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Authentication of Herbal Samples of Indian Medicinal Plants in Trade using DNA Barcoding

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Abstract:

Medicinal plants are the prime source of herbal materials, with 1178 species traded in India and 242 having high trade volumes. Most of the medicinal plants are collected from the wild indiscriminately, as very few of these plants are cultivated, leading to overexploitation and decline of species. Scarcity of these often results in adulteration and substitution, making traditional identification difficult. DNA barcoding has proved to be an accurate and reliable alternative for identification of such herbals but before being used for this purpose, species-specific barcodes for the species of interest should be available. The barcode library developed was used for authenticating the botanical identities of 163 herbal samples, procured from different markets or online, supposed to be belonging to 54 species, including 41 species of high trade volume by phylogenetic tree (NJ) method and BLAST1 analysis. Herbal samples (147) of 54 species could be tested and 92 (62.6%) of the tested samples were found to be authentic by using any of the four barcode loci. Of these, number of samples identified by ITS2 (69) was the highest, followed by *rbcL* (51), *matK* (26) and ITS (17) individually. BLAST1 search of the sequences of the samples not found to be authentic revealed that some of the herbal samples were substituted following the ayurvedic principle "*Abhava Pratinidhi Dravyas*" with their known substitutes or other unrelated medicinal plants, while few by totally unrelated plant species, such as, 'Besan' (*Cicer arietinum*) in place of 'Vachhnag' (*Aconitum ferox*) and an obnoxious weed, *Parthenium hysterophorus* substituting for 'Pashanbheda' (*Bergenia ligulata*).

Keywords: Herbals, Medicinal Plants, DNA Barcoding, Adulteration.

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1. INTRODUCTION

It was probably unimaginable that barcodes could be developed for plants and animals until Paul Hebert of University of Guelph, based on the study on 200 closely allied Lepidopteran species, proposed the concept of DNA Barcoding in 2003 [14]. A single gene or only a part of this is sequenced to be used as a DNA barcode. The main purpose of DNA barcoding is not to build the tree of life or to perform molecular taxonomy instead, it aims to produce a simple diagnostic tool based on strong taxonomic knowledge utilising a DNA barcode reference library [15]. The results of DNA barcoding are expressed or represented in the form of genetic distances, through phylogenetic trees [14] and/or the analysis is done using BLAST (Basic Local Alignment Search Tool) in which, just a raw similarity score is used to determine the nearest neighbour to the query sequence[13]. Previous studies, performed on the search for a universal DNA barcode for plants, revealed that a single universal locus barcode does not exist for plants and, rather it needs to be a multilocus one [12]. Of the seven chloroplast genomic regions of plants compared by CBOL Plant working group [11], a combination of *matK* and *rbcL* was found to provide maximum species resolution of only 72% in closely related species. Chen et al. [10] and Pang et al. [10] proposed ITS2 (Internal Transcribed Spacer 2) and ITS/ITS2 from nuclear genome as potential barcodes for plants, respectively. Medicinal plants provide 80% of the raw materials for herbal drugs and its effectiveness depends on the authenticity of the species used [9]. A majority of the pharmaceutical drugs used today are based on medicinal plants mentioned in the traditional medicine systems [7,8]. The herbal product market is expanding rapidly due to globalisation of trade[6]. The use of medicinal plants after the COVID-19 pandemic has elevated the use of medicinal plants[5]. However, authenticity of herbals is generally at stake with increase in unscrupulous activities of adulteration/substitution, where the original herb is mixed and/or replaced by some unlabelled fillers and contaminants[3,4]. Herbal samples of medicinal plants are generally traded as dried roots/rhizomes, bark, seeds, leaves or in processed forms like, powders, tablets, tinctures that have lost their morphological identities [1,2].

India harbours an estimated 17,000–18,000 flowering plant species, of which 7,000 are recognized for their medicinal value in traditional systems such as Ayurveda, Siddha, Unani, and Homoeopathy (Introduction | National Medicinal Plants Board|Government of India). The National Medicinal Plant Board (NMPB) has identified 1178 species actively traded, with 242 having annual trade volumes exceeding 100 metric tonnes [16,17].

DNA barcoding provides a reliable alternative for species authentication as it is independent of morphology, requires minimal tissue, and can be applied to processed materials [22,23,24,25]. The process involves: (i) developing a reference barcode library of authenticated species, and (ii) comparing DNA sequences from unknown samples using phylogenetic tree-based or sequence similarity analyses such as BLAST and BOLD [18,19,20,21].

The present study applies this molecular approach to authenticate the botanical identities of herbal market samples by comparing DNA sequences from key barcode loci with reference sequences in the established barcode library using phylogenetic and sequence similarity analyses**.**

2. MATERIALS AND METHODS

2.1.*In silico* analysis and preparation of reference barcode library

The work of *in silico* analysis for the preparation of reference barcode library was used from Priya et al., 2018 [26].

Procurement of herbals

Herbal samples of the selected medicinal plants were purchased using their vernacular names from wholesale markets of Khari Baoli (New Delhi), local shops of Patna (Bihar) and Amritsar (Punjab) and through online sellers (Table 1).

Table 1: List of medicinal plants herbal market samples of which were procured for authentication (+: sample available, -: sample not available)

S.No.	(Herbal procured as) Botanical name, Family	Listed among traded (T)/highly traded species	Plant part used	Place of Collection			
				New Delhi	Patna	Amritsar	Online
1.	(Gunja) *Abrus precatorius,* Fabaceae	HT	Seeds	+	+	+	-
2.	(Vachhnag) *Aconitum ferox,* Ranunculaceae	HT	Root	+	+	+	-
3.	(Atis) *Aconitum heterophyllum,* Ranunculaceae	HT	Root (tuber)	+	+	+	+
4.	(Vach) *Acorus calamus,* Araceae	HT	Root (Rhizome)	+	+	+	-
5.	(Akarkara) *Anacyclus pyrethrum,* Asteraceae	T	Root	+	+	-	+
6.	(Adusa) *Justicia adhatoda,* Acanthaceae	HT	Leaves, root	+	+	-	+
7.	(Kalmegh)*Andrographis paniculata,* Acanthaceae	HT	Whole plant	+	-	+	+
8.	(Neem) *Azadirachta indica*, Meliaceae	HT	Whole plant	+	+	+	-
9.	(Brahmi) *Bacopa monnieri,* Scrophulariaceae	HT	Whole plant	+	-	+	+

S.No.	(Herbal procured as) Botanical name, Family	Listed among traded (T)/highly traded species	Plant part used	Place of Collection			
				New Delhi	Patna	Amritsar	Online
10.	(Raktapushpa) *Barleria cristata,* Acanthaceae	HT	Whole plant	+	+	+	-
11.	(Daruhaldi) *Berberis aristata,* Berberidaceae	HT	Root, bark	+	+	-	+
12.	(Pashanbheda) *Bergenia ligulata,* Saxifragaceae	HT	Rhizome	+	+	+	-
13.	(Salmali) *Bombax ceiba,* Bombacaceae	HT	Exudate of bark, flower, root	+	-	+	+
14.	(Patthimugam) *Caesalpinia sappan,* Caesalpiniaceae	HT	Wood	+	-	+	+
15.	(Bhang) *Cannabis sativa,* Cannabinaceae	T	Seed, whole plant	+	+	+	-
16.	(Kalazira) *Carum carvi,* Apiaceae	T	Fruit	+	+	+	-
17.	(Kusum phool)*Carthamus tinctorius,* Asteraceae	T	Fruit (Seed)	+	+	+	-
18.	(Jyotismati) *Celastrus paniculatus,* Celastraceae	HT	Seeds	+	+	-	+
19.	(Brahmi booti)*Centella asiatica,* Apiaceae	HT	Leaf, whole plant	+	-	+	+
20.	(Kasani) *Cichorium intybus,*Asteraceae	HT	Flower, leaf, fruit,root	+	-	+	+

S.No.	(Herbal procured as) Botanical name, Family	Listed among traded (T)/highly traded species	Plant part used	Place of Collection			
				New Delhi	Patna	Amritsar	Online
21.	(Prasarni) *Convolvulus arvensis,*Convolvulaceae	T	Leaf	+		+	+
22.	(Dhatura) *Datura stramonium,* Solanaceae	T	Leaf, flower, fruit (seed)	+	+	+	-
23.	(Salparni) *Desmodium gangeticum,* Fabaceae	HT	Root, whole plant	+	+	-	+
24.	(Bhringraj) *Eclipta prostrata,* Asteraceae	HT	Whole plant	+	+	-	+
25.	(Vaividang) *Embelia ribes,* Myrsinaceae	HT	Seeds	+	+	-	+
26.	(Shankhpushp) *Evolvulus alsinoides,* Convolvulaceae	T	Whole plant	+	+	+	
27.	(Mulethi) *Glycyrrhiza glabra,* Fabaceae	HT	Root	+	+	+	-
28.	(Gurmar) *Gymnema sylvestre,*Apocynaceae	HT	Leaf	+	+	-	+
29.	(Kantapalci/Kapur kachri) *Hedychium coronarium,* Zingiberaceae	T	Flower	+		+	+
30.	(Marorphali) *Helicteres isora,* Sterculiaceae	HT	Roots, fruits, bark (stem)	+	+	+	-
31.	(Anantmool) *Hemidesmus indicus, Apocynaceae*	HT	Root	+	+	-	+

S.No.	(Herbal procured as) Botanical name, Family	Listed among traded (T)/highly traded species	Plant part used	Place of Collection			
				New Delhi	Patna	Amritsar	Online
32.	(Hauber) *Juniperus communis,* Cupressaceae A	HT	Fruit	+	+	+	-
33.	(Maida lakri) *Litsea glutinosa,* Lauraceae	HT	Leaf, bark	+	+	+	-
34.	(Nagkesar) *Mesua ferrea,* Clusiaceae	HT	Flower	+	-	+	+
35.	(Jatamansi) *Nardostachys jatamansi,* Caprifoliaceae	HT	Root (rhizome)	+	-	+	+
36.	(Shyonaka) *Oroxylum indicum,* Bignoniaceae	HT	Bark, root	+	+	+	-
37.	(Pippali) *Piper longum,* Piperaceae	HT	Root, fruit	+	-	+	+
38.	(Kali mirch) *Piper nigrum,* Piperaceae	HT	Stem, fruit	+	+	-	+
39.	(Lahuriya) *Plantago major,* Plantaginaceae	HT	Fruit (seed)	+	+	+	-
40.	(Chitraka) *Plumbago zeylanica,* Plumbaginaceae	HT	Root	+	+	-	+
41.	(Manjishtha) *Rubia cordifolia,* Rubiaceae	HT	Root	+	+	-	+
42.	(Chandan) *Santalum album,* Santalaceae	HT	Wood	+	+	-	+
43.	(Bala) *Sida cordifolia,* Malvaceae	T	Root, seed, whole plant	+	+	-	+
44.	(Chopchini) *Smilax aspera,* Smilaceae	T	Root	+	-	+	+
45.	(Kuchla) *Strychnos nux-vomica,* Loganiaceae	HT	Seed	+	+	+	-

S.No.	(Herbal procured as) Botanical name, Family	Listed among traded (T)/highly traded species	Plant part used	Place of Collection			
				New Delhi	Patna	Amritsar	Online
46.	(Dudhi) *Taraxacum officinale,* Asteraceae	T	Root (rhizome)	+		+	+
47.	(Arjuna) *Terminalia arjuna,* Combretaceae	HT	Fruit, bark	+	+	+	-
48.	(Baheda) *Terminalia bellirica,* Combretaceae	HT	Fruit	+	+	+	-
49.	(Harad) *Terminalia chebula,* Combretaceae	HT	Fruit, Galls	+	-	+	+
50.	(Ajwain) *Trachyspermum ammi,*Apiaceae	HT	Fruit	+	+	-	+
51.	(Musakbala) *Valeriana jatamansi,* Caprifoliaceae	HT	Root, whole plant	+	+	+	-
52.	(Dhaiphool)*Woodfordia fruticosa,* Lythraceae	HT	Bark, flower	+	+	+	-
53.	(Tejbal) *Zanthoxylum armatum,* Rutaceae	T	Fruit	+	+	+	-
54.	(Sonth) *Zingiber officinale,* Zingiberaceae	T	Root, whole plant	+	-	+	+

2.2.DNA Isolation, Amplification and Sequencing of the Selected Loci

Genomic DNA was isolated from herbal market samples using the modified CTAB method [27] and its quality was assessed electrophoretically on 0.8% TAE agarose gel. Four loci- two chloroplast (rbcL, *matK*) and two nuclear (ITS, ITS2) were amplified using locus-specific primers (Table 2). Locus-specific thermal cycling conditions were followed; for non-amplifying samples, variant annealing temperatures were optimized. PCR products were resolved on 1% TAE agarose

gels containing ethidium bromide (4 × 10^{-4} mg/ml) at 5 V/cm and visualized under UV illumination (Alpha-Imager Pvt. Ltd., Bengaluru). Purified amplicons were bi-directionally sequenced using Sanger's dideoxy method with the same primers as amplification. Sequencing was performed at the Central Instrumentation Facility (CIF), University of Delhi South Campus, and AgriGenome Labs Pvt. Ltd., Kerala. Sequence assembly and quality trimming were performed in CodonCode Aligner v5.0.1 (CodonCode Corporation, USA).

Table 2: The primers used and the thermal cycles employed for the amplification of the targetted loci.

Locus	Primer Name	Primer Sequence	Thermal Cycle
rbcL Fay et al. (1997) Olmstead et al. (1992)	rbcL 1F rbcL 724R	5'-ATGTCACCACAAACAGAAAC 5'-TCGCATGTACCTGCAGTAGC	95°C 2 min 94°C 1 min 50-55°C 30 sec (35 cycles) 72°C 1 min 72°C 7 min
matK Ki-Joong Kim, pers. comm. (Fazekas et al. 2008)	3F KIM 1R KIM	5′-CGTACAGTACTTTTGTGTTTACGAG 5′-ACCCAGTCCATCTGGAAATCTTGGTTC	94°C 1min 94°C 30sec 51-56°C 20sec (35 cycles) 72°C 50sec 72°C 5min
Cuenoud et al. (2002)	390F 1326R	5'-CGATCTATTCATTCAATATTTC 5'-TCTAGCACACGAAAGTCGAAGT	94°C 3 min 94°C 1 min 48-50°C 30 sec (26 cycles) 72°C 1 min 72°C 7 min
ITS White et al. (1990)	ITS 5F ITS 4R	5′-TCCTCCGCTTATTGATATGC 5′-GGAAGTAAAAGTCGTAACAAGG	94°C 5min 95°C 30sec 50-53°C 40sec (30 cycles)

			72°C 1min 72°C 5min
ITS2 Chen et al. (2010)	ITS 2F ITS 3R	5'-ATGCGATACTTGGTGTGAAT 5'-GACGCTTCTCCAGACTACAAT	94°C 5 min 94°C 30 sec 50-55°C 30 sec (40 cycles) 72°C 45 sec 72°C 10 min

2.3. Validation of the Herbal Market Samples

DNA barcodes from the reference barcode library were used for validating the identity of the sequences generated from the herbal samples. Neighbor Joining trees [31] based on K2P model with 1000 bootstrap value were constructed for each locus, ITS, ITS2, *matK* and *rbcL*, respectively using sequences retrieved from the herbals along with the sequences belonging to species of the genera to which the herbal probably belonged. ITS2 NJ trees were constructed using annotated ITS2 region from complete ITS sequences, using the annotation tool available on the ITS2 database[30]. The sequences were then aligned using MUSCLE[29] on MEGA 7 [28]. The herbal market sample was considered authentic if its sequence clustered with the sequence of the taxonomically identified specimens. In cases, where the botanical identity of the herbals could not be deciphered using the tree-based method or the species, itself was not specific, BLAST1 analysis was performed on NCBI and sequence query for *matK* and *rbcL* sequences were performed on the BOLD identification engine to determine their probable identities.

3. RESULTS

Procurement of Herbal Samples

A total of 163 herbal trade samples, representing 54 medicinal plant species of 50 genera belonging to 36 families, were purchased from four markets of India. At least three herbal samples of each species were available for checking their authenticity (Table 1). The herbal samples purchased from the wholesale markets and local shops were in the form of roots, barks, seeds, dried flowers, leaves and fragments, while those purchased online were in powder form (Fig. 1). Of 54 selected species, 41 were from among the 242 species having high trade volume and the remaining 13 species were from the list of 1178 traded medicinal plants, as per National Medicinal Plant Board of India (NMPB). The selected medicinal plants are also included in the Ayurvedic pharmacopoeia of India (http://www.ayurveda.hu/api/API-Vol-1.pdf, http://www.ayurveda.hu/api/API-Vol-2.pdf http://www.ayurveda.hu/api/API-Vol-3.pdf, http://www.ayurveda.hu/api/API-Vol-4.pdf, http://www.ayurveda.hu/api/API-Vol-5.pdf).

Fig. 1: Dried bark, roots of a) *Aconitum heterophyllum*, b) *Aconitum ferox*, c) *Berberis aristata*, d) Seeds of *Mesua ferrea* and powder samples of e) *Carthamus tinctorius,* f) *Bombax ceiba,* g) *Berberis aristata*, h) *Desmodium gangeticum.*

DNA Extraction, Amplification and Sequencing

A modified CTAB method[27] yielded better quality DNA. However the DNA isolated from the herbals often appeared as smears, were present in low quantities and thus could not be detected on 0.8% agarose gel. The concentration of DNA from the herbal samples ranged from 0.30-400ng/µl. Among the four loci tested, ITS2 exhibited the highest (92.0%) amplification success, followed by *rbcL* (81.5%), ITS (25.7%) and *matK* (23.3%). The sequencing success rate exhibited by ITS2 was also the highest, being 80.3%, followed by *rbcL*, ITS and *matK* with 66.8%, 19.6% and 17.1%, respectively. The total number of sequences retrieved was 300. The number of ITS, ITS2, *matK* and *rbcL* sequences retrieved from 163 herbal samples representing 54 medicinal species were 32, 131, 28 and 109 from 19, 51, 18, 48 species, respectively.

Validating the Botanical Identities of the Herbal Samples

Of the 163 herbal samples procured, authenticity of 147 herbals, presumably belonging to 54 species could be checked by (i) constructing individual phylogenetic NJ trees for each individual locus and BLAST analysis. The botanical identities of the herbals not authenticated as the species to which these were supposed to belong were determined by BLAST1 on NCBI and on identification engine on the BOLD data systems.

On the basis of the phylogenetic tree (NJ) constructed, for the herbals and ITS sequences of the corresponding botanically authentic species, 16 herbals belonging to eight species were authentic. On the basis of the NJ tree constructed for the herbals and ITS2 sequences, 36 herbals belonging to 17 species were authentic. The phylogenetic tree (NJ) based on *matK* sequences of the herbals and botanically authentic species could authenticate 18 herbals belonging to 11 species. On the basis of the Phylogenetic tree (NJ) constructed between the herbals and *rbcL* sequences of the corresponding botanically authentic species, 26 herbals belonging to 18 species were authentic.

The botanical identity of the herbals sequence of any of the four loci of which did not match the corresponding sequence(s) of the authentic sample was checked by BLAST search performed on NCBI and BOLD identification system for *matK* and *rbcL*. Therefore, total number of samples that were authentic by any of the two methods of identification, viz., phylogenetic tree-based and BLAST1 search on NCBI and BOLD identifications, for the four barcode loci, ITS, ITS2, *matK* and *rbcL* were 92. Of these, number of samples identified by ITS2 (69) was the highest, followed by *rbcL* (51), *matK* (26) and ITS (17), respectively (Fig.: 2). Thus, in the present study, 62.6% of the herbals were true to their identity.

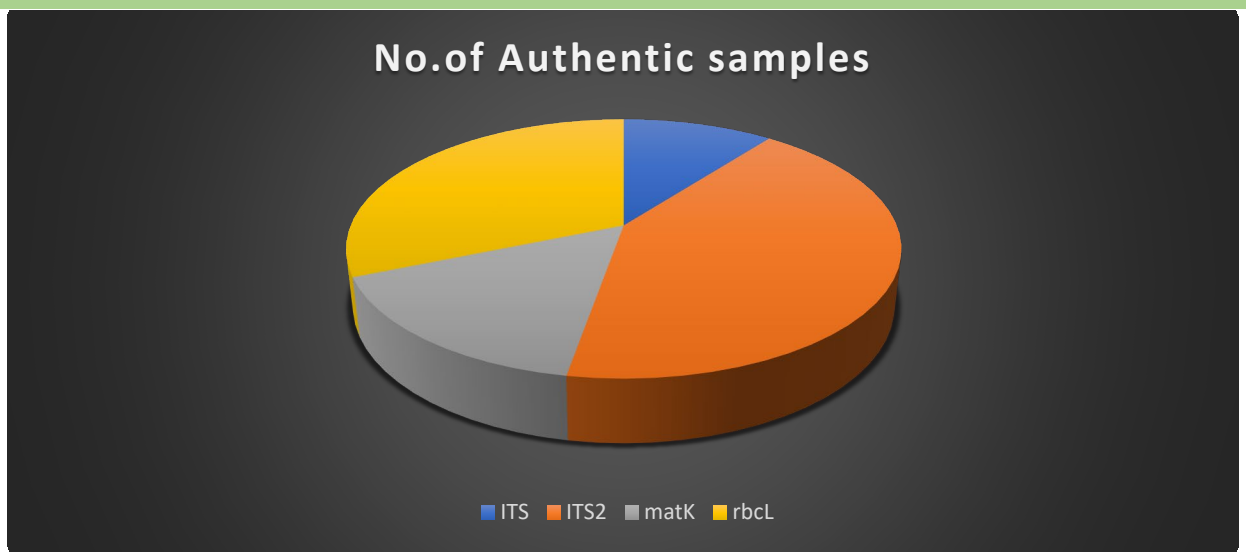

Fig.2: Number of authentic samples for the four barcode loci used.

DISCUSSION

Medicinal plants have played a significant role in man's life, be it for their health benefits or their usage in socio-cultural aspects. Plant based raw drugs are either obtained from the whole plant or a part of the plant is used for its medicinal value[41]. Most of the medicinal plants are declining in population due to their increased demand[39,40]. To fill in this lacuna, cases of adulteration and substitution are likely to increase. This would definitely lead to reduced or no efficacy of the drug[38]. Thus, it is essential that the medicinal plants used either as raw or in the processed form, should be botanically authentic and conservation practices for them should be advocated. Botanical identity of such herbal samples can be well authenticated using DNA Barcoding, in which, a small amount of a living material is sufficient enough to provide identity to a particular organism, with ease and accuracy without being influenced by age of the samples, storage conditions, environmental effects etc.[37]. However, availability of reference barcodes is a prerequisite for species level identification. DNA barcoding has revived the identification of herbal medicinal materials in trade[36]. The application of this technology has been accepted and realized by the Ayurvedic Pharmacopoeia of India [35], United States Pharmacopoeia- National Formulatory [34], Chinese Pharmacopoeia [33], British and Korean Pharmacopoeias [32]. The reference barcode library or a reference database should consist of DNA sequences generated from botanically authentic specimens of the medicinal plants and in some cases, also from their known adulterants and substitutes. The Canadian Centre for DNA Barcoding (CCDB) established the first database for barcoding, i.e. Barcode of Life Database (BOLD), developed by Ratnasingham and Hebert in 2007. In the present study, 62.6% of the herbals were true to their identity. Some of the herbal samples were substituted with their known substitutes or other unrelated medicinal plants, while few by totally unrelated plant species, such as, 'Besan' (*Cicer arietinum*) in place of 'Vachhnag' (*Aconitum ferox*) and an obnoxious weed, *Parthenium hysterophorus* substituting for 'Pashanbheda' (*Bergenia ligulata*). This study demonstrates and re-affirms the relative efficacy of the four loci as barcodes for validating the identity of herbal samples of medicinal plant species belonging to diverse taxonomic groups, with ITS2 being the best followed by *rbcL, matK*, and ITS

in decreasing order of efficacy and need for a multilocus approach and the inclusion of ITS/ITS2 in the core barcode along with earlier suggested combination of *matK* and *rbcL.* In the initial years, DNA barcoding relied on Sanger sequencing for species identification and characterization but the recent advances in next-generation sequencing (NGS) have expanded DNA barcoding applications to quality control, biomonitoring of protected species, and biodiversity assessments.

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