Molecular Authentication of the Traditional Medicinal Plant “Lakshman Booti” (Smithia conferta Sm.) and Its Adulterants through DNA Barcoding

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ABSTRACT

Background: Smithia conferta Sm. is an annual herb widely used in Indian traditional medical practice and commonly known as “Lakshman booti” in Sanskrit. Morphological resemblance among the species of genus Smithia Aiton. leads to inaccurate identification and adulteration. This causes inconsistent therapeutic effects and also affects the quality of herbal medicine. Aim: This study aimed to generate potential barcode for authentication of S. conferta and its adulterants through DNA barcoding technique. Materials and Methods: Genomic DNA extracted from S. conferta and its adulterants was used as templates for polymerase chain reaction amplification of the barcoding regions. The amplicons were directed for sequencing, and species identification was conducted using BLASTn and unweighted pair-group method with arithmetic mean trees. In addition, the secondary structures of internal transcribed spacer (ITS) 2 region were predicted. Results: The nucleotide sequence of ITS2 provides species-specific single nucleotide polymorphisms and sequence divergence (22%) than psbA-trnH (10.9%) and rbcL (3.1%) sequences. The ITS barcode indicates that S. conferta and Smithia sensitiva are closely related compared to other species. Conclusion: ITS is the most applicable barcode for molecular authentication of S. conferta, and further chloroplast barcodes should be tested for phylogenetic analysis of genus Smithia.

Key words: DNA barcode, internal transcribed spacer, single nucleotide polymorphism, Smithia

SUMMARY

- The present investigation is the first effort of utilization of DNA barcode for molecular authentication of S. conferta and its adulterants. Also, this study expanded the application of the ITS2 sequence data in the authentication. The ITS has been proved as a potential and reliable candidate barcode for the authentication of S. conferta.

INTRODUCTION

The genus Smithia (Fabaceae) is widely distributed in temperate and tropical regions of the Old World consisting of thirty species.1 It is represented by 18 species native to Indian subcontinent2,3 and of these, 11 are endemic to India. Smithia conferta Sm. is an annual herb used in Indian Pharmacopoeia as crude drug. It is commonly known as “Lakshman booti” in Sanskrit4 and widely used in the traditional medical practice.

The Lakshman booti is used in the treatment of biliousness, ulcer, rheumatism, laxative, tonic, uterine trouble such as gravel along with regulating fertility, and also in sterility problems in women.5,6 The leaf powder mixed with honey was advised for treating cholera and its ointment is used to cure elephantiasis.7,8 It is also used to remove the effects of old age and wrinkles.9,10 The morphological resemblance among the species of genus Smithia Aiton. leads to inaccurate identification and adulteration. This causes inconsistent therapeutic effects and also affects the quality of herbal medicine. Raising its demand in traditional and folk medicines consequently, issues such as substitution, adulteration, and low-quality products create bottleneck toward authenticity. Therefore, there is an urgent need to develop a reliable and quick method for its identification. The DNA-based approach seems to be appropriate which provides a means of assessing the accuracy of the taxonomic treatment. The multi-focus approach based on the chloroplast and nuclear genome has been proposed and accepted for barcoding of land plants.11,12 The

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choice of barcoding regions is very crucial, but the DNA regions such as internal transcribed spacer (ITS), psbA-trnH, and rbcL are suggested and proposed for constructing phylogeny and authentication of medicinal plants.[14-16]. The aim of the present study was to test the discrimination power of these barcodes and establish reference database to facilitate future identification and authentication of S. conferta.

**MATERIALS AND METHODS**

**Taxon sampling**

A total of nine species of *Smithia* were collected from the Western Ghats of India. The plant material was identified, and voucher specimens have been deposited in the herbarium, Department of Botany, Shivaji University, Kolhapur, Maharashtra, India [Table 1].

**Genomic DNA isolation, amplification, and sequencing**

Genomic DNA was extracted from dried leaves using cetyltrimethylammonium bromide method[17] with minor modifications. Polymerase chain reaction (PCR) amplification of three candidate DNA barcodes was carried out in an Eppendorf Master Cycler Pro (Eppendorf India Ltd., India) employing about 20 ng of genomic DNA as a template in 25 µl reaction mixture, which contains 1 × PCR buffer, 2.0 mM MgCl₂, 0.2 mM of each dNTPs, 1 µM of each primer (Synthesized by Xcelris Labs Ltd., India), and 1.0 U of Taq DNA Polymerase (Fermentas, Thermo Fisher Scientific, India). We used three primer pairs corresponding to the barcode regions, namely, ITS (5fwd-CCCTATCATTTAGAAAGAGG; 4rev-TCCTGGATTATGATGTC; trnH rev-GCGCATGTGGATTCAAAATC), and rbcL (1 fwd-CCTATCATCTGGAATACTTAG; 724 rev-GCTAIGGAAATGGTGATATGC), and trnH (5fwd-CCCTATCATTTAGAAAGAGG; 4rev-TCCTGGATTATGATGTC; trnH rev-GCGCATGTGGATTCAAAATC), and rbcL (1 fwd-CCTATCATCTGGAATATGGTGATATGC), and rbcL (1 fwd-CCTATCATCTGGAATACTTAG; 724 rev-GCTAIGGAAATGGTGATATGC). All the PCR products were purified with Axy Prep PCR Cleanup Kit (Axygen Biotechnology, USA). DNA sequencing was performed with Z-Big Dye Terminator Cycle Sequencing version 3 (Sci Genom Labs Pvt. Ltd., Kerala, India). Sequenced product was analyzed on automated ABI 3730XL analyzer (Sci Genom Labs Pvt. Ltd., Kerala, India) using forward primer. The low-quality sequences were subjected to re-sequencing.

**Sequence alignment and analysis**

The sequences were analyzed with BLASTn of the National Centre for Biotechnology Information to confirm the identity of the samples (http://blast.ncbi.nlm.nih.gov). Raw sequence data were analyzed with the Ridom Trace Edit version 1.1.0 (Ridom GmbH, Germany). Sequences were preliminarily aligned with multiple sequence alignment tool ClustalX. The secondary structure of the ITS2 was predicted according to Koetsch et al.[18] The ITS2 region was identified and delimited based on hidden Markov model which was performed through the web server (http://its2.bioapps). For authentication and assessment of relationships among the nine *Smithia* species, unweighted pair-group method with arithmetic mean (UPGMA)-based phylogenetic tree was constructed using MEGA6 software version 6.0.6. The genetic distances were calculated using Kimura 2-parameter distance model. The bootstrap test with 1000 replicates was applied to assess the reliability of phylogenetic trees.

**RESULTS**

**Efficiency of amplification for each DNA barcode**

The efficiency of the PCR amplification of ITS, psbA-trnH, and rbcL regions was 100%, and all the three candidate DNA regions were successfully sequenced. Sequences were submitted to the GenBank database (EMBL) and their accession numbers were listed in Table 1.

**Sequence analysis and interspecific variation among Smithia species**

The DNA sequences of the ITS regions among *S. conferta* and its adulterants were highly conservative. The sequence length varied from 676 to 678 bases, and aligned length was 681 bp [Table 2]. Out of which, 529 (77.6%) were constant, 100 (14.6%) were parsimony informative, and 52 (7.8%) were variable sites. Two single nucleotide polymorphisms (SNPs) specific to *S. conferta* were detected at the aligned positions of 234 (C → T) and 283 (A → G) [Figure 1]. The sequences of *S. conferta* and *S. sensitiva* were identical at aligned positions 49 (T), 122 (C), 165 (C), 173 (T), and 210 (C) but showed SNPs when compared with other *Smithia* species. The nucleotide frequency of G + C (56%) was observed in *S. conferta* and *S. sensitiva*. Based on these SNPs, *S. conferta* and its adulterant species were differentiated. The interspecific divergence between *S. conferta* and other *Smithia* species varied from 9.3% to 13.5%, with an average interspecific distance of 8.4%. Together with other species of genus *Smithia*, the UPGMA tree based on Kimura 2-parameter model of ITS sequence data accurately discriminated *S. conferta* and its adulterants with high bootstrap support [Figure 2a].

**Table 1: Smithia species used in this study with voucher information and GenBank accession number of barcodes**

| Taxa                  | Locality          | Voucher specimens | Latitude/longitude | Altitude (ft) | GenBank accession number |
|-----------------------|-------------------|--------------------|--------------------|---------------|-------------------------|
| *S. agharkarii* Hemadri | Borbet, Kolhapur, Maharashtra | PRK-37 | N16°30′45″/E73°33′31″ | 3207 | LM644013, LM644029, LM644045 |
| *S. bigemina* Dalzell  | Kolhapur, Maharashtra | PRK-34 | N16°40′31″/E71°15′01″ | 2231 | LM644010, LM644026, LM644042 |
| *S. conferta* Sm.      | Kolhapur, Maharashtra | PRK-33 | N16°40′24″/E74°13′09″ | 2068 | LM644009, LM644025, LM644041 |
| *S. hirsuta* Dalzell   | Borbet, Kolhapur, Maharashtra | PRK-40 | N16°30′56″/E73°53′32″ | 3207 | LM644016, LM644032, LM644048 |
| *S. purpurea* Hook     | Bhemashankar, Pune, Maharashtra | PRK-31 | N19°04′25″/E73°33′51″ | 3301 | LM644007, LM644023, LM644039 |
| *S. purpurea* Hook     | Bhemashankar, Pune, Maharashtra | PRK-32 | N19°04′25″/E73°33′81″ | 3301 | LM644008, LM644024, LM644040 |
| *S. racemosa* Wight and Arn | Kolhapur, Maharashtra | PRK-36 | N16°40′49″/E74°15′45″ | 2136 | LM644012, LM644028, LM644044 |
| *S. salsuginea* Hance  | Kargat, Thane, Maharashtra | PRK-35 | N19°08′19″/E73°25′47″ | 587 | LM644011, LM644027, LM644043 |
| *S. sensitiva* Aiton   | Gaganawada, Kolhapur, Maharashtra | PRK-38 | N16°31′57″/E73°49′50″ | 1871 | LM644014, LM644030, LM644046 |
| *S. setulosa* Dalzell  | Gaganawada, Kolhapur, Maharashtra | PRK-39 | N16°31′54″/E73°49′59″ | 2021 | LM644015, LM644031, LM644047 |
| *D. sissoo* Roxb. ex DC | -                  | -                  | -                  | -             | JX856444, JX858674, GU135159 |

ITS: Internal transcribed spacer; *S. agharkarii*; *Smithia agharkarii*; *S. bigemina*; *Smithia bigemina*; *S. conferta*; *Smithia conferta*; *S. hirsuta*; *Smithia hirsuta*; *S. purpurea*; *Smithia purpurea*; *S. racemosa*; *Smithia racemosa*; *S. salsuginea*; *Smithia salsuginea*; *S. sensitiva*; *Smithia sensitiva*; *S. setulosa*; *Smithia setulosa*; *D. sissoo*; *Dalbergia sissoo*
The sequence variations of \textit{psbA-trnH} among \textit{S. conferta} and other \textit{Smithia} species varied from 1.4\% to 7.6\%, with an average interspecific distance of 4.8\%. The sequence length varied from 338 bp to 354 bp, with aligned length of 356 bp and 10.9\% nucleotide divergence. The remarkable nucleotide frequency of G + C (32\%) was found in \textit{S. conferta}. Within \textit{psbA-trnH}, 39 (10.9\%) sites were variable, 309 (86.8\%) were conserved, 13 (3.6\%) were singleton, and 26 (7.3\%) were parsimony informative. Among the aligned sequences, two SNPs at 238 (A \rightarrow G) and 286 (T \rightarrow G) were found specific to \textit{S. conferta}. Results revealed unique 6 bp insertion (ATTTTT) between 44\textsuperscript{th} and 49\textsuperscript{th} positions. \textit{S. conferta} is distinctly recognized from its adulterant \textit{Smithia} species in UPGMA tree based on Kimura 2-parameter model [Figure 2b].

The DNA sequences of \textit{rbcL} region of \textit{S. conferta} and its adulterants were not showed much variation than the ITS and \textit{psbA-trnH} regions. The identical sequences were observed in \textit{S. conferta} and \textit{S. sensitiva}. Four SNPs, 186 (C \rightarrow T), 388 (C \rightarrow T), 634 (A \rightarrow C), and 649 (G \rightarrow A), specific to \textit{S. conferta} and \textit{S. sensitiva} were detected at the aligned sequences of \textit{rbcL}, and the sequence length was 705 bases [Table 2]. Out of which, 683 (96.8\%) were constant, 13 (1.8\%) were parsimony informative, 9 (1.2\%) were singleton, and only 22 (3.1\%) were variable sites. Very low sequence divergence (1.1\%–1.8\%) was observed among \textit{S. conferta} and its adulterants with an average interspecific distance of 1.1\%. The nucleotide frequency of G + C (42\%) was reported in \textit{S. conferta} [Table 2].

Remarkably, no single insertion or deletion was observed in all species under investigation. According to the UPGMA tree based on Kimura 2-parameter model, \textit{S. conferta} and its adulterants were placed in two different clusters with high bootstrap support and differentiated from \textit{S. sensitiva} by least SNPs [Figure 2c]. The \textit{rbcL} sequence data were unable to distinguish \textit{S. conferta} from its adulterant \textit{Smithia} species.

\textbf{Secondary structure of the internal transcribed spacer 2 sequences}

For authentication of \textit{Smithia} species, we focused not only on the divergence of the sequences of nuclear ITS region, but also used
variations in the secondary structures of ITS2. All the Smithia species had a typical 4-helix folding structure, a central ring, and four similar helices: Helices I, II, III, and IV. Moreover, helix III was longer than other helices [Figure 3]. It revealed that their variation was mainly present in helices I, III, and IV in ITS2 secondary structure. The helix II was most stable and showed pyrimidine-pyrimidine base pairs. The secondary structure of S. conferta shows that helices I and II were different from the others and the secondary structure of S. sensitiva is close to S. conferta, but it can still distinguish them at helix IV. Thus, we can distinguish S. conferta from its adulterants by comparing ITS2 secondary structures.

**DISCUSSION**

Traditionally, *S. conferta* is used in Ayurveda and has reported a wide range of biological activities, namely, laxative, diuretic, antifungal, antimicrobial, anti-inflammatory, antirheumatic, tonic, antibilious, headache, ulcers, reduces wrinkles, reduces sterility problems in women, and stimulates central nervous system. Similarly, the worm leaf paste is used to reduce bodyache.\[^{18}\] Pulliah and Naidu[^{20}] reported anti-diabetic activity of *S. conferta*. Furthermore, rendering the authentication of *Smithia* species based on morphological methods is very difficult. In recent years, adulteration of medicinal plant species is a matter of
The current investigation is the first effort of utilization of DNA standard DNA barcodes were proposed for the authentication of species. In the present investigation, unique indels were observed in S. conferta. However, indels are common and often more common than SNPs which is a limitation of rbcL. Although rbcL offers high universality, good PCR amplification, and sequencing, it was demonstrated to have insufficient sequence variation to distinguish closely related species. In this study, the lowest sequence divergence was observed in rbcL sequence data. The discriminating power of plastid regions was significantly lower than the nuclear region (ITS) because of slow rate of evolution. Results showed that ITS is one of the most appropriate and suitable barcodes with highest discrimination potential. Similarly, a previous study on authentication of medicinal plants also proved that ITS region has the potential as universal barcode. In the present study, it was reported that ITS sequences of S. conferta and S. sensitiva are identical except unique SNPs in S. conferta. S. conferta is an unique medicinal plant according to Indian Pharmacopoeia, and S. sensitiva, Smithia hirsuta, Smithia racemosa, and Smithia setulosa are used as adulterants. Based on DNA barcoding studies, it is appropriate to treat S. conferta and S. sensitiva as a distinct species. In the present investigation, the precise and standard DNA barcodes were proposed for the authentication of Smithia species. The current investigation is the first effort of utilization of DNA barcode for molecular authentication of S. conferta and its adulterants. Furthermore, this study expanded the application of the ITS2 sequence data in the authentication of medicinal plant research. Several studies used DNA barcoding methods to identify Indian medicinal plants, such as Swertia L. and Sida L. In addition, they had verified the utilization of ITS sequence for the authentication of herbal medicinal plants.

CONCLUSION

The ITS region provides enough variability over psbA-trnH and rbcL for authentication of S. conferta and its adulterants. This study provided a scientific approach, based on DNA barcoding, to accurately and rapidly identify Smithia species. The ITS has been proved as a potential and reliable candidate barcode for the authentication of S. conferta and its adulterants. The present findings also suggest that further chloroplast barcodes should be tested for phylogenetic analysis of genus Smithia.

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Conflicts of interest

There are no conflicts of interest.

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