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Original Research Article

DNA Barcoding of *Withania somnifera* (L) Dunal Using *Trnh-Psba* Gene Sequences

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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 (CO1) 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/I [9]. Four plant DNA barcode markers, rbcL, matK, trnH- psbA, and ITS2, have been developed in

the last decades [10]. The Consortium for the Barcode of Life (CBOL) Plant Working Group suggested, the chloroplast gene rbcL and matK as the core barcodes for plant species, as well as the intergenic sequence *trnH-psbA* and the nuclear gene ITS as supplemented barcodes [11]. In green plants, the chloroplast genome is much simpler in structural complexity than the nuclear genome and typically has a highly conserved structure and gene content across organisms [12]. DNA barcoding can provide an alternative means of estimating species diversity in field identification skills and in a much shorter time frame without high-level expertise. It is an effective, simple, low-cost, and standard method for assessing and identifying various plant species [13].

The three vital principles of DNA barcoding are minimalism, standardization, and scalability [14]. Most important studies have shown that chloroplast genomes are characterized by different clusters of mutations known as "hotspots" or wildly varying regions that may serve as DNA markers for the accurate identification of plant species [15-16]. DNA barcoding, next-generation sequencing, and microarray technology have developed as potential techniques and promising tools to elucidate and conserve plant genetic diversity. They are extremely helpful in authenticating valuable medicinal plants for the preparation of herbal drugs [17]. The internal transcribed spacer region and the plastid *trnH-psbA* intergenic spacer are potentially usable DNA regions for applying barcoding to flowering plants. [18]. Compared to the difficulty and subjective biases associated with morphology-based taxa identification, DNA barcoding is currently becoming popular due to its simplicity and high accuracy [19].

Withania somnifera (L.) Dunal, a power herb of the family *Solanaceae*, has multiple medicinal properties. *W. somnifera* is one of India's 36 highly commercialized medicinal plants [20]. *W. somnifera,* an indigenous plant of India widely used various indigenous medicinal systems such as Siddha, Ayurveda, Unani, and Allopathy [21]. *W. somnifera* roots are compared with Ginseng roots because of its restorative properties and have been given the name Indian Ginseng [22]. An extensive range of biological activities have been reported from *W. somnifera* rhizome, root and leaves namely, antimicrobial [23], antifungal and antioxidant [24], antiobesity [25], anticancer [26], anti-inflammatory [27], antiarthritic [28], neuroprotective [29], antiepileptic [30-32], antidepressant, [33] and anti-anxiety [34]. Its other restorative properties include hepatoprotective [34], hypoglycaemic and hypolipidemic [35-37], sexual vitality as an adaptogen [38]. *W. somnifera* seeds also possess unique medicinal benefits includes anti-helminthic, eliminate white corneal spots, increase the count of sperm, and increase the development of the testicles [39].

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 polymerase 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 94^0 C for 45 seconds, Primer annealing at 55^0 C for 45 seconds, Extension at 72^0 C for 1 minute 20 seconds for 35 cycles, Final extension @ 72^0 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	ACTGCCTTGATCCACTTGGC
trnH-psbA R	CGAAGCTCCATCTACAAATGG

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).

Descriptions | Graphic Summary | Alignments | Taxonomy

Sequences producing significant alignments Download Manage Columns Show 100

select all 100 sequences selected GenBank Graphics Distance tree of results

Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
Withania somnifera PsbA (psbA) gene, partial cds; psbA-trnH intergenic spacer, complete sequence; and tRNA-His (trnH) gene, partial seque	802	802	99%	0.0	97.46%	MG947139.1
Withania somnifera PsbA gene, partial cds; and psbA-trnH intergenic spacer, partial sequence; chloroplast	802	802	97%	0.0	98.06%	KY494846.1
Withania somnifera trnH-psbA intergenic spacer, partial sequence; chloroplast	802	802	99%	0.0	97.46%	KR735722.1
Withania somnifera trnH-psbA intergenic spacer, partial sequence; chloroplast	802	802	99%	0.0	97.46%	KR735678.1
Withania somnifera trnH-psbA intergenic spacer, partial sequence; chloroplast	802	802	99%	0.0	97.46%	KR735244.1
Withania somnifera trnH-psbA intergenic spacer, partial sequence; chloroplast	802	802	99%	0.0	97.46%	KR735216.1
Withania somnifera isolate Tu189 PsbA (psbA) gene, partial cds; and psbA-trnH intergenic spacer, partial sequence; plastid	802	802	99%	0.0	97.46%	HQ216190.1
Withania somnifera clone 154386 chloroplast, complete genome	791	791	97%	0.0	97.62%	MK142783.1
Withania coagulans PsbA (psbA) gene, partial cds; psbA-trnH intergenic spacer, complete sequence; and tRNA-His (trnH) gene, partial seque	791	791	99%	0.0	97.04%	MG947167.1
Withania coagulans PsbA (psbA) gene, partial cds; psbA-trnH intergenic spacer, complete sequence; and tRNA-His (trnH) gene, partial seque	791	791	99%	0.0	97.04%	MG947166.1
Withania frutescens PsbA (psbA) gene, partial cds; psbA-trnH intergenic spacer, complete sequence; and tRNA-His (trnH) gene, partial seque	780	780	99%	0.0	96.62%	MG947168.1
Withania sp. OA11 chloroplast DNA containing psbA-trnH IGS, specimen voucher OA11	780	780	97%	0.0	97.19%	HE687509.1
Withania sp. OA8 chloroplast DNA containing psbA-trnH IGS, specimen voucher OA8	780	780	99%	0.0	96.62%	HE687507.1
Iochroma lehmannii plastid, complete genome	763	763	97%	0.0	96.54%	KJ310654.1
Dunalia brachyacantha psbA-trnH intergenic spacer, partial sequence; chloroplast	763	763	97%	0.0	96.54%	KP267791.1
Dunalia brachyacantha chloroplast, complete genome	763	763	97%	0.0	96.54%	KP308151.1
Saracha punctata chloroplast, complete genome	763	763	97%	0.0	96.54%	KP280050.1
Withania adpressa PsbA (psbA) gene, partial cds; psbA-trnH intergenic spacer, complete sequence; and tRNA-His (trnH) gene, partial seque	760	760	99%	0.0	95.62%	MG947165.1

Fig-1: BLAST result of *W. somnifera*

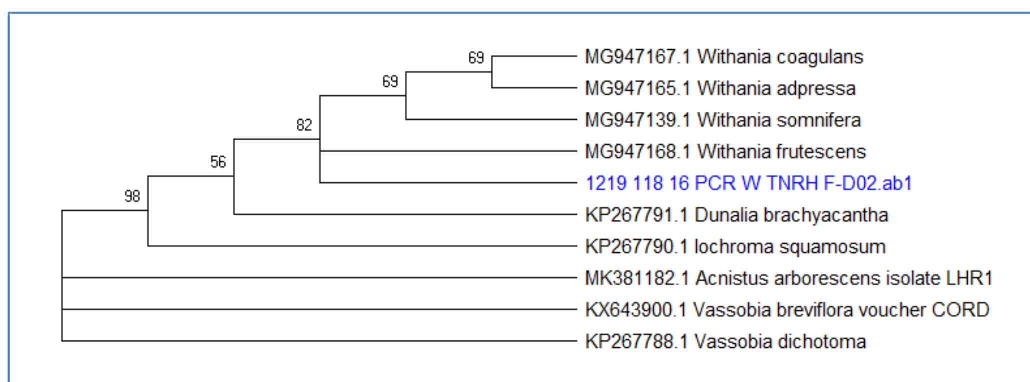

Fig-2: Maximum Likelihood Phylogenetic Tree of *W. somnifera* using *trnH-psbA* gene Sequence

PHYLOGENETIC TREE

The phylogenetic tree constructed showed maximum resolved topology for internal branches of 82% bootstrap value with species-specific clusters with *W. somnifera*.

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

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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