DNA Barcoding of Withania somnifera (L) Dunal Using Trnh-Psba Gene Sequences
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DOI: 10.36348/sjls.2020.v05i10.002 | Received: 26.09.2020 | Accepted: 06.10.2020 | Published: 10.10.2020

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Abstract

Objective: In this present study, the DNA barcoding method has been applied to define and authenticate collected plant material as W. somnifera from its closely linked species at the molecular level and the organization of trnH-psbA in W. somnifera and its potential as a DNA barcode. Method: The plant samples were collected from Pannapatti, Salem district. The tissue from the leaf was extracted. Isolation of the genomic DNA was carried out using CTAB technique. Polymerase chain reaction (PCR) was carried out and amplified a specific region of the chloroplast using trnH-psbA and analyzed PCR products by gel electrophoresis. The Basic Local Alignment Search Tool (BLAST) was used to identify sequences in databases. The sequence information was used to construct a phylogenetic tree by Maximum Likelihood Method using MEGA X. This tree-building tool is used to analyze the phylogenetic relationship of W. somnifera. Results: DNA yield was good with 50 ng. The purity of the DNA was also calculated and it was 1.7. Phylogenetic tree constructed showed maximum resolved topology for internal branches of 82% bootstrap value with species-specific clusters with W. somnifera. Conclusion: W. somnifera are an indigenous plant of India and an important plant in the Indian Traditional Medicinal System. To improve and enhance the utilization of this plant species for further study as medicine, accurate, proper identification and authentication is very important.

Keywords: Cetyltrimethylammonium bromide (CTAB); DNA Barcoding; Chloroplast; molecular phylogeny; trnH-psbA.

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INTRODUCTION

The world has an estimated 300,000 plant species (IUCN, 2012), but relatively few of them can be identified based on traditional methods of plant identification [1]. Even for expert taxonomists, correct classification and description of this large number is a significant challenge. DNA barcoding has had a positive impact on the classification and recognition of species [2]. DNA barcoding is an innovative method aimed to provide a quick, accurate, and automated identification of species through the use of short and standardized gene regions [3]. The theory of DNA barcoding is derived from the small, uniform sequence of DNA can also be used to classify and differentiate between species in the tree of life. DNA barcoding does not guarantee full taxonomic resolution by using a single gene region, but it does promise proximity [4]. DNA barcoding is a powerful tool to help classify organisms with broad applications in conventional taxonomy, ecology, food analytics, forensics, and environmental science [5]. For animals, a 648-bp long Folmer region of the Cytochrome C Oxidase 1 (COI) mitochondrial gene is the barcode used with effectiveness. Nevertheless, no such universal barcode has yet been found in plants [6, 7]. Low substitution rates of mitochondrial DNA have led to the search for alternative barcoding regions. From initial investigations of plastid regions 7 leading candidates have emerged [8].

Numerous diverse plant barcode sequences were suggested by different researchers based on a single chloroplast namely rpoC1 , rpoB , rbcL, matK, trnH-psbA , atpF and some combinations sequences were also acclaimed which include rpoC1 + matK+ trnH-psbA or rpoC1 + rpoB + matK, rbcL+ trnH-psbA , matK + atpF/H + trnH-psbA and matK + atpF/H + psbK/l [9]. Four plant DNA barcode markers, rbcL, matK, trnH- psbA, and ITS2, have been developed in
In this context, the *W. somnifera* plant has been used in the world for decades to preserve health and cure more chronic diseases. However, the adulteration and use of counterfeit products as replacements have become a major concern for consumers and industry on safety and efficacy grounds. Thus, medicinal plant authentication is of the highest concern [40, 41]. Therefore, the accurate identification and collection of this medicinal herb are vital to enhancing the drug’s efficacy and biosafety. The objective of the present study is to evaluate an ideal barcode candidate for distinguishing and authenticating the species of *W. somnifera* using the *trnH-psbA* intergenic spacer region.

### METHODOLOGY

DNA Isolation, Amplification, and Sequencing of the *trnH-psbA* gene: The DNA from the sample was extracted by using modified CTAB according to Aboul-Maaty *et al.* using 100 mg of fresh leaves. The quality of isolated genomic DNA was checked electrophoretically on 0.8% TAE (Tris-acetate-EDTA) Agarose gel. The *trnH-psbA* intergenic region was amplified using the primers listed in Table 1. The PCR chain reaction was performed in 20 µl reaction mixture contained 1-X buffer, 1U Taq DNA polymerase, 0.2mM of dNTPs, 0.5µM each of the forward and reverse primers, and 50 ng of genomic DNA were added. The PCR programmed for initial denaturation at 94°C for 5 minutes, denaturation at 94°C for 45 seconds, Primer annealing at 55°C for 45 seconds, Extension at 72°C for 1 minute 20 seconds for 35 cycles, Final extension @ 72°C for 7 minutes. The PCR products were run on 1% TAE Agarose gels containing Ethidium bromide (EtBr) and visualized on a UV trans-illuminator. The sequencing was done by applied biosytems 3500 genetic analyzer using Sanger sequencing.

### Table 1: Primer Details

| Primer     | Sequence                  |
|------------|---------------------------|
| *trnH-psbA* F | ACTGCCCTTGATCCACTTGGC |
| *trnH-psbA* R | CGAAGCTCCCATCTACAAATGG |

### Data Analysis and Species Identification

The chromatograms obtained after sequencing were base called and forward and reverse sequences were trimmed-and assembled. Similarity search is an important methodology in DNA barcoding. Nucleotide blast (BLASTn) using BLAST program and Genbank nucleotide database with default parameters was performed to determine the identity and the closest known relatives of the sequences obtained. The sequences were aligned using the MUSCLE algorithm in MEGA X. The interspecific and intraspecific divergences were calculated using MEGAX software. A phylogenetic tree was constructed using Maximum Likelihood Method. Insertion and deletion were treated as missing data. All characters were equally weighted and unordered. The evaluation of the internal support of clades was conducted by bootstrap analysis using 1,000 replicates.
RESULTS
Genomic DNA Extraction and PCR Amplification: Isolated genomic DNA and PCR amplicons for trnH-psbA were resolved using the Agarose gel. Barcode regions must be relatively short in length to facilitate easy PCR amplification and DNA sequencing. The trnH-psbA was amplified using a single pair of universal primers that resulted in efficient amplification and sequencing. This sequence was used as a query sequence in BLAST at NCBI to find a similar sequence. The trnH-psbA sequences of W. somnifera had 96% similarity with other sequences of W. somnifera, available on BLAST at NCBI (Fig. 1).

DISCUSSION
DNA barcoding has been proven to help identify recently diverged species in some cases, whereas in other, barcodes of the species under study were not distinctive. Since 1957, several studies conducted on W. somnifera have highlighted intra-specific variations and differences between the wild and cultivated plants of this species. The commercial value of W. somnifera has received more attention due to its unique medicinal properties. Its accurate identification is therefore of great importance. Yet it is extremely difficult to identify this plant-based on morphological characteristics. Recent molecular approaches, such as

Fig-1: BLAST result of W. somnifera

Fig-2: Maximum Likelihood Phylogenetic Tree of W. somnifera using trnH-psbA gene Sequence

PHYLOGENETIC TREE
The phylogenetic tree constructed showed maximum resolved topology for internal branches of 82% bootstrap value with species-specific clusters with W. somnifera.
DNA barcoding have been commonly used for species identification, diversity, forensic medicine, and ecological science. It also plays a significant role in distinguishing common medicinal herbs [43]. The success of an individual locus as the barcode depends not only on its ability to discriminate against species but also on its success in amplification and sequencing. In the present work, we used the chloroplast trnH-psbA region for barcoding W. somnifera plant.

A similar pattern has been showing for other land plant groups, with trnH-psbA PCR amplification and sequencing rates high enough to be considered as barcode loci [44]. The chloroplast trnH-psbA demonstrated good amplification across land plants with a single pair of primers and high levels of species discrimination. The trnH-psbA is an intergenic spacer with high discriminatory power with high universality.

The second method used in the present study was based on phylogenetic trees, which are constructed based on distances obtained in the sequences of the candidate loci. It provides a graphical representation that is easy to analyze. The species discriminatory power of locus is determined based on the visualized percent monopoly. Based on this method, the loci trnH-psbA discriminated the accessions of the species clustered on the same clade. BLAST results unequivocally demonstrated the discriminatory power of the tested loci for species. These results also confirmed that the sequences generated in the present study were of only the targeted loci. As of trnH-psbA sequences of W. somnifera matched 100% with the sequence of W. somnifera. The character-based analysis of sequence revealed species-specific characters, i.e. distinctive bases at the corresponding positions of the accession of the species compared.

CONCLUSION

The basic purpose of this study was to improve and simplify the DNA extraction protocol and to authenticate plant material collected from the Salem district for further studies as food and medicine. This study develops DNA barcodes for W. somnifera species of the Solanaceae family. The results of this study do not only support species identification and analysis of genetic relationships among some species of the Solanaceae family but also contributed to the conservation and commercialization of this economically important medicinal plant.

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