Molecular Authentication of the Ethnomedicinal Plant *Sabia parviflora* and Its Adulterants by DNA Barcoding Technique

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Key words
- *Sabia parviflora*
- Sabiaceae
- DNA barcoding
- ethnomedicinal plant
- authentication

Abstract

Sabia parviflora Wall. ex Roxb. is a traditional herb widely used by Chinese people, especially by the Buyi ethnic group which resides in Guizhou and Yunnan provinces. According to the Chinese Ethnic Pharmacopeia, the species is commonly used for soothing the liver and for the treatment of icteric hepatitis, hemostasis, and inflammation. However, due to the similar morphological characters of *Sabia* species and higher market demands, there are many substitutes and adulterants of *S. parviflora*. In this study, the differential identification of 6 *Sabia* species and 7 adulterants were investigated through DNA sequence analysis of three candidate DNA barcodes (*trnH-psbA, rbcL-a, matK*). Based on sequence alignments, we concluded that not only the *trnH-psbA* spacer sequence can distinguish *S. parviflora* from other *Sabia* species, but the *matK* + *rbcL-a* sequences also can differentiate it from the substitutes and adulterants. The classification tree of all samples based on *rbcL-a* sequences indicated that the *rbcL* region can identify samples into a family/genus level. Our results suggest that the three candidate barcodes can be used for the identification of *S. parviflora* and to distinguish it from common substitutes or adulterants.

Abbreviations

rbcL:	ribulose-1,4-bisphosphate carboxylase large subunit
trnH-psbA:	*trnH-psbA* intergenic spacer
matK:	ribosomal RNA maturase
SNP:	single nucleotide polymorphism
MSA:	multiple sequence alignment

Supporting information available online at http://www.thieme-connect.de/ejournals/toc/plantamedica

received August 5, 2010
revised Sept. 25, 2010
accepted October 1, 2010

Bibliography
DOI http://dx.doi.org/10.1055/s-0030-1250468
Published online October 26, 2010
Planta Med 2011; 77: 492–496

ISSN 0032-0943

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Introduction

Ethnomedicine refers to the medical plants or other organisms used by indigenous people. It is an essential component of cultural diversity and cultural heritage and plays a significant role in biodiversity conservation and sustainable uses [1]. However, the traditional medicine knowledge is vanishing dramatically because of the impact from modern medicine and economic development in Chinese minority regions [2,3]. According to the Chinese Ethnic Pharmacopeia, *Sabia parviflora* Wall. ex Roxb. (Sabiaceae) is a traditional medical plant used by the Buyi ethnic group and called Qing-feng-teng. This ethnomedicine is an important herb to deal with icteric hepatitis, hemostasis, inflammation and rheumatism [4], and traumatic injury as well as for soothing the liver [5]. However, *S. fasciculata, S. latifolia, S. yunnanensis, S. dielsii, S. swinhoei, Cocculus trilobus, Euonymus fortunei, E. fortunei* var. *radcans, Hedera nepalensis* var. *sinensis, Paederia yunnanensis, P. scandens* var. *tomentosa*, and *Sinomenium acutum* [6] are often misused or intentionally introduced as *S. parviflora* in Buyi societies due to the morphological similarity and high herb market demands, particularly in the form of dried slices. A few criteria and methods have been developed to authenticate *S. parviflora*, which rely mainly on morphological [7], physical, and chemical assays such as histology, micrography [4], and analysis of chemical compounds [8,9]. The identification guidelines of these methods are based on genetic phenotypes such as appearance, histological structure, and chemical components, which are susceptible to intrinsic and extrinsic factors [10]. Moreover, these methods depend on the availability of experts and on too

many or expensive laboratory equipments [11]. Therefore, a reliable authentication of *S. parviflora* is not only essential for the prevention of misuse, but also critical to the conservation of the ethnomedicinal culture.

DNA barcoding involves sequencing a standard region of DNA as a tool for species identification [12]. The mitochondrial gene cytochrome *c* oxidase I (COI) has already provided a reliable, cost-effective, and accessible solution to animal species identification [13]. In plants, several candidate DNA barcodes have been proposed, four are portions of a coding gene (*matK, rbcL, rpoB*, and *rpoC1*), three are noncoding spacers (*atpF-atpH, trnH-psbA*, and *psbK-psbI*), and some are multiregion approaches to barcoding plants, such as the combinations of *rbcL* and *matK* [12] and *trnH-psbA* + *rbcL-a* [14]. Recently, DNA barcoding techniques began to be applied in traditional Chinese medicine (TCM) authentication [15,16]. Therefore, selecting candidate DNA barcodes for medicinal plant authentication has been demonstrated to be an effective approach.

In this paper, we describe the analysis of three candidate barcodes: the noncoding *trnH-psbA* intergenic spacer, the coding *matK* gene, and a subset of the *rbcL* molecule (termed *rbcL-a*) of *S. parviflora* and its substitutes and adulterants by PCR and subsequent sequence analysis. The results show that the alignment of the *trnH-psbA* spacers should allow the discrimination of the target plant species from its close relatives and possible adulterants. In addition, plastid regions *rbcL-a* combined with *matK* can also distinguish them. The classification tree based on *rbcL-a* sequences of all samples is also discussed.

Materials and Methods

Plant material

The plant samples were collected from Yunnan, Sichuan, Guizhou, and Shaanxi provinces (**Table 1**). All specimens were examined and identified by Dr. G. W. Hu and the authors. The specimens were registered before being deposited in the Herbarium of the Kunming Institute of Botany, Chinese Academy of Sciences (KUN).

Table 1 Samples used in this study.

Sample	Scientific name	Family	Sample origin	Voucher
SP	*Sabia parviflora*	Sabiaceae	Guizhou, China	XY01-XH (W)
SF	*Sabia fasciculata*	Sabiaceae	Yunnan, China	XY02-CH (W)
SL	*Sabia latifolia*	Sabiaceae	Yunnan, China	XY03-KY (W)
SD	*Sabia dielsii*	Sabiaceae	Yunnan, China	XY05-PF (W)
SY	*Sabia yunnanensis*	Sabiaceae	Yunnan, China	XY04-YN (W)
SS	*Sabia swinhoei*	Sabiaceae	Sichuan, China	XY06-JY (W)
HNS	*Hedera nepalensis* var. *sinensis*	Araliaceae	KIB, China	WZ02-ZH (C)
EFR	*Euonymus fortunei* var. *radcans*	Celastraceae	KIB, China	WZ01-PX (C)
EF	*Euonymus fortunei*	Celastraceae	KIB, China	TY01-FF(C)
PY	*Paederia yunnanensis*	Rubiaceae	KIB, China	WZ03-JST (C)
PST	*Paederia scandens* var. *tomentosa*	Rubiaceae	Yunnan, China	WZ03-JS (W)
SA	*Sinomenium acutum*	Menispermaceae	Shaanxi, China	XY08-Q (C)
CT	*Cocculus trilobus*	Menispermaceae	Yunnan, China	XY07-F (C)

KIB stands for Kunming Institute of Botany, Kunming, China; "W" represents wild samples while "C" represents cultivated samples

Table 2 Primers and reaction conditions used in the study.

Locus	Name of primer	Primer sequence 5′ – 3′	Reaction condition
matK	KIM 3F	CGTACAGTACTTTT-GTGTTTACGAG	94 °C 1 min 94 °C 30 s, 52 °C 20 s, 72 °C
	KIM 1R	ACCCAGTCCATCTGGA-AATCTTGGTTC	50 s, 35 cycles 72 °C 5 min
rbcL-a	rbcLa_f	ATGTCACCACAAACAG-AGACTAAAGC	95 °C 4 min 94 °C 30 s, 55 °C 1 min, 72 °C
	rbcLa_rev1	GTAAAATCAAGTCCAC-CRCG	1 min, 35 cycles 72 °C 10 min
trnH-psbA	psbA3′f	GTTATGCATGAACG-TAATGCTC	95 °C 4 min 94 °C 30 s, 55 °C 1 min, 72 °C
	trnHf	CGCGCATGGTGGA-TTCACAATCC	1 min, 35 cycles 72 °C 10 min

DNA extraction, amplification, and sequencing

Genomic DNA was extracted from fresh or silica gel-dried leaves by use of the modified CTAB method according to Doyle and Doyle (1987) [17]. Each sample was repeated twice.

Amplifications of the *trnH-psbA, matK*, and *rbcL-a* regions of the cpDNA were obtained. The primer pair names, primer sequences, and reaction conditions used in the present study are listed in **Table 2** according to the references [12,14].

Polymerase chain reaction (PCR) amplifications of the three candidate DNA barcode genes carried out in a Peltier Thermal Cycler (BioRad Lab, Inc.) were performed in a 20 µL reaction mixture containing 1 × *Taq* buffer [50 mM $(NH4)_2SO_4$; 75 mM Tris-HCl (pH 8.3); 50 mM KCl; 0.001 % gelatin]; 2.5 mM $MgCl_2$, 0.4 mM of dNTPs in equimolar ratio, 0.5 µM of each primer (Synthesized by Sangon Co.), 1.0 U of *Taq* DNA Polymerase (TaKaRa Biotechnology Dalian Co., Ltd.), and 1 µL of genomic DNA (25–30 ng). The *matK* region PCR conditions were the same as the former conditions except for the addition of 4 % DMSO and 0.2 µL 0.1 mg/mL BSA.

PCR products were examined using 2 % agarose gel electrophoresis in 1 × TBE (Tris-Boric acid-EDTA) buffer with 2 µL SYBR Safe (Invitrogen) DNA stain at 70 V for ~ 45 min and analysis in a Bio-Rad Illuminator with ChemiDocXRS Camera and Quantity One software. Purifying and bidirectional sequencing were completed by Sangon Co., Ltd.

Sequence alignment and analysis

Sequences were assembled and aligned by the CLUSTALX program (Version 1.83) and adjusted manually in BioEdit (Version 7.0.5). The nucleotide sequences data of the partial *trnH-psbA* spacer, *rbcL-a* gene, and *matK* gene were deposited in the Genbank nucleotide sequence databases with the PCR product size and accession numbers reported in **Table 3**. All sequence distances were calculated with MEGA (4.0 Version). Additionally, a bootsrap NJ (Neighbor-Joining) tree was calculated according to Kimura's 2-parameter method with bootstrap testing of 1000 replicates.

Supporting information

Sequence divergences among 6 *Sabia* species and multiple sequence alignment (MSA) of the *trnH-psbA, matK*, and *rbcL-a* sequences are available as Supporting Information.

Table 3 PCR product size and accession number of *Sabia parviflora* and other related species.

Sample	*trnH-psbA*		*rbcL-a*		*matK*	
	Size (bp)	Accession number	Size (bp)	Accession number	Size (bp)	Accession number
SP	421	HM755921	503	HM755929	734	HM755902
SF	418	HM755919	503	HM755931	708	HM755904
SL	428	HM755920	494	HM755933	725	HM755906
SY	413	HM755923	509	HM755932	744	HM755905
SD	429	HM755918	495	HM755934	723	HM755907
SS	430	HM755922	506	HM755930	722	HM755903
CT	576	HM755913	558	HM755925	747	HM755911
EF	422	HM755915	480	HM755928	741	HM755909
EFR	439	HM755914	507	HM755927	736	HM755908
HNS	436	HM755916	501	HM755937	715	HM755910
PY	310	HM755917	486	HM755936	–	–
PST	–	–	514	HM755935	–	–
SA	656	HM755924	480	HM755926	741	HM755912

A hyphen (–) indicates that sequencing of the PCR product failed

Results

For a DNA-based identification of *Sabia parviflora*, three candidate DNA barcode sequences were submitted to multiple sequence alignment (MSA): *trnH-psbA, matK*, and *rbcL-a*. The alignment of all sequences can be found in the Supporting Information.

The *trnH-psbA* intergenic spacers of all samples were successfully amplified from total DNA and sequenced (except for the sample *Paederia scandens* var. *tomentosa*). The results showed that the *trnH-psbA* intergenic spacer of all species were 310 to 656 bp in length (**Table 3**). The interspecies percentages of nucleotide differences in the *trnH-psbA* intergenic spacers of all six *Sabia* species range from 0.24% to 7.70%, with an average of 4.11%. DNA sequence of *S. parviflora* and other related species were determined to obtain a spectrum wide enough to differentiate species of various sections as well as to show interspecific polymorphisms (**Fig. 1**; **Fig. 1S A**, Supporting Information). Therefore, it is feasible to use sequence alignment to accurately distinguish *S. parviflora* from the adulterants and closely related species.

The *matK* sequences of all samples, except for *Paederia scandens* and *P. scandens* var. *tomentosa* sequences, ranged between 747 and 708 bp in length (**Table 3**). The sequence divergence among *Sabia parviflora* and its substitutes varied from 0.00% to 0.86%. In contrast, sequence divergence among *S. parviflora* and its adulterants were from 0.29% to 24.45%. The sequence divergence among different *Sabia* species indicated that the *matK* gene sequences are highly conserved. However, there are nine base substitutions located at positions 12, 22, 77, 367, 497, 516, 520, 581, and 733 (**Fig. 1**; **Fig. 1S B**, Supporting Information).

For the *rbcL-a* region, various tested samples showed approximately an equal size of the PCR product. Excluding the primer flanking sites, the sizes of the *rbcL-a* region were from 480 to 558 bp (**Table 3**). The multiple sequence alignment with a total of 565 examined sites, revealed 88 variable sites (**Fig. 1**; **Fig. 1S C**, Supporting Information). The sequence divergence among *Sabia parviflora* and its substitutes were from 0.00% to 0.82%. In contrast, sequence divergence among *S. parviflora* and its adulterants were from 0.42% to 12.06%. The spacer domain among all *Sabia* species is also highly conserved (~ 99.68%; **Table 1S**, Supporting Information). However, sequence variations were also revealed: there are five base substitutions located at positions 269, 399, 403, 429, and 561. Combining with five SNP of the *rbcL-a* region and nine SNP of the *matK* region of *Sabia* species sequences, *S. parviflora* could be distinguished at the DNA level.

For the purpose of finding a quick, easily used, and accurate system of identification and to determine if the candidate barcode regions can be used for species identification, a NJ (Neighbor-Joining) tree was calculated including the *rbcL-a* sequences of all sample and loci. Based on the NJ tree, the 13 species were divided into five major clades (**Fig. 2**), and *Sabia parviflora, S. fasciculata, S. latifolia, S. yunnanensis, S. dielsii*, and *S. swinhoei* were found to be closely related (bootstrap value is 100%). On the other hand, *Cocculus trilobus* and *Sinomenium acutum* together with *Euonymus fortunei* and *E. fortunei* var. *radcans; Puederia yunnanensis* and *P. scandens* var. *tomentosa* together with *Hedera nepalensis* var. *sinensis* were separated with bootstrap support of 70% and 85%, respectively.

Discussion

Recently, the applicability of DNA barcoding has been widely used in phylogenetic research, cryptic plant species identification, traditional medical plant authentication, and culture diversity conservation.

A suitable barcode must exhibit high interspecific but low intraspecific divergence [18]. Our research shows that a single-region *trnH-psbA* can distinguish *Sabia parviflora* from its substitutes and adulterants. This was supported by sequence alignment analyses, which revealed the high sequence variation to be enough for species identification. Previously, Yao et al. identified successfully 17 *Dendrobium* species and one adulterant by the investigation of the *trnH-psbA* region [16]. Song et al. reported that the *trnH-psbA* region could distinguish the 18 species of the Polygonaceae family in Chinese pharmacopoeia [15]. Vongsak et al. used the *trnH-psbA* sequencing analysis to differentiate the Thailand medical plant *Stemona tuberosa* from 5 related species [19]. Kress et al. studied the *trnH-psbA* spacer of angiosperms (for a total of 99 species, 80 genera, and 53 families), suggesting that the *trnH-psbA* region had the potential to discriminate among the largest number of plant species for barcoding purposes [20]. Our barcoding results support those from other researches who con-

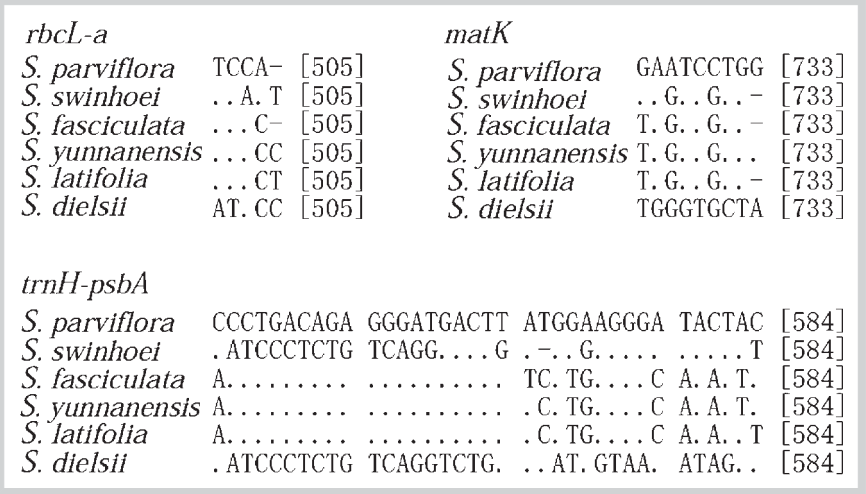

Fig. 1 Representative sequence alignment of the three barcode regions of 6 different *Sabia* samples. Dots indicate identical nucleotides, dashes indicate gaps.

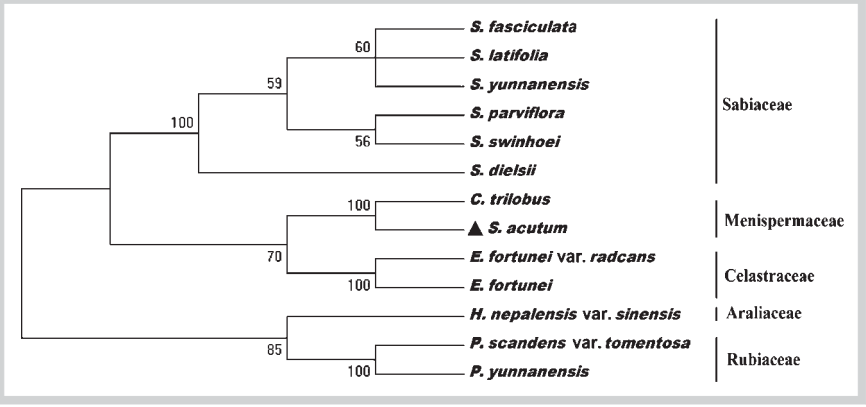

Fig. 2 Classification tree of partial *rbcL* gene using the NJ method. Branch length was calculated by Kimura's 2-parameters method. Bootstrap (1000 replications) analysis was performed to estimate the confidence of the topology of the consensus tree. Capital letters denote different genus: S = *Sabia*, C = *Cocculus*, H = *Hedera*, E = *Euonymus*, ▲ S = *Sinomenium*, and P = *Paederia*. Bootstrap support values are show above branches.

sidered the *trnH-psbA* spacer to be the potential land plant barcode for species discrimination.

On the other hand, a multilocus approach may be an effective strategy for species identification in plants. Kress and Erickson [14] have recommended a two-locus barcode based on *rbcL* and the *trnH-psbA* intergenic spacer. The CBOL Plant Working Group [12] investigated the seven leading candidate plastid DNA regions, demonstrating the 2-locus combination of *rbcL* + *matK* as the plant barcode. Newmaster and Radupathy [21], in a research that investigated the utility of the *rbcL*, *matK*, and *trnH-psbA* regions of 19 *Biophytum* species, demonstrated that DNA barcoding validated several new cryptic species which were previously recognized by the aboriginal knowledge. Comprehensive studies indicated that combining more variable plastid markers provided clear benefits for resolving species, but all combinations assessed using four to seven regions had only marginally better success rates than some two or three region combinations [22]. In our study, by combining the coding gene *matK* with a portion of the coding gene *rbcL*, it was demonstrated that the sequence nucleotide variation can distinguish *S. parviflora* from other related species.

When presented with a completely unknown sample, it would be highly desirable to place it in a smaller group of taxa (i.e., within a genus). Therefore, the successful pair(s) of primers should be well designed [23]. The *rbcL* region, with the advantages of being universal, easily amplified, and sequenced in most land plants, could be applied in forensics and economic uses [24], or serve as a baseline for species discrimination [23].

In this paper, the classification tree of those relative species based on the *rbcL-a* region showed that all samples had different botanical origins. The 6 *Sabia* species, all belonging to the family Sabiaceae, were clustered into a clade, which divided into three subclades. *S. parviflora* and *S. swinhoei* clustered in one subclade, which might be ascribed to their botanical morphological similarities, i.e., foliage shape; *S. yunnanensis* and *S. latifolia* clustered together, since *S. latifolia* is a subspecies of *S. yunnanensis* [25]. *Euonymus fortunei* and *E. fortunei* var. *radcans* (Celastraceae), *Cocculus trilobus* and *Sinomenium acutum* (Menispermaceae), *Paederia yunnanensis* and *P. scandens* var. *tomentosa* (Rubiaceae) as well as *Hedera nepalensis* var. *sinensis* (Araliaceae) belong to different families. They were clustered into different clades. This result supports the evidence that the *rbcL* region can place an unidentified specimen into a family or genus [23,24].

In conclusion, the ethnomedicinal plant *S. parviflora* can be identified by a unique DNA barcoding sequence or a combination of multiple DNA barcodes. This technology is useful in providing a reliable and effective means for the differentiation of *S. parviflora* from its substitutes and adulterants.

Acknowledgements

We thank Mr. Z. Wang (Southwest Forestry University, China) for sample collection and identification and Dr. B. Long (Kunming Institute of Botany, China) for comments on the manuscript. This research was supported by the Ministry of Education of China through its 985 and 111 projects (MUC 98503-001006 & B08044), the Chinese Academy of Sciences (O92441112F & KSCX2-YW-Z-0925), the National Science Foundation of China (31070288), the Japan Society for the Promotion of Science (JSPS/AP/109080), and the Ministry of Science and Technology of China (2008FY110400-2-2).

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