes are bootstrap values (BS) ≥50%; scale bar at the lower left corner indicates 10 substitutions per 100 bases; different clades are given on the right.

Clade II consisted of *S. aegyptiaca* and *S. aristata*, and these are distributed in the Mediterranean region (Old World). Clade III comprised the 3 species of *S. apiana*, *S. patens*, and *S. splendens*, and all of these are from the Americas (New World).

In Clade I, it was obvious that 3 subclades (Subclade A, Subclade B, and Subclade C) were formed. Subclade A consisted of 13 species, and all of them were in subgen. *Salvia* (100% PP and 83% BS). These species were: *S. tricuspis*, *S. roborowskii*, *S. omeiana*, *S. castanea*, *S. cyclostegia*, *S. brevilabra*, *S. digitaloides*, *S. cynica*, *S. flava*, *S. aerea*, *S. pauciflora*, *S. przewalskii*, and *S. evansiana*. Subclade B included species of subgen. *Sclarea* (100% PP and 96% BS), such as *S. cavaleriei* var. *simplicifolia*, *S. yunnanensis*, *S. bowleyana*, and *S. miltiorrhiza* f. *alba*, together with 7 accessions of *S. miltiorrhiza* from different distributions. *S. plebeia* alone formed Subclade C.

Discussion

Phylogenetic relationships of *Salvia* in different distributions

The genus *Salvia* consists of species that are distributed widely all over the world and a large number of endemic species. Alziar (31) recognized

that *Salvia* has undergone marked species radiations in 3 regions of the world: Central and South America, Central Asia/Mediterranean, and Eastern Asia. In this study, all of the *Salvia* accessions formed 3 major clades based on nrDNA ITS sequences. The 3 *Salvia* clades were distinguished by 3 main geographical distributions. Based on the results of this study, all species in Clade I were distributed in China, and these species belonged to subgenera *Salvia* and *Sclarea*, which are Old Word representatives. The individual clade indicated that the native species in China had close relationships (100% PP). The species in Clade II were only from the Mediterranean region and were also Old World. There were closer relationships between Clade I and Clade II as compared with Clade III. The species of *S. apiana*, *S. patens*, and *S. splendens* were grouped in Clade III from the Americas (New World). The results show that *S. miltiorrhiza* in China can be clearly distinguished from the species from abroad.

An earlier phylogenetic study (19) suggested that *Salvia* was not monophyletic but comprised at least 2 and possibly 3 distinct lineages. Epling (32) found that the New World group also had a high proportion of endemic small species groups, although these apparently originated from a common Old World ancestor. However, the derivations of the Asian species were much less clear because there is not as much information available about the species in this region (33). Pertaining to systematic and evolutionary issues, our study showed that *Salvia* is polyphyletic; the Old World species (Clade I and Clade II) have common origins and the New World species have another origin.

Phylogenetic relationships of the *Salvia* species in China

There were a few studies on the phylogeny of *Salvia* species from China based on nrDNA ITS sequences; however, most of the *Salvia* species in China have not been studied on this basis (21-23). According to BI analysis in the present study, there were obvious differences in molecular phylogeny between subgen. *Salvia* and subgen. *Sclarea*. In Subclade A, *S. evansiana*, *S. przewalskii*, *S. pauciflora*, *S. aerea*, *S. flava*, *S. cynica*, *S. digitaloides*, *S. brevilabra*, *S. cyclostegia*, *S. castanea*, *S. omeiana*, *S. roborowskii*, and *S. tricuspis* were clustered together; all belong to subgen. *Salvia*. It is suggested that there are closer relationships among these species. There was slight diversity between the samples of *S. miltiorrhiza* from different areas and *S. miltiorrhiza* f. *alba*; they clustered together first, and then clustered with *S. bowleyana*, *S. cavaleriei* var. *simplicifolia*, and *S. yunnanensis*. All were in Subclade B, which belonged to section Drymosphace of subgen. *Sclarea*. The high PP (100%) was in agreement with the morphological classification. The accessions of *S. miltiorrhiza* from different regions in China and *S. bowleyana* were clustered together, which suggested that *S. bowleyana* was close to *S. miltiorrhiza*. *S. plebeia* belonged to section Notiosphace of subgen. *Sclarea* and formed Subclade C alone. The phyletic positions of subgenera inferred by ITS analysis were comparable with those of traditional classifications. This differs from the opinion of Xu et al. (23). However, the taxonomy of *S. plebeia*, which is significantly different morphologically from other species in subgen. *Sclarea*, is still doubtful due to limited ITS sequence information, and further study is required.

Germplasm candidates for *S. miltiorrhiza*

S. miltiorrhiza, known as Danshen in China, is regarded as an important Chinese traditional herbal medicine with a history of use stretching back over 2000 years. Danshen could address many coronary heart diseases, particularly angina pectoris and myocardial infarction (34). With the decline in herbal resources, finding, identifying, and evaluating new germplasm resources from the closely related taxa in *Salvia* is necessary (8). In this study, ITS phylogenetic analysis clearly showed that *S. miltiorrhiza* f. *alba*, *S. bowleyana*, *S. cavaleriei* var. *simplicifolia*, and *S. yunnanensis* were the taxa most closely related to *S. miltiorrhiza*. These species could be regarded as new gene germplasm candidates for Danshen.

In recent years, more studies on chemical composition have been carried out on *S. przewalskii*, which belongs to subgen. *Salvia*. Researchers found many compounds that were similar to those in *S. miltiorrhiza*, and a number of new compounds were separated from *S. przewalskii* (35-37). In addition, many studies showed that the contents of the active components in *S. miltiorrhiza*, such as total tanshinone and cryptotanshinone (38), were sometimes present in smaller amounts than in *S. przewalskii*, a plant

recorded in literature documenting folk usage. In fact, according to information from the ITS sequences, *S. przewalskii* should be treated as a new breed, and an investigation of its chemical constituents, medicinal functions, and cytological and genetic character are being carried out in our laboratory.

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