Development of an Authentication System for Genuine Radix Salviae Miltiorrhizae (*Salvia miltiorrhiza*) Using SNP Markers

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Abstract

Radix Salviae Miltiorrhizae (*Salvia miltiorrhiza*) is one of the most widely used traditional Chinese medicinal materials. However, the dried roots of *Salvia przewalskii, Salvia yunnanensis, Salvia sinica*, and *Salvia digitaloides* are usually mistaken for Radix Salviae Miltiorrhizae due to their similarity in appearance. Although several efforts have been made to develop molecular markers for *Salvia* species, the discrimination of Radix Salviae Miltiorrhizae from its adulterants is still an unresolved problem. In order to develop a simple and reliable method for identifying genuine Radix Salviae Miltiorrhizae, the nuclear internal transcribed spacer region was analyzed to exploit DNA polymorphisms, and a host of single nucleotide polymorphism sites were discovered among five *Salvia* species. By introducing additional mismatches, allele-specific primers were designed and a multiplex polymerase chain reaction was conducted for authentication of Radix Salviae Miltiorrhizae. The established multiplex allele-specific polymerase chain reaction system enabled the simultaneous identification of four *Salvia* species in one reaction. Therefore, a simple and reliable method for molecular authentication of *S. miltiorrhiza* from its adulterants was established, and the present method should be an important tool to complement morphological and chemical analysis for quality control of Radix Salviae Miltiorrhizae.

Abbreviations

ITS:	internal transcribed spacer
SNP:	single nucleotide polymorphism
RAPD:	random amplified polymorphic DNA
ISSR:	inter-simple sequence repeats
RFLP:	restriction fragment length polymorphism
AFLP:	amplified fragments length polymorphism
SRAP:	sequence related amplified polymorphism
CoRAP:	conserved region amplification polymorphism

Key words
Salvia miltiorrhiza · Lamiaceae · Radix Salviae Miltiorrhizae · ITS · SNP · allele-specific PCR

Salvia miltiorrhiza Bunge (Lamiaceae) is one of the most widely used traditional Chinese medicinal plants. The dried root and rhizome of *S. miltiorrhiza*, known as Radix Salviae Miltiorrhizae (Danshen in Chinese), have been used for the treatment of various cardiovascular and cerebrovascular diseases for hundreds of years in Asian countries [1]. Modern pharmacologic studies have demonstrated that Radix Salviae Miltiorrhizae has additional biological activities including anticancer, anti-inflammatory, antioxidant, antimicrobial, and antivirus [2]. Due to its comprehensive and remarkable pharmacological activities, especially its antioxidant capacity, Radix Salviae Miltiorrhizae has become a widely accepted health-promoting product and worldwide consumption is rapidly increasing [3].

A common problem existing in traditional Chinese medicine materials is that substitutes and adulterants are often introduced intentionally or accidentally, and Radix Salviae Miltiorrhizae is no exception. In the *Salvia* genus, only *S. miltiorrhiza* is regarded as the botanical source of Radix Salviae Miltiorrhizae in the Pharmacopoeia of People's Republic of China [4]. However, the dried roots of *Salvia przewalskii* Maxim., *Salvia yunnanensis* C.H. Wright, *Salvia sinica* Migo, and *Salvia digitaloides* Diels are usually mistaken for Radix Salviae Miltiorrhizae in some local areas in China [5]. Therefore, a simple and reliable method is clearly needed for the accurate identification of Radix Salviae Miltiorrhizae in order to ensure its clinical efficacy and safety as well as to protect consumer's rights.

Traditional methods based on morphological characteristics for authentication of Radix Salviae Miltiorrhizae are subjective and error-prone, as Radix Salviae Miltiorrhizae and its adulterants resemble each other in shape and color [6]. Chemical constituents such as tabshinone and cryptotanshinone are used as identification markers as well [7], but their contents may be affected by physiological conditions and many *Salvia* species contain similar chemical components [8]. With the development of molecular biology techniques, DNA markers have become a popular means for authentication of Radix Salviae Miltiorrhizae. Although several efforts have been made to develop molecular markers for *Salvia* species, the discrimination of Radix Salviae Miltiorrhizae from its adulterants is still an unresolved problem. In this study, we present a simple and reliable method for identifying genuine Radix Salviae Miltiorrhizae from its adulterants by using SNP markers and multiplex allele-specific PCR.

Proven to be an ideal DNA barcode for species identification [9, 10], sequences of the nuclear ribosomal ITS region have been evaluated for genotyping *Salvia* species [7, 11, 12]. But specific authentication of *S. mitiorrhiza* from its adulterants was scarcely studied, especially the use of many *Salvia* species going under the name of Radix Salviae Miltiorrhizae. Therefore, we tried to establish a simple and reliable DNA method for Radix Salviae Miltiorrhizae authentication.

The compiled ITS sequences of five *Salvia* species were registered in GenBank with accession numbers of KJ397256–KJ397260. From the multiple sequence alignment result, as shown in **Fig. 1**, a host of SNP sites exist in the ITS region among five *Salvia* species. However, an SNP site specific for *S. mitiorrhiza* was discovered neither in the ITS1 region nor in the ITS2 region among five species. Accordingly, the authentication of Radix Salviae Miltiorrhizae was conducted using the combination of mutation sites common to two or three species via multiplex allele-specific PCR.

At the nucleotide positions of 75 bp and 76 bp in ITS1, *S. miltiorrhiza, S. sinica,* and *S. yunnanensis* contain nucleotide A and T, while *S. przewalskii* and *S. digitaloides* were replaced with T and C in the same positions. These two mutation sites were chosen to design primer SMR for the former three species mentioned above. Primer SYR was designed for both *S. miltiorrhiza* and *S. yunnanensis* based on the SNP site at the nucleotide position of 538 bp, with an additional mismatch of A for T. Similarly, primer SSR was designed for specific authentication of *S. sinica*, with a substitution of G for T, to ensure reliable discrimination (**Table**

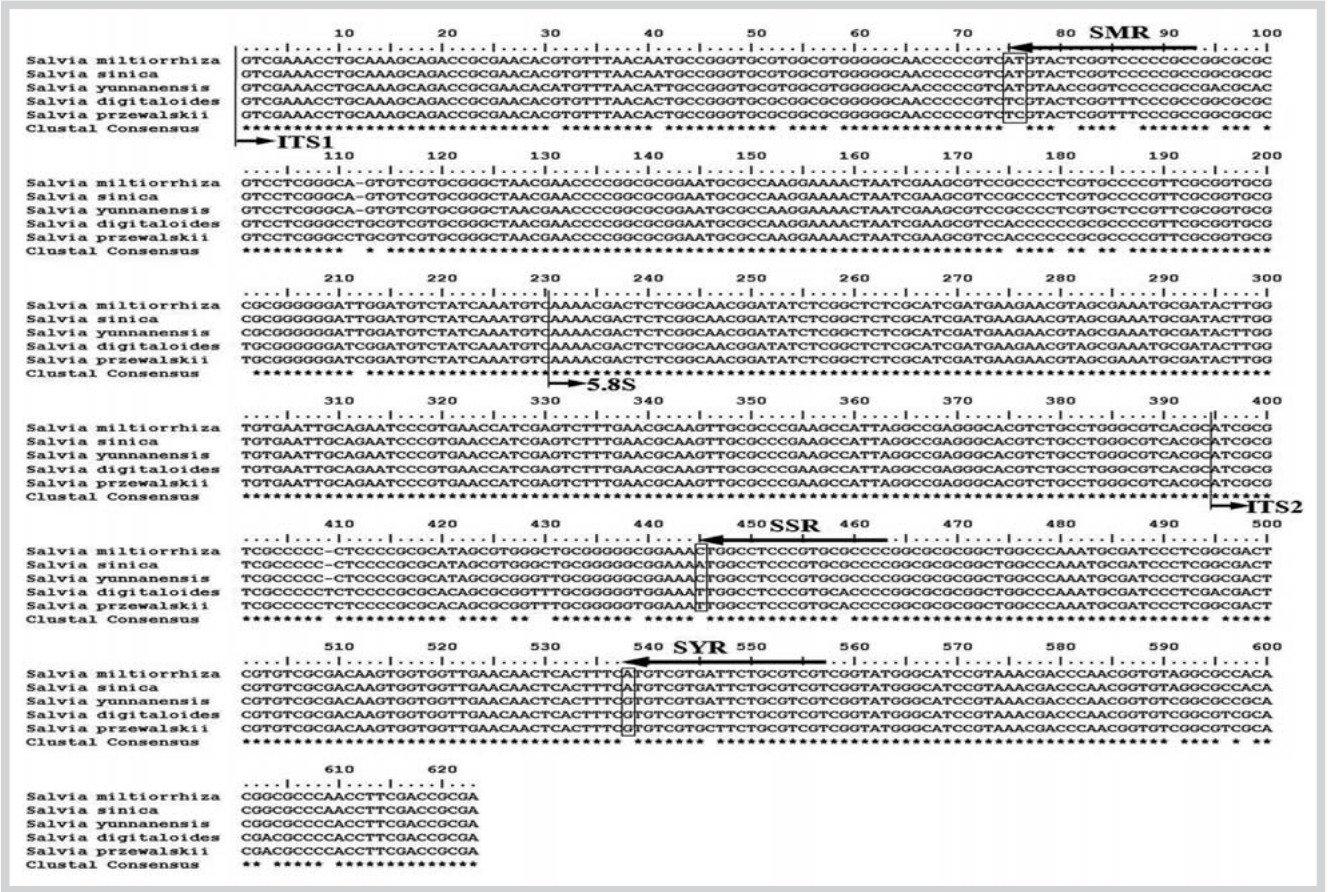

Fig. 1 Comparison of ITS sequences of five *Salvia* species.

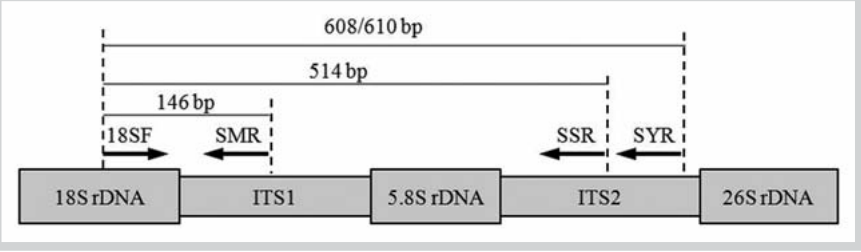

Fig. 2 Positions of primers used in this study.

1). Additionally, dimmers, hairpins, and false primings are avoided as far as possible to minimize false positive results and ensure amplification efficiency [13].

With the combination of these three primers, as well as the forward primer 18SF, multiplex allele-specific PCR was performed to identify *S. miltiorrhiza* from its adulterants. As expected, the combination of three primer pairs generated different fragment patterns for different *Salvia* species (**Fig. 3**). *S. miltiorrhiza, S. sinica,* and *S. yunnanensis* yielded their specific amplicons of 146 bp, which represents their AT allele. Likewise, only *S. yunnanensis, S. przewalskii*, and *S. digitaloides* could be amplified by primer SYR, with their bands of 608 bp, 610 bp, and 610 bp, respectively. The fragments of 514 bp, from which *S. sinica* can be differentiated among the other four species, were produced by primer SSR, representing its specific A allele. From the fragment patterns, *S. miltiorrhiza* can be easily discriminated from its four adulterants by its single 146 bp fragment, which differs from the 610 bp specific bands of *S. przewalskii* and *S. digitaloides*.

Table 1 Primers used in this study.

Primer name	Nucleotide sequence (5′→3′)	Position in ITS
18SF	GGAAGTAAAAGTCGTAACAAGG	
26SR	TCCTCCGCTTATTGATATGC	
SMR	GGCGGGGGACCGAGTACAT	93–75
SSR	GGGTCGCACGGGAGGC**T**AT (G→T)	463–445
SYR	ACGACGCAGAATCACGAC**T**C(A→T)	557–538

Bold nucleotides are additional mismatches introduced intentionally

Although *S. sinica* and *S. yunnanensis* also generated the 146 bp fragment, they can be clearly identified by their specific amplicons of 514 bp and 608 bp, respectively. Therefore, the established multiplex allele-specific PCR system is effective for authentication of *S. miltiorrhiza* from its adulterants.

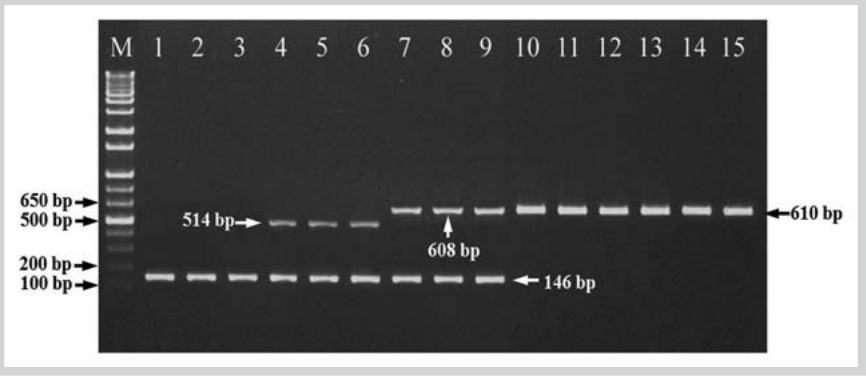

Fig. 3 Multiplex PCR products of five *Salvia* species. Lane M: 1 kb DNA ladder; lanes 1–3: *S. mitiorrhiza*; lanes 4–6: *S. sinica*; lanes 7–9: *S. yunnanensis*; lanes 10–12: *S. digitaloides*; lanes 13–15: *S. przewalskii*.

Table 2 Plant samples used in this study.

Species	Locations	Voucher specimen	Genbank accession no.
S. miltiorrhiza	Shandong, China	SM201301	KJ397256
S. miltiorrhiza	Henan, China	SM201302	
S. miltiorrhiza	Sichuan, China	SM201303	
S. sinica	Anhui, China	SS201301	KJ397257
S. sinica	Zhejiang, China	SS201302	
S. sinica	Hubei, China	SS201303	
S. yunnanensis	Yunnan, China	SY201301	KJ397258
S. yunnanensis	Yunnan, China	SY201302	
S. yunnanensis	Sichuan, China	SY201303	
S. digitaloides	Yunnan, China	SD201301	KJ397259
S. digitaloides	Yunnan, China	SD201302	
S. digitaloides	Yunnan, China	SD201303	
S. przewalskii	Sichuan, China	SP201301	KJ397260
S. przewalskii	Yunnan, China	SP201302	
S. przewalskii	Yunnan, China	SP201303	

Due to the potential confusion over which variety of the medicinal plant is called for in a given situation, accurate authentication is critical for its effective and safe application. This is particularly true in the context of the *Salvia* genus in which many species are usually mistaken for Radix Salviae Miltiorrhizae in some local areas in China. Nowadays, molecular technology provides an independent approach for the authentication of medicinal plants. Several DNA molecular markers have been developed for *Salvia* species, including RAPD [14], AFLP [15], PCR-RFLP [16], SRAP [17], CoRAP [18], and ISSR [19]. However, these methods are not suitable for developing a simple and reliable method to identify Radix Salviae Miltiorrhizae,mainly due to their sensitivity to PCR temperature or tedious visualization procedures.

Although SNP site specific for *S. miltiorrhiza* does not exist in the ITS region, molecular authentication of Radix Salviae Miltiorrhizae from its adulterants was achieved by SNP genotyping with a multiplex allele-specific PCR. The introduction of additional mismatches ensured a reliable discrimination between alleles and multiplex PCR-enabled identification of Radix Salviae Miltiorrhizae. Besides, the established multiplex allele-specific PCR system enables the simultaneous identification of four *Salvia* species in one reaction, requires no sequencing analysis of PCR products, and the authentication of Radix Salviae Miltiorrhizae only needs a simple agarose gel-based assay after PCR. Compared with the developed DNA markers for *Salvia* species, the present method is simple, reliable, and cost-effective. The ITS region appears in multiple copies in the nuclear genome, and PCR amplification is thus not appreciably affected by DNA degradation. Therefore, this method is strongly recommended for the authentication of processed Radix Salviae Miltiorrhizae materials. In this study, a simple and reliable method for molecular authentication of *S. miltiorrhiza* from its adulterants was established by SNP genotyping and multiplex PCR. Because chemical compositions are usually affected by environmental and processing factors, the present method should be an important tool to complement morphological and chemical analyses for quality control of Radix Salviae Miltiorrhizae.

Materials and Methods

Plant materials and DNA isolation

The plant samples listed in **Table 2** were collected from different regions of China and identified by Prof. Haizhu Jin, College of Life Science, Yantai University. All the voucher specimens were deposited in the Research Institute of Food Science and Technology, Yantai University. The plant leaves and medicinal materials were frozen in liquid nitrogen and ground into a fine powder. Genomic DNA was then isolated and purified using a plant DNA isolation kit (Exgene Tissue SV, GeneAll), according to the manufacturer's instructions.

Polymerase chain reaction amplification of internal transcribed spacer region and multiple sequence alignment

The ribosomal ITS region was amplified using forward primer 18SF (5′-GGAAGTAAAAGTCGTAACAAGG-3′) and reverse primer 26SR (5′-TCCTCCGCTTATTGATATGC-3′). The 20 µL PCR reaction mixture contained 20 ng of template DNA, 0.5 µM of each primer,

and 10 µL of 2X PreMix DNA polymerase (Genotech). The PCR amplification profile consisted of 1 predenaturation cycle of 4 min at 94 °C, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s, extension at 72 °C for 30 s, and a final extension at 72 °C for 5 min. PCR products were analyzed by electrophoresis on 1.0% agarose gel and then purified with a PCR DNA Purification Kit (GeneAll) as described in the manufacturer's instructions. DNA was sequenced in both directions on an automatic DNA sequencer (ABIPRISM 3700) by using a BigDye Terminator Cycle Sequencing Kit (Applied Biosystems). DNA sequences were assembled using SeqMan software, and multiple sequence alignments were conducted using the ClustalW2.0 program [20].

Design of specific primers

Primers were designed based on the SNP sites detected from the multiple alignment result. The substitutions of G for T in primer SSR and A for T in primer SYR were introduced deliberately to ensure absolute specificity and reliable discrimination between alleles [21]. Primer SMR was also designed to perform multiplex PCR with SSR and SYR for Radix Salviae Miltiorrhizae authentication. The locations of primers used in this study are shown in **Fig. 2**.

Multiplex allele-specific polymerase chain reaction

Three new designed primers, together with the ITS forward primer 18SF, were used for authentication of Radix Salviae Miltiorrhizae by multiplex PCR. The 20 µL PCR reaction mixture consisted of 50 ng of DNA template and 10 µL of 2X Premix DNA polymerase (Genotech). The concentrations of four primers, 18SF, SMR, SSR, and SYR, were 0.5 µM, 0.125 µM, 0.3 µM, and 0.5 µM, respectively. Multiplex PCR was performed using 1 cycle of 4 min at 94 °C, 33 cycles of 30 s at 94 °C, 30 s at 62 °C, and 30 s at 72 °C with a final extension at 72 °C for 5 min.

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Conflict of Interest

The authors declare of no conflict of interest.

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