

DNA barcoding of authentic and substitute samples of herb of the family *Asparagaceae* and *Asclepiadaceae* based on the ITS2 region

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ABSTRACT

Background: Herbal drugs used to treat illness according to *Ayurveda* are often misidentified or adulterated with similar plant materials. **Objective:** To aid taxonomical identification, we used DNA barcoding to evaluate authentic and substitute samples of herb and phylogenetic relationship of four medicinal plants of family *Asparagaceae* and *Asclepiadaceae*. **Materials and Methods:** DNA extracted from dry root samples of two authentic and two substitutes of four specimens belonging to four species were subjected to polymerase chain reaction (PCR) and DNA sequencing. Primers for nuclear DNA (nu ITS2) and plastid DNA (*matK* and *rpoC1*) were used for PCR and sequence analysis was performed by Clustal W. The intraspecific variation and interspecific divergence were calculated using MEGA V 4.0. **Statistical Analysis:** Kimura's two parameter model, neighbor joining and bootstrapping methods were used in this work. **Results:** The result indicates the efficiency of amplification for ITS2 candidate DNA barcodes was 100% for four species tested. The average interspecific divergence is 0.12 and intraspecific variation was 0.232 in the case of two *Asparagaceae* species. In two *Asclepiadaceae* species, average interspecific divergence and intraspecific variation were 0.178 and 0.004 respectively. **Conclusions:** Our findings show that the ITS2 region can effectively discriminate *Asparagus racemosus* and *Hemidesmus indicus* from its substitute samples and hence can resolve species admixtures in raw samples. The ITS2 region may be used as one of the standard DNA barcodes to identify closely related species of family *Asclepiadaceae* but was noninformative for *Asparagaceae* species suggesting a need for the development of new markers for each family. More detailed studies involving more species and substitutes are warranted.

Key words: *Asparagus gonoclados*, *Asparagus racemosus*, *Decalepis hamiltonii*, DNA barcoding, *Hemidesmus indicus*, ITS2 region

INTRODUCTION

Traditional herbal medicines are gaining popularity worldwide and about 80% of the world's population utilizes traditional medicines for well-being and healthcare.^[1] The

increased demand for botanical products is accomplished by an expanding industry but also accompanied by calls for assurance of quality, efficacy, and safety.^[2] Studies on adulteration of herbal medicines or misidentification of plants have health consequences which may vary from life threatening and even to death.^[3] Biological species are authenticated traditionally according to their morphological features. Morphological keys still are main basis of taxonomy. But, the traditional taxonomy study usually requires the expertise of an experienced professional taxonomist. In the case that the diagnostic morphological trait of a specimen is lacking, it becomes difficult even for the specialists to recognize a species correctly. Authentication using DNA barcodes overcomes these problems. The DNA barcode is a short fragment of the genome and species identification using DNA barcoding has been completed in several studies when morphological characteristics are absent. The DNA barcoding technique is a useful tool for taxonomists and is likely to usher the taxonomic research into a new era. In plants, several candidate DNA barcodes have attracted the

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attention of many researchers such as *trnH-psbA*, *rbcL*, *matK*, *rpoB*, *rpoC1*, *ndhJ*, *accD*, *YCF5* in the plastid genome and rDNA ITS in the nuclear genome to classify the medicinal plants.^[4,5] The advent of DNA barcoding to identify plant species in herbal medicine appears to be promising but remains to be fully evaluated.^[6]

The species across families *Asparagaceae* and *Asclepiadaceae* possess important medicinal properties and proven healthcare benefits. *Asparagus racemosus* Willd. is a popular ayurvedic ingredient where the dried roots are used in different formulations. This plant has been described as a *rasayana* herb and has been extensively used as an adaptogen to increase the nonspecific resistance of organisms against a variety of stresses. In a recent literature, it has been examined for various pharmacological conditions such as antiinflammatory,^[7] immune modulatory,^[8] neuroprotective,^[9] and several others. *Asparagus gonocladus* Baker is another plant of the same genera and is considered as a substitute to *A. racemosus*.^[10] These two species are closely related and there is no marked difference in the flower and fruit structure, but there is difference in the leaf structure and in the distribution of the plant. The identification of the plant, especially the dry roots, becomes a difficult task and it can be easily adulterated with substitutes. Plant *Hemidesmus indicus* R. Br. is used to treat various medicinal conditions such as skin disease, diarrhea, fever, and rheumatism in Eastern part of India. The aromatic, woody, and deep roots are extremely important in *Ayurveda* and are used in various skin care formulations.^[11] Due to scarcity of this plant in the wild in the recent past and the cumbersome extraction procedures, few other plants are now being used as substituents to *H. indicus*. The most common species used to substitute or adulterate *H. indicus* is *Decalepis hamiltonii* Wight and Arn. also a member of family *Asclepiadaceae*. Since the identification of these dried plants especially from roots is difficult, we

explored utility of DNA barcoding to evaluate intraspecies specific variations and interspecies specific divergence in two species each of family *Asparagaceae* (*A. racemosus* and *A. gonocladus*) and family *Asclepiadaceae* (*H. indicus* and *D. hamiltonii*).

MATERIALS AND METHODS

Plant materials

H. indicus and *D. hamiltonii* roots were collected from a medicinal plants garden in Bangalore, and roots of *A. gonocladus* and *A. racemosus* were collected from Thirumala Hills (Andhra Pradesh) and Denkanikotta (Tamil Nadu). Botanical identification of all the four plants species was confirmed by in-house taxonomist (R&D, Natural Remedies, Bengaluru, India). Voucher specimens have been preserved with the authors at R&D of Natural Remedies. The voucher numbers for *H.indicus*, *D. hamiltonii*, *A. gonocladus*, and *A. racemosus* are Sariva I, Sariva II, AGR31, and AGR30 respectively. These samples hereafter referred to as reference material were used to develop DNA barcode. Roots were washed, chopped, and dried in sunshine.

Genomic DNA isolation, PCR amplification, and sequencing of DNA barcode

Dried roots were thoroughly rinsed in sterile distilled water and cut into small pieces. DNA extraction was performed according to the protocol of Warude *et al.*,^[12] Primer sequences for three candidate DNA barcodes (ITS2, *rpoC1*, *matK*) were taken from the previous study.^[5] PCR amplification was performed in 25 µl reaction mixtures containing approximately 40 ng template DNA, 1 X PCR buffer, 1.5 mM MgCl₂, 0.2 mM of each dNTP, 0.1 µM of each primers (Sigma Prologo and Sigma Life Science, India), and 1.0 U *Taq* DNA Polymerase (Invitrogen) in a thermal cycler (Eppendorf, Germany). The primer sequences and PCR reaction conditions used for PCR amplification are

Table 1: Primers and reaction conditions used in the present study

Maker	Name of primer	Primer sequence 5'--3'	Reaction conditions
ITS2	Forward	5'-ATGCGATACTTGGTGTGAAT-3'	95 °C 5 min
	Reverse	5'-GACGCTTCTCCAGACTACAAT-3'	95 °C 30 Sec
<i>matK</i>	Forward	5'-CGATCTATTCATTCAATATTTTC-3'	56 °C 30 Sec, 72 °C 1.5 min, 40 cycles
	Reverse	5'-TCTAGCACACGAAAGTCTGAAGT-3'	72 °C 10 min
<i>rpoC1</i>	Forward	5'-GGCAAAGAGGGGAAGATTCG-3'	94 °C 5 min
	Reverse	5'-CCATAAGCATATCTTGAGTTGG-3'	94 °C 1min
			48 °C 30 sec, 72 °C 1min, 30 cycles
			72 °C 10 min
			94 °C 5 min
			94 °C 30 sec
			53 °C 40 sec, 72 °C 40 sec, 40 cycles
			72 °C 10 min

listed in Table 1. Purified PCR products were sequenced in both directions on ABI3130 sequencer (Applied Biosystem, USA).

Sequence alignment and genetic analysis

To estimate the quality of the generated DNA sequence traces, the original forward and reverse sequences of PCR products were assembled using sequence alignment tools (BLAST) from NCBI. The data analysis was done for all the four plant species of the two families, *Asparagaceae* and *Asclepiadaceae* for which the sequences are available in the GenBank and with the sequence generated in the present study to find the intraspecific divergence and interspecific variations. GenBank accession numbers of candidate DNA barcodes generated in the present study are *H. indicus* (JN712767), *D. hamiltonii* (JN712768), *A. gonoclados* (JN712766), and *A. racemosus* (JN712765), respectively. The GenBank accession numbers of candidate DNA barcodes of database taxa used in this study were *A. densiflorus* (HM357929), *A. officinalis* isolate A5 (HM35793), *A. officinalis* (GU474425), *A. racemosus* (GU474426), *A. setaceus* (GQ202238), *A. filicinus* (HM357928), and *H. indicus* (DQ916851).^[13] Multiple sequence alignment was performed by Clustal W.^[14] The basic sequence statistics including nucleotide frequencies and variability in different regions of the sequence were computed using the Kimura 2-parameter method by MEGA V 4.0 software. The sequence data were analyzed by neighbor joining and bootstrapping methods.^[15-18]

RESULTS AND DISCUSSION

DNA barcodes must simultaneously contain enough variability to be used for species identification and adequate conservative regions for the universal primers.^[19] Previous studies have suggested that ITS2 sequences are relatively easy to amplify using universal primers and that the ITS2 region possesses high interspecific divergence.^[5] In this study, ITS2 DNA barcode candidate sequences in samples of *Asparagaceae* and *Asclepiadaceae* family tested were obtained through PCR amplification with universal primers followed by DNA sequencing and multiple sequence alignment. The success rate of PCR amplification of ITS2

sequences was 100% Figure 1 and alignment of sequences was performed with ease for all the four species studied in accordance with previously published studies.^[20] Sequences were aligned using Clustal W and final alignment adjustments were made manually in order to remove artificial gaps. We assessed divergences within and between species using software MEGA V 4.0. In the species of *Asparagaceae* and *Asclepiadaceae*, average distances were computed based on pairwise comparisons of all sequences. For each species, of *Asparagaceae* average interspecific nucleotide differences was 0.198 and differences between individuals of the same species sequences was 0.233. The average interspecific percentage difference in sequence homology was 3.6% and the percentage difference between individuals of the same species is 5%. The distance between generated ITS2 gene sequence of *A. gonoclados* and *A. racemosus* is 0.011 and the distance between generated sequence of *A. gonoclados* and database sequence of *A. racemosus* was 0.217. Further we observed that *A. gonoclados* is much closer to *A. densiflorus* (distance 0.012) than *A. racemosus* sequence from database [Tables 2 and 3]. In *Asclepiadaceae* species, gene sequences for ITS2 are available only for one species in the GenBank. The average interspecific nucleotide differences

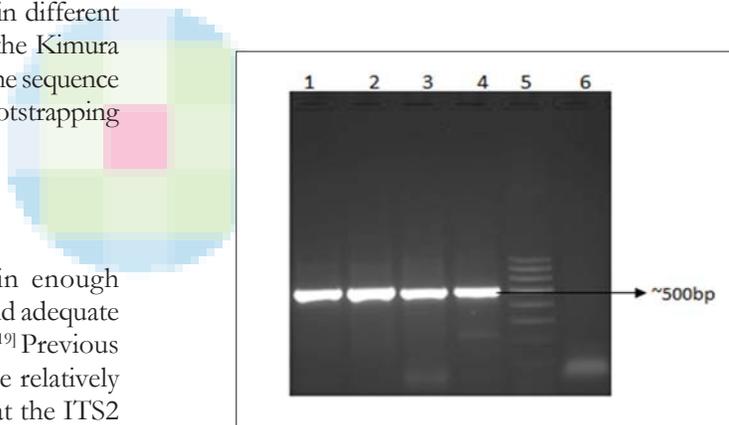


Figure 1: PCR for the ITS2 region of root DNA samples of family *Asparagaceae* (*Asparagus racemosus* and *Asparagus gonoclados*) and *Asclepiadaceae* (*Hemidesmus indicus* and *Decalepis hamiltonii*) Detection of the ITS2 region of ~500 bp PCR product on a 1.5% agarose gel. Lane 1. *Asparagus racemosus*, Lane 2. *Asparagus gonoclados*, Lane 3. *Hemidesmus indicus*, Lane 4. *Decalepis hamiltonii*, Lane 5. 100bp ladder, Lane 6. Negative control

Table 2: Homology and distance matrix of *Asparagus gonoclados* plant ITS2 gene nucleotide sequences with ITS2 sequences of other *Asparagus* species

<i>Asparagaceae</i> species	Genetic distance	Homology in percentage
<i>Asparagus Aethiopicus</i> [#]	0.024	98
<i>Asparagus densiflorus</i> [#]	0.0119	99
<i>Asparagus officinalis</i> isolate A5 [#]	0.5367	94
<i>Asparagus officinalis</i> [#]	0.0807	96
<i>Asparagus racemosus</i> [#]	0.2167	95
<i>Asparagus setaceus</i> [#]	0.0488	95
<i>Asparagus filicinus</i> [#]	0.0239	94
<i>Asparagus racemosus</i> [*]	0.011	97

^{*}Present study ITS2 sequences, [#]GenBank ITS2 sequences

Table 3: Estimated distance matrix for *Asparagaceae* species

<i>Asparagaceae</i> species	1	2	3	4	5	6	7	8	9
<i>A. Aethiopicus</i> #									
<i>A. densiflorus</i> #	0.036								
<i>A. officinalis isolate A5</i> #	0.561	0.535							
<i>A. officinalis</i> #	0.100	0.080	0.560						
<i>A. racemosus (data base)</i> #	0.224	0.232	0.571	0.248					
<i>A. setaceus</i> #	0.074	0.055	0.548	0.107	0.265				
<i>A. filicinus</i> #	0.048	0.023	0.512	0.080	0.240	0.042			
<i>A. racemosus</i> *	0.0364	0.017	0.549	0.094	0.232	0.061	0.036		
<i>A. gonocladus</i> *	0.024	0.011	0.536	0.080	0.216	0.048	0.023	0.01	

*Present study ITS2 sequences, #GenBank ITS2 sequences

Table 4: Homology and distance matrix of *Decalepis hamiltonii* ITS2 gene nucleotide sequences with ITS2 sequences of other species of the *Asclepiadaceae* family

<i>Asclepiadaceae</i> species	Genetic distance	Homology in percentage
<i>Hemidesmus indicus</i> #	0.1786	87%
<i>Hemidesmus indicus</i> *	0.1846	87%

*Present study ITS2 sequences, #GenBank ITS2 sequences

was 0.123 and differences between individuals of the same species sequences was 0.004. The average interspecific percentage difference in sequence homology was 13% and the percentage difference between individuals of the same species was 2% [Tables 4 and 5]. The distance between *H. indicus* and *D. hamiltonii* sequences of the present study was 0.1854 and the distance between the sequences of *H. indicus* from database was 0.008. Phylogenetic analyses were computed using the MEGA V 4.0 software. Phylogenetic inference was performed by the neighbor-joining method. Bootstrapping tests were conducted to examine the reliability of the interior branches and the validity of the trees. The optimal tree with the sum of branch length 0.805 was shown for *Asparagaceae* family species and the branch length was 0.629 for *Asclepiadaceae* family species. The percentage of replicate trees in which the associated taxa clustered together in the bootstrapping test (1000 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The distances were computed and are in the units of the number of base substitutions per site. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated from the dataset. There were a total of 170 positions in the final dataset of *Asparagaceae* family species and of 232 positions in the final dataset of *Asclepiadaceae* family species [Figures 2 and 3]. The intraspecific percentage differences among *H. Indicus* species were less than the interspecific variations; hence the ITS2 region can be potentially used as a standard DNA barcode to identify medicinal plants of

Table 5: Estimation of distance matrix for *Asparagaceae* species

<i>Asclepiadaceae</i> species	1	2
<i>H.indicus</i> #	-	-
<i>H.indicus</i> *	0.0043	-
<i>D.hamiltonii</i> *	0.1786	0.1846

*Present study ITS2 sequences, #GenBank ITS2 sequences

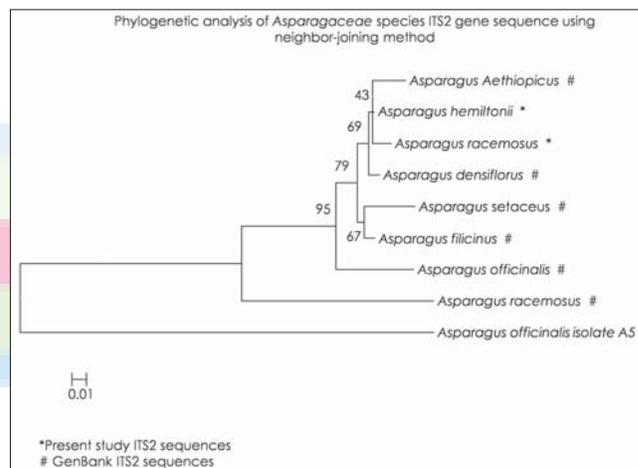


Figure 2: Phylogenetic analyses of the *Asparagaceae* species ITS2 gene sequence using the neighbor-joining method *Present study ITS2 sequences #GenBank ITS2 sequences

Asclepiadaceae family. But in the case of *Asparagus* species we found that the intraspecific percentage differences among the *Asparagus* species were greater than the interspecific variations, which suggest that the ITS2 gene is not an effective candidate barcode for *Asparagaceae* species.^[21] All the samples were also tested using chloroplast-specific primers (*rpoC1* and *matK1*) to amplify any contaminating chloroplast containing parts. No amplification products were obtained suggesting that the materials used primarily originated from the roots of the plants. Despite the potential benefits of DNA barcoding as a novel system designed to provide rapid and accurate species identifications by using short, standardized gene regions as internal species tags, but may at times barcoding may fail as sequences for some taxa are not as robust and broadly applicable.^[22] In such cases new genomic markers may need to be adopted.

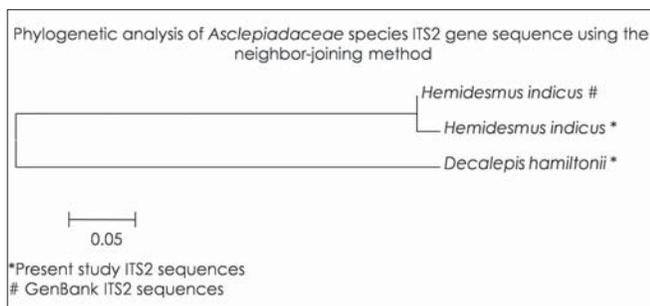


Figure 3: Phylogenetic analyses of the *Asclepiadaceae* species ITS2 gene sequence using the neighbor-joining method *Present study ITS2 sequences #GenBank ITS2 sequences

In this study, DNA sequence alignment and sequence variation between species within the ITS2 region was examined for its usefulness as the candidate DNA barcode for species variation and species identification of *Asparagaceae* and *Asclepiadaceae* families. With 100% PCR amplification efficiency, straightforward sequence alignment, and ability to distinguish closely related species indicates that DNA sequences of the ITS2 region is an effective and powerful tool for identification of medicinal plants of *Asclepiadaceae* families in traditional Indian *Ayurveda* medicine and their contaminants. Although the ITS2 region of *Asparagaceae* species showed 100% PCR amplification efficiency, straightforward sequence alignment and had the ability to discriminate authentic *Asparagus racemosus* from its substitute sample, but the interspecific variation was low and intraspecific divergence was high which suggests that the ITS2 region is not an effective marker loci for *Asparagaceae* species. More studies are warranted with samples of most commonly available species and substitutes of these two families examined.

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