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Review Article

DNA Based Molecular Markers: A Tool for Differentiation of Ayurvedic Raw Drugs and their Adulterants

Manasi Malgaonkar[1], Amey Shirolkar[1], Soma Narasimha Murthy[1], Anupam K Mangal[2], Sharad Daulatrao Pawar[1]*

ABSTRACT
Recent advances in molecular techniques have generated several typing methods based on PCR for genetic assessment of genetic relatedness of plant species. Considering the medicinal importance of plants, it is essential to explore, discover and conserve genetic diversity of these plant species. Levels of polymorphisms represent genetic distance at intra-species level and method used to detect it is efficient marker for assessment of genetic relatedness. With this background, an attempt has been made to summarize the applicability of different DNA based molecular Markers for differentiation of Ayurvedic raw drugs and their adulterants considering the current practices and future perspectives.
Key words: Ayurveda, Adulterants, Molecular biomarkers, DNA barcoding.

Manasi Malgaonkar[1], Amey Shirolkar[1], Soma Narasimha Murthy[1], Anupam K Mangal[2], Sharad Daulatrao Pawar[1]*

[1]Regional Ayurveda Institute of Fundamental Research, Under CCRAS; Min. of AYUSH, Nehru Garden, Gandhi Bhavan Road, Kothrud, Pune, Maharashtra, INDIA.
[2]Central Council for Research in Ayurvedic Sciences, Under Min. of AYUSH, Janakpuri, New Delhi, INDIA.

Correspondence

Dr. Sharad Daulatrao Pawar,

Research Officer (Pharmacology), Regional Ayurveda Institute of Fundamental Research, Under CCRAS; Min. of AYUSH, Nehru Garden, Gandhi Bhavan Road, Kothrud, Pune-411038, Maharashtra, INDIA.

Phone no : +91 020 25380326

E-mail: sd_pawar@yahoo.com

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Ayurveda

Traditional herbal medicines those originating from China, Korea and India incorporate a huge variety and wide range of plant species with beneficial medicinal properties that have conventionally been relied to treat ailments and disease. Ayurvedic medicine is one of the world's oldest medical systems that originated in India, more than 5,000 years ago and has evolved over thousands of years. These medicines are getting popularity in recent years, as they are used as alternatives to the conventional pharmaceutical drugs / product, mainly because; such products are natural and have less or no side effects.

The term "Ayurveda" combines the Sanskrit words Ayur (life) and veda (science or knowledge), thus 'Ayurveda' means "the science of life and art of living". Ayurveda, has uniqueness in providing treatment to humanity. As this system believes in not only treating the diseases, but also has shown various methods and procedures in protecting the health of every healthy individual (*Swasthasya swasthya rakshanam*).

In the United States, Ayurveda is a system of Indian traditional form of alternative medicine or considered as Complementary and Alternative Medicine (CAM). In 20th and 21st century, there is an increasing interest in Ayurvedic proprietary due to the side effects of synthetic drugs.

Ayurveda is considered to be the oldest healing science and traces its roots to the Vedic period and is known as fifth Veda and an upaveda of Atharva Veda. According to Hindu Mythology, Brahma is regarded as the Adya (first) Guru of Ayurveda. It stems from the ancient Vedic culture and the knowledge was taught in an oral tradition from accomplished masters to their disciples for thousands of years.

Some of this knowledge was set to print a few thousand years ago, but much of it is inaccessible. A structured presentation or making a treatise on Ayurveda had taken place only between 2nd century B.C. and 10th century A.D. The recreation of such lost data from the experiments that spread over years produced perfect results which are described in concentrated forms in ancient books like *Charaka Samhita, Sushruta Samhita* and *Ashtangahridaya*. The most popular and authentic *Samhitas* which are still in use, are *Charaka* and *Susrutha Samhitas*.

Ayurveda deals with special concepts to maintain the health as well as to cure the diseases. As per Ayurveda, health is a balance between dosha, dhatu and malas. Imbalance in these factors may lead to disease condition. Thus, Ayurvedic concepts / treatments are focused towards maintaining / bringing the health factors to normal levels. Ayurveda classical texts have mentioned many herbal and mineral compounds and their formulations to treat various disease conditions. Ayurveda has described tetrads of therapeutic management of diseases in which Bheshaja (Medicine) stands next to the physician for successful management of the diseases. Dravyagun vignan is fundamental inseparable branch of Ayurveda which deals with study of such dravyas (Bheshajas) and their properties (Guna), actions (Karma), dose, time of administration and various preparations of these drugs. The utility of dravya in Ayurved depends on the 'Rasa', 'Guna', 'Veerya', 'Vipaka' and 'Prabhava' which can be considered as pharmacodynamic principles of drug/ Dravya through which drug action can be assessed.

The texts describe eight branches of ayurvedic medicine: Internal medicine; Surgery; Treatment of head and

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neck disease; Gynecology, obstetrics and pediatrics; Toxicology; Psychiatry; Care of the elderly and rejuvenation; and Sexuality vitality.

Ayurvedic herbs and raw drugs

An ayurvedic herb can be defined as any plant "with leaves, seeds or flowers used for flavouring food, medicine or perfume".[1] As per the definition provided by the European Parliament and Council, "Herbal medicinal product is exclusively containing as active ingredients one of more herbal substances or one or more herbal preparations".

Ayurveda and Traditional Chinese Medicine (TCM) utilize naturally occurring herbal and animal ingredients in combination with meditation, dietary regulation and hygienic living with holistic approach. These treatments have been trusted for more than thousands of years and are the intrinsic part of Asian cultures.[2]

Ayurvedic medicinal products are used popularly by Western people, either as alongside with prescribed medicine or relied completely for ailments ranging from acne to cancer. A survey commissioned by MHRA, in 2008, reported that 35% adults had used herbal medicines purchased over the counter, on the internet or from a herbal practitioner or clinic in 2006-07. The global market value for such product was estimated US $83 billion in 2008.[3]

Adulteration and Substitution

The traditional systems of medicine utilize medicinal plants to cure various ailments but the herbal medicine industry suffers from various substitution and adulteration of medicinal herbs with closely related species. The adulteration and substitution of the ayurvedic drugs is the major concern of herbal medicinal industry which requires immediate attention to maintain the quality and standards of Ayurvedic medicines for the betterment of human kind.

Herbal drugs used to treat illness, are often misidentified or adulterated with similar plant materials. Overuse of the raw material in the form of roots, barks has made most of the species to be extinct or made them to fall under the category of RETO (Rare, endangered, threatened and overexploited). The deforestation and extinction of many species as well as incorrect identification of medicinal plants has resulted in adulteration and substitution of raw drugs. Due to adulteration faith in herbal drugs has declined over the period of time.[4]

Ayurvedic plants which are usually grown worldwide may not face the problem of substitution or adulteration like *Anethum graveolens*, *Foeniculum vulgare*, *Linum usitatissimum* etc., as these plants have history of cultivation and are widely grown around the world. Identification of such plant may pose no problems at the time of collection, however it may be difficult to distinguish leaf and seed material once it is dried and packaged, for example, *Anethum graveolens* and *Foeniculum vulgare*.

Production of ayurvedic drugs or product is often not strictly regulated, which leads to quality control problems. Identification of the plant material used for the preparation of drugs after processing or grinding becomes difficult.

Substitutions

Ayrurvedic Acharyas (Scholars of Ayurvedic) found and identified the substitute which is context specific; in such condition principle of the drug is taken into consideration. This provided physician a huge scope for selection of the drug, which is most appropriate and easily available. The most essential criteria for substitution are the pharmacological activity rather than morphology or phytoconstituents. (Table 1)

Need for Substitution

There are various needs for the substitution of raw drug as mentioned by Sarin, 1996;[5] Mishra *et al*. 2002;[6] Mukherjee, 2002;[7] and Shastri *et al*. 2005[8] are as follows:

I. Non-availability of the drug

Example - Substitution for *Ashtavarga Dravyas* (group of 8 highly potent drugs mentioned in ayurvedic literature).

II. Uncertain identity of the drugs

Example - For the drug *Lakshmana* different species such as *Arlia quinquefolia*, *Ipomea sepiaria* are considered.

For the drug *Agnimantha* different species of *Clerodendrum* sps and *Premna* sps are considered.

III. Cost of the drug

Example - *Kumkuma* being costly herb, it is substituted by *Kusumbha*

IV. Geographical distribution of the drug

As Rasna, *Pluchea lanceolata* is used in Northern India while *Alpinia galanga* is considered as Rasna in Southern parts of India.

V. The adverse reaction of the drug

Vasa (*Adhatoda vasica*) is well known Rakta-Pittahara drug (useful in bleeding disorders), but due to its abortificiant activity its utility in pregnant women is limited and instead of that *Laksha* (*Laccifera lacca*), *Ashoka* (*Saraca asoka*) etc are substituted.

Criteria for substitution

As per the Aushadhi vignana (Science of medicinal plant), a drug to be considered as a substitute should fulfill the following criteria

- They should possess similar phytochemically active principles – Ex. *Bharangi* (*Clerodendrum indicum*) and *Kantakari* (*Solanum xanthocarpum*).
- Substitute should exhibit similar therapeutic effects – Pashanbheda (*Bergenia ligulata* and Aerva lanata); Ativisha (*Aconitum heterophyllum*) and *Musta* (*Cyperus rotundus*).
- In the formulation of any drug, the major ingredient should never be substituted.

A. Substitutions can be with totally different drug

Ex. *Bharangi* (*Clerodendrum indicum*) and Kantakari (*Solanum xanthocarpum*) produce glycosides and have shown antihistaminic activity. Both of these are commonly associated and used in the treatment of respiratory problems.[9]

B. Substitutions can be from different families

This is mainly because of the same local / ayurvedic names to two different plant species. Ex. Gokshura – *Tribulus terrestris* (Zygophylaceae) has chemical constituents like chlorogenin, diosgnin, rutin, rhamnose, alkaloids and *Pedalium murex* (*Pedaliaceae*) has sitosterol, ursolic acid, vanillin, flavanoids and alkaloids. Both these are known to have nephroprotective, lithotriptic, diuretic and hepatoprotective activities, therefore both appear to be appropriate.

C. Substitution from same Genus

Datura metal and *Datura stramonium* have chemical constituents like alkaloids, scopolamine, atropine, hyocymin, lyoscine; alkaloids are proved as bronchodialatory and inhibitor of secretion of mucus membrane. Alcoholic extracts is useful in this case. Thus, as far as respiratory tract diseases are concerned both the drugs are beneficial.

D. Substitution of different parts of the same plant

The roots of *Sida cordifolia* is usually substituted by the whole plant of *Sida cordifolia*. Root has sitoindoside, acylsteryglycoside and whole plant has alkaloid, hydrocarbons, fatty acids, ephidrin. Various extracts showed antibacterial, antioxidant, hypoglycemic and cardio tonic activities. These are found to be equally effective when used as medicinal drug.

Manjishta (*Rubia cordifolia*) roots are often substituted with whole plant due to less availability of the roots.

Table 1: Commonly substituted drugs in Ayurveda.

Sr. No.	Common name	Botanical name	Substitute drug	Botanical name
1	Ahimsa	*Capparis sepiaria*	Manakanda	*Alocasia indica*
2	Amlavetas	*Garcinia pedunculata*	Chukra	*Garcinia indica*
3	Ativisha	*Aconitum heterophyllum*	Mustaka	*Cyperus rotundus*
4	Bakula	*Mimusops elengi*	Kamala	*Nelumbo nucifera*
5	Bakula (bark)	*Mimusops elengi*	Babul bark	*Acacia arabica*
6	Bhallataka	*Semecarpus anacardium*	Nadi Bhallataka	*Semecarpus travancorica*
7	Bharangi	*Clerodendrum serratum*	Kantakari	*Solanum xanthocarpum*
8	Chavya	*Piper chaba*	Pippali(root)	*Piper longum*
9	Chitrak	*Plumbago zeylanica*	Danti	*Baliospermum montanum*
10	Dadim	*Punica granatum*	Vrikshamla	*Garcinia indica*
11	Dhanavayasa	*Fagonia cretica*	Duralabha	*Alhagi pseudalhagi*
12	Draksha	*Vitis vinifera*	Kashmari phala	*Gmelina arborea*
13	Ikshu	*Saccharum officinarum*	Nala	*Arundo donax*
14	Jatipatra (Aril)	*Myristica fragrans*	Lavanga	*Syzigium aromaticum*
15	Jatipatra (Aril)	*Myristica fragrans*	Jatiphala (fruits)	*Myristica fragrans*
16	Kakoli	*Lilium polyphyllum*	Asvagandha	*Withania somnifera*
17	Karpua	*Cinnamomum camphora*	Granthi parna	*Leonotis nepetafolia*
18	Kshirakakoli	*Fritillaria roylei*	Asvagandha	*Withania somnifera*
19	Kusha	*Desmostachya bipinnata*	Kasha	*Saccharum spontaneum*
20	Kutherika	*Ocimum basilicum*	Gramya tulasi	*Ocimum sanctum*
21	Murva	*Marsdenia Tenacissima*	Jinghini	*Lannea coromandelica*
22	Nagapuspa	*Mesua ferrea*	Padma kesar	*Nelumbo nucifera*
23	Puskar mool	*Inula racemosa*	Kustha	*Saussrea lappa*
24	Puskar mool	*Inula racemosa*	Eranda (root)	*Ricinus communis*
25	Riddhi and Vriddhi	*Hobenaria* spp	Varahikanda	*Dioscorea bulbifera*
26	Tagar	*Valeriana wallichii*	Kustha	*Saussrea lappa*
27	Tulasi	*Ocimum sanctum*	Nirgundi	*Vitex negundo*

- Source: (Sastry, 2002; Chunekar, 2004; Poornima 2010).[10-12]

Adulteration

It is the substitution of the original crude drug partially or fully with some other substances, which may have inferior or may not have therapeutic and chemical properties at all,[12] or low grade or spoiled drugs or entirely different drug similar to that of original drug substituted with an intention of gaining profits.[7,13]

Therefore, adulteration may also be defined as mixing or substituting the original drug material with other spurious, inferior, defective, spoiled, useless other parts of same or different plant or harmful substances or drug which do not confirm with official standards.[14]

Types of adulteration

I. Adulteration with inferior or substandard commercial varieties

When adulterants resembles the original crude drug morphologically, chemically, therapeutically but is of low standard in nature and cheaper in respect with cost, this is the most common type of adulteration observed with market samples.[13,14]

Ex. *Piper nigrum* (Maricha) adulterated by Papaya seeds.

II. Adulteration with artificially manufactured substitutes

The drug is adulterated with the substance which has been prepared artificially and resembling the crude drug. This method is usually followed for the costlier drugs.[13,14]

Ex. Adulteration by invert Sugar for Honey

III. Adulteration by exhausted drugs

In this type same drug is admixed, but it is devoid of the mechanically active substances, as usually it has been extracted already. Mainly volatile oil containing drugs like clove, coriander, fennel are adulterated by this method. As it is devoid of colour and taste due to extraction, natural colour and taste is manipulated with additives.

Ex. Use of Clove, Fennel

IV. Adulteration by heavy metals

Heavy metals are added in the drug to increase the volume and weight of the samples and such adulteration is always harmful.

Ex. Addition of pieces of limestone in *Asafoetida* (Hing),

Use of lead in Opium

V. Adulteration by synthetic preparations / principles

This is carried out when synthetic chemicals are used to enhance natural character of the exhausted drug.[13,14]

Ex. Using Citral oil in place of lime oil or orange oil.

VI. Vegetative matter adulterant

Some miniature plants growing with the medicinal plants are added, knowingly or un-knowingly due to their colour, odour and constituents.[14]

VII. Harmful adulterants

Some harmful materials as the adulterant are collected from market waste materials and admixed with drug. This is usually done for the liquid drugs.[14]

Ex. *Argemon mexicana* seed oil mixed with mustard (*Brassica nigra*) oil can cause harmful effects like nephrotic syndrome, skin rashes etc

VIII. Adulteration of powders

The drugs which are in powder form are usually adulterated.

Ex. Fruit hair powder of Kampillaka (*Mallotus philippinensis*) is of brick red colour, having the best vermicidal effect and is often adulterated with brick powder,[15] Dextrin is added in ipecacuanha (dried root powder of *Cephaelis ipecacuanha*),

Red sandlers wood in capsicum powder

Powder drug of Kapikachu (*Mucuna pruriens*) is often adulterated with *Mucuna cochinsinensis* or *Mucuna utilis*, depending upon the availability of the species. *Mucuna pruriens* (L) DC. Is available all over India and is quoted as *Kapikachu* by many research scholars / physicians and modern textbooks of Ayurveda. In most of markets of India especially in northern parts *Mucuna cochinchinensis* (Lour.) A. Chev. (White variety) plant seeds are being used as *Kapikachu*. *Mucuna cochichinensis* (Lour.) A. Chev. (Black variety) seed are used as *Kapikachu* in the southern parts of the country, especially in Kerala by Ayurvedic physicians. Seeds of *Mucuna pruriens* are adulterated with *Mucuna utilis* and *Mucuna deeringiana* due to the similarity in morphology. Apart from this, *Mucuna cochinchinensis*, *Canavalia virosa* and *Canavalia ensiformis* are also sold in Indian markets.[5,14] All these varieties have been estimated to have varied level of L-dopa. L-dopa is a one of the major component of *Mucuna* sps responsible for spermatogenesis, due to which *Kapikachu* is used in ayurvedic practices for the treatment of infertility.[16,17]

Causes / reasons of Adulteration and Substitution

The deforestation and extinction of many species and incorrect identification of many plants has resulted in this adulteration and substitution of raw drugs. The concept of substitution ages back found in Ayurveda in the treatises like *Bhavaprakasha* and *Yogaratnakara* mentioning the substitute plants / drugs. This article enlightens the present scenario of the concepts of substitution and trends of adulteration. The lack of cultivation practices, the possibility of inaccurate taxonomic identification at the time of collection and long supply chain from harvesting site to market provides many opportunities for substitution or adulteration of the raw drugs.

In particular, adulteration with morphologically indistinguishable species arise the problem in identification of the material. Ambiguous taxonomy due to confusion between Latin nomenclature and local or vernacular naming / terminology also enhances the problem. Fraudulent substitution using less potent / valuable species or masking of endangered species is responsible for less potency of medicines in curing the disease.

In Ayurveda, 'Parpatta' refers to *Fumaria parviflora* and in Siddha, it refers to *Mollugo pentaphylla*. Owing to the similarity in the names in traditional system of medicine, these two herbs are often interchanged or adulterated or substituted. Usually in Southern parts of India *Mollugo pentaphylla* is used as Parpatta due to the popularity of Siddha system of medicine and in Northern parts, *Fumaria parviflora* is used.[14] *Casurina equisetifolia* for *Tamarix indica* and *Aerva lanata* for *Berginia ciliate* are some other example for adulterations due to confusion in names.[5]

Due to the lack of knowledge about authentic source of the drug, adulteration in Ayurveda is often observed. 'Nagakesar' is one of the important drugs in Ayurveda, used to treat the bleeding disorders as a coagulant. The authentic source of 'Nagakesar' is *Mesua ferrea*, however, market samples are often adulterated with flowers of *Calophyllum inophyllum*. Another reason of its adulteration could be availability of the *Mesua ferrea* in the Western Ghats and Himalayan regions.[5]

Increasing demand of the ayurvedic raw drugs and unstable financial environment have provided the impetus behind adulteration of the product with other species of a plant, heavy metals and pharmaceutical substances. The booming traditional Chinese medicine and Ayurvedic industry has resulted in the scarcity of wild species which are commonly incorporated in the drug preparations. Familiar example for this statement is, American ginseng (*Panax quinquefolius*) and Asian ginseng (*Panax ginseng*) are at the risk of extinction due to unregulated trade and were recently included in the Convention on International Trade in Endangered Species (CITES), appendix II.[18,19] *Panax ginseng* is highly effective than *Panax quinquefolius*, but roots are not distinguishable when sliced or processed, which leads to the fraudulent substitution practices.[19]

Lack of authentic plant in a particular geographic region is also one of the important reasons for adulteration. *Hypericum perforatum* is cultivated and sold in European markets. In India, availability of this species is limited; however *Hypericum patulum* is abundant in Indo-Nepal region and is sold in place of *Hypericum perforatum*.[5] In place of *Berberis aristata*, a plant available at the high altitude (Northern parts of India) locations is being adulterated with *Coscinium fenestratum*, which is available in plains (Southern parts of India).

With the course of time, drug materials get changed to or substituted with other plant species. In past, roots of *Ventilago madraspatana* were collected in the past as a raw material for the drug 'Ratanjot', while recently *Arnebia euchroma-vareuchroma* is the present source for 'Ratanjot'.[5]

Carelessness of herbal field collectors and suppliers of the herbal plants is the cause of adulteration. For the instance, *Parmelia perlata* is used in Ayurveda, Unani and Siddha, is most of the time admixed with *Parmelia perforate* and *Parmelia cirrhata*; mainly due to the identification of the herb is by their thallus nature.[14]

Drawbacks of substitution and adulteration

The efficacy of the drug decreases if substituted and in some cases, can be lethal if adulterated with toxic adulterants. Substituting one species for another may have minimal effect on product's efficacy but in some cases the beneficial effect of product may be lost completely. Many times substitution within certain plant families could be fetal, especially Apiaceae and Solanaceae, as medicinal and culinary plant species may look very similar to the poisonous species. Correct formulation is important for the medicinal herb for being effective in curing or in the treatment of any disease.

Adulteration of herbal medicines can put consumers at risk of unknown chemical interactions with other drugs and can be allergic to the patients.

Identification and barcoding of Ayurvedic herbs

Medicinal plants which are widely used throughout the world are often in the form of packaged herbal preparations manufactured by either different pharmaceutical companies of manufacturers. For such preparations raw material is predominantly collected from the wild source. Barcoding of raw ingredients can confirm or disprove the identity of medicinal plants before they are processed.

Traditional approaches for identification of herbal products include morphological examination of phenotypic characteristics, microscopy and chemical analysis. These traditional methods of medicinal plant identification include organoleptic methods (*i.e* identification by shape, colour, texture); and chemical profiling (*i.e.* Thin Layer Chromatography; High Performance Liquid Chromatography- UV; High Performance

Liquid Chromatography-Mass Spectrocopy). Morphological and microscopic assessment requires skills and experience that are increasingly scarce as well as these are proven to be subjective. Such identification technique also has disadvantage that experts do not always agree upon the same species identity. Current chemical detection methods identify the contents of herbal medicines using absorbance spectra of chemically characteristic metabolites. High Performance Liquid Chromatography (HPLC) is most common method for analysis of ayurvedic and herbal medicine components. HPLC in tandem with mass spectroscopy could resolve such identification problems in better way chemically on the basis of molecular weight. Though chemical analysis is used for primary detection of the compounds present in the sample, it lacks the accuracy when species or population determination is required.[20]

Chemical fingerprinting can often be heterogeneous within one species, as it depends on the age of plant or soil effect on the plant, or altitude at which plant is grown etc. These methods are reliable to detect the presence of heavy metal or other pharmaceuitical compound; however these are not sufficient in detection of trace amounts of a single herbal species in a mixed sample.[21]

However, neither of the above-mentioned methods can identify the related species easily in processed products because the former method requires trained personal for macroscopic and microscopic examinations. In the later method, chemical profile of markers may be affected by physiological and storage conditions.

Solution to resolve both the ambiguity of species identification using morphological examination and the chemical analysis may lie in the study of the molecular composition of a herbal plant.

Molecular biomarkers

The product barcode has become a universal feature of modern life. A barcode is a machine-readable digital tag, usually a series of stripes, which encodes information about the item to which it is attached. The barcode can include some systematic or 'taxonomic' information yielding data such as origin, major classification, date, type etc. Similar universal system is used in publishing, where the ISSN / ISBN uniquely identify the book, publisher and edition.

Molecular barcoding methods are reliable tools for the identification of medicinal plants, their substitutes and adulterants at the genus, species and many times up to varietal level. DNA barcoding provides consistency and reliability in terms of results regardless of the age, plant part or environmental conditions where plant grows. DNA is ideal to analyte as it is present in all parts of the plant and has the potential to be detected even after mechanical processing and heat treatment.

Due to rapid increase in demand of herbal remedies, there is need for accuracy in identification and authentication of the Ayurvedic plant material. Therefore, it is necessary to provide a sole, extensive database with DNA data for easy and scientifically correct identification.

Taxonomy of the medicinal plant at DNA level provides more reliable tool, as in contrast to RNA, DNA is stable macromolecule that is not affected by external factors and is found in all tissues. Therefore, development of DNA-based markers is important for authentication of medicinal plants.

The increased availability of accurate species-specific DNA sequence information facilitated by the establishment of open access database has laid the foundations for DNA molecular approaches to impart specific, sensitive and reliable identification of herbal medicinal plants and their products.[2]

A DNA barcode derived from the sequence of a part of the genome of the organism theoretically can carry both specific as well as systematic data. Evolutionary aspect qualitatively differentiates DNA barcodes from others.

DNA barcoding

It is a method of identifying an organism based on sequence data from one or several gene regions. DNA barcoding involves the generation of DNA sequencing data from particular genetic regions in an organism and the use of these sequence data to identify or "barcode" that species and distinguish it from others. The term DNA barcode as taxon identifiers was first proposed by Paul Hebert of University of Guelph in 2003.[22] There are several challenges to the successful implementation of plant DNA barcoding despite of which, DNA barcoding has potential to uniquely identify medicinal plants and provide quality control and standardization of the plant material supplied to the pharmaceutical industry. DNA barcoding is a desirable tool for ayurvedic product authentication.

The novel technique of identifying biological specimens using short DNA sequences from either nuclear or organelle genomes is called DNA barcoding. Main principle of barcoding is matching the sequence data from a query sample (may be an unknown specimen) to a reference sequence (from voucher specimen and/or allied specimens).

Molecular methods for detection and identification of species in herbal medicinal products have clear potential to be fit for purpose, as samples can be identified regardless of appearance and chemical constituents (which may vary according to the tissue type, growth conditions or age of the sample) and the sample can be taxonomic mixture. DNA molecule is ubiquitous in nature and it is expressed at all developmental stages of a cell's life cycle, as well as DNA molecule is robust enough to remain relatively intact even when subjected to high temperatures and highly processed chemical reactions. DNA barcoding is completely specific and demonstrate reliable sensitivity and can be used even at the low concentrations of herbal samples.

Barcoding has multiple applications and has been used for ecological surveys,[23] cryptic taxon identification[24] and confirmation of medicinal plants.[25] DNA barcoding not only helps in the identification of species but also defines species boundaries and species delimitation.[26,27]

In plants the mitochondrial genes are slowly evolving, with very low substitution rates and were not suitable for barcoding, as they are commonly used in animals and fungal taxonomy. Due to these reasons search for plant barcode shifted to chloroplast and nuclear genomes with high substitution rates.

Methods used in DNA barcoding

PCR based DNA barcoding methods include RFLP, AFLP, RAPD, SSR, ISSR, hybridization, microarrays, sequencing of genomic regions as well as DNA chip technology used to identify traditional Chinese medicinal materials.[28] DNA fingerprinting can be achieved using various methods as they yield barcode-like data, but these are less optimal for a molecular taxonomy because of problems with high variability within taxon and lack of confident assignment of orthology between markers. (Figure 1)

DNA sequencing can overcome these hurdles. With sequence based molecular taxonomy, a single technique is applicable to all taxa: extract DNA, PCR and sequence. Different gene regions can be used for DNA barcoding of the plant, for which various studies have been carried out and are discussed in the chapter.

RFLP (Restriction Fragment Length Polymorphism) technique

Advent of Restriction Fragment Length Polymorphism (RFLP) was a significant historical milestone in the hunt for molecular polymorphism.

This technique exploits the presence of nucleotide substitutions or insertions or deletions within the restriction sites of enzymes isolated from bacteria and archaea. These restriction endonucleases amplify the genomic products after digesting the DNA at the restriction site and produce fragments of varying length. When samples are compared using this technique and if they are different from each other then polymorphism will be observed. This polymorphism can be visualized by gel electrophoresis and for large genomes, by Southern blot.[29] This technique is highly reproducible, but it is time consuming and labour intensive.

AFLP (Amplified Fragment Length Polymorphism) technique:

Vos *et al*.[30] Combined RFLP with selective PCR amplification to create AFLP. In this technique, adapters are ligated to the ends of the restriction site of newly cleaved fragments of DNA after digestion with restriction endonucleases. Primers are designed to anneal to these fragments and extend into restriction site so as to get selective amplification sequences.

Ghosh *et al*.[31] Designed an AFLP assay successfully to distinguish and discriminate between *Zingiber officinale* and its common adulterant *Zingiber* sps. In their study, they found that seven set of primers can produce 99.7% polymorphism.

RAPD (Random Amplified Polymorphic DNA) technique:

RAPD was developed by Williams *et al*.[32] To overcome the economic and temporal losses of RFLP. Amplification of DNA is carried out by using single arbitrary primer, which will produce varying sized fragments for different species. Primers which are used in the RAPD are less stringent than that of restriction enzymes therefore this technique lacks reproducibility.

Shinde *et al*.[33] Identifies the three main components of an ayurvedic medicine using RAPD technique. For this success they screened 120 sets of primers and then one was selected for discriminating between three known species.

This technique has been used by Shirolkar *et al*.[34] To differentiate the *Marsilea minuta* from *Marselia quadrifolia* which are difficult to identify when dried. Similar results were obtained when different species of *Desmodium* were analyzed and differentiated using RAPD technique.[35]

RAPD technique has been used for differentiating the varieties of *Kapikachu*, as it possess the spermatogenesis property and used in Ayurveda for treating the related ailments. This is carried out by the authors because *Mucuna pruriens* is often adulterated with the *Mucuna cochinsinesis* as per the availability of the raw drug. Shah *et al*.[36] Created the baseline data to study the genetic markers of *Mucuna* species as the information on the genetic diversity of *Mucuna*.

ISSR (Inter Simple Sequence Repeats) technique

ISSR is stretches of DNA that are present between repeat motifs in DNA. For this technique PCR primers are designed to anneal the repeat motifs and amplify the intervening genomic regions. Level of polymorphism will decide the number of PCR products and the method is often referred as DNA fingerprinting.[37]

Geographically distinct *Asparagus acutifolins* plants were analyzed using ISSR technique and found that it is appropriate for closely related accessions.[38]

SSR (Simple Sequence Repeat) technique

In this technique primers are designed to amplify the repetitive DNA sequence which can vary in size between closely related plant varieties based on number of repeat motifs. It is obligatory for the method to sequence the information for plant species. It is a genetic mapping technique which utilizes the fact that microsatellite sequences "repeat" (*i.e.* appear repeatedly in sequence within the DNA molecule) in a manner enabling them to be used as "markers". Genetic characterization of *Rhodiola rosea* L. was performed by Soni *et al*.[39] using SSR markers and their results showed a significant gene flow between the populations of studied plant.

SCAR – PCR (Sequence Characterized amplified region – Polymerase Chain Reaction) technique

Polymorphic fragments produced by RAPD and ISSR can be sequenced to design primers for SCAR-PCR. It is technique which has become popular for the detection of adulterants in herbal medicines.[28] This technique is more reproducible than RAPD and ISSR methods, as amplification of a single target results in a clear presence or absence, therefore this method is used in the detection of single species.

Gene regions used as DNA barcodes

Molecular DNA approaches for speciation and taxonomic studies often capitalize upon genes that are universally present in eukaryotic genomes, such as 18S ribosomal RNA subunit gene. Within these sequences highly conserved DNA regions are present, which can be easily used for species differentiation.[40] Use of such universal primers often facilitates rapid and detailed species information from DNA sample. But as per the name, "Universal" primers do not exclusively target species specific DNA sequences and often create confusion when closely related species are studied.

The consortium for the Barcode of Life Plant Working Group (GBOL) evaluated seven chloroplast genomic regions across the plant kingdom and proposed a combination of maturase K (*matK*) and ribulose 1,5-bisphosphate carboxylase/oxygenase large subnut (*rbcL*) as plant barcodes.[41] High universality but less species resolution is provided by *rbcL* whereas *matK* affords high resolution but less universality.[25]

Plastid genes are uniparentally inherited, so reliance on these sequence may lead to incomplete analysis when alloploid (an organism containing the genetic information of two or more species) or hybrid species are encountered.[42,43] The Internal Transcribed Spacer regions (ITS and ITS2) in ribosomal DNA have great deal of interest as alternatives or supplements to the plastid genes.

A combination of *matK* and *rbcL* can help to achieve maximum species discrimination and differentiation. According to Cameron *et al*.[44] and Singh *et al*.[16] in closely related species discrimination ability of these two markers is low. Therefore, China Plant BOL Group proposed the addition of nuclear ITS (Internal Transcribed spacer) as plant barcode, in order to achieve maximum accuracy in identification of medicinal plants.[45]

Along with maturase K (*matK*) and ribulose 1, 5-bisphosphate carboxylase/oxygenase large subnut (*rbcL*) several chloroplast gene regions are typically used as plant barcodes.[46] The *matK* is located in the large single copy region of chloroplast genome, nested between the 5' and 3' exons of *trnK, t-RNA*-lysin. The space region between tRNA-His and photosystem II protein D1 (*trnH-psbA spacer*) and nuclear internal transcribed spacer 2 (ITS2) are also widely used.[20,4748] The *trnH-psbA* spacer is among the most variable plastid region in angiosperms. It is popular tool for plant population genetic and species level authentication.[49,50] The combination of *rbcL, trnH-psbA* provided the most accurate and efficient barcoding tool for the authentication of *Cassia* medicinal plant products.[51,52]

Many barcode regions (*matK, rbcL,* ITS, ITS2, *psbA-trn*H, *atp*F, *ycf*5, *psb*K-I, *psb*M *trn*D, *cox*I, *nad*1, *rps*16, *trnL-F, rpo*B, *rpo*C1, *atp*F-*atp*-H) of medicinal plants were recently reported to aid in the authentication

and identification of medicinal plant materials.[53,25] According to Techen *et al.*[25] the majority of barcoding regions mentioned in the literature were ITS (26 references), ITS2 (9 references), *psbA-trnH* (21 references), *matK* (19 references), *rpoC1* (6 references), *rpoB* (4 references), *trnL-F* (3 references) and *rbcL* (14 references).

Twelve genomics regions of 95 samples were analyzed by Zuo *et al.*[54] And demonstrated that the *psb-trnH* and ITS combination would be sufficient for the identification, which was true for the samples they used in their study. Whereas, for other medicinal plant samples analyzed by Guo *et al.*[54] and Sui *et al.*[55] it was observed that the combination up to three genomic regions (*matK, rvcL,* ITS, *psb-trnH*) provided accuracy in the identification.

Chen *et al.*[56] analyzed more than 6600 plant samples belonging to 4800 species from 753 distinct genera using different genomic regions such as: *psb-trnH, matK, rbcL, rpoC1, ycf5,* ITS and ITS2. From their data it could be concluded that, ITS2 of nuclear ribosomal DNA is the most suitable (92.7% successful) region for DNA barcoding applications.

He *et al.*[57] and Selvaraj *et al.*[58] also analyzed multiple genomic barcode regions and suggested that ITS or ITS2 show the highest discrimination rate among the samples. But, as per the contrasting opinions of Asahina *et al.*[59] Ma *et al.*[60] Tezcan *et al.*[61] Sun *et al.*[62] and Liu *et al.*[63] the highest discrimination and accuracy was found to be from *psbA-trnH* and *matK*.

Theodoridis *et al.*[64] when analyzed the medicinal plants of Lamiaceae family, using *matK, rbcL, psb-trnH* and showed that *matK* and *psb-trnH* were useful as in discriminating the species.

Single barcode-regions for identification have been reported for *matK*.[50,61,63,65-82]

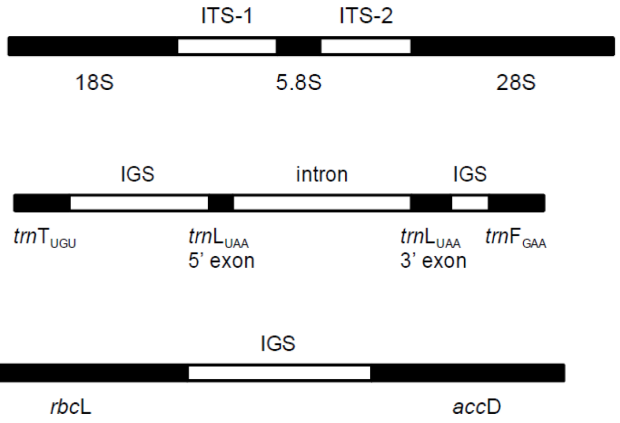
Multi-region approach to the barcoding is supported by most of the literature.[43,83-87] Such multi-gene (*matK* and *trnH-psbA*) approach was explored in the identification of ethnomedicinal plants (*Catharanthus roseus* (L.) G. Don., *Alstonia scholaris* (L.) R.Br.., *Thevetia peruviana* (Pers.) Merrill, *Calotropis gigantean* (L.) R.Br. Ex Ait) from Apocynaceae family through barcoding.[88]

An attempt was made to identify the medicinal plant genus *Paris*, using DNA barcoding technique by Zhu *et al.*[56] Five chloroplast sequences, *psb-trnH, rpoB, rpoC1, rbcL, matK* and one nuclear marker, ITS2 were amplified and sequenced. They concluded that ITS2 can be used for accurate identification of *Paris*. Importance of ITS2 as barcode was also supported by Pang and Chen.[89]

The identification of various species using Internal Transcribed Spacer (ITS) as DNA barcode was successfully conducted for *Rauvolfia serpentina* (L.) Bth., *Crotalaria juncea* L., *Jatropa curcas* L., *Jatropa gossypifolia* L., *Pongamia pinnata* L.) Pierre., *Turnera ulmifolia* L., *Butea monosperma* (Lam.) Taub Var. Monosperma, *Ricinus communis* L. and *Xanthimum indicum* Koen.[90] ITS region is shown to distinguish the medicinal plant Boerhavia diffusa from its adulterants and potential of the ITS region as barcoding region was confirmed.[58]

DNA barcoding for Apiaceae family of angiosperm was carried out by Liu *et al.*[91] Mainly to identify the medicinal plant species from this family and their adulterants. A total of 6032 sequences representing 1957 species in 385 diverse genera were sampled, of which 211 sequences from 50 individuals (representing seven species) were newly obtained. For the barcoding *rbcL, matK, psbA-trnH* ITS and ITS2 markers were used. Their results confirmed that ITS or ITS2 could be better option when incorporated into the core barcode for Apiaceae and combination of ITS/ITS2 + *psbA-trnH* has much potential value as a powerful standard DNA barcode for Apiaceae identification.

Vassou *et al.*[92] conducted a case study to authenticate the market samples of *Sida cordifolia* using DNA barcoding method (*rbcL, matK, psbA-trnH* and ITS markers). Their observation suggested that, it is wrong belief



Name	Primer sequence 5'-3'	Taxonomic group	Reference
rbcL primers		**Vascular plants**	
rbcLa-F	ATGTCACCACAAACAGAGACTAAAGC		Levin, 2003
rbcLa-R	GTAAATCAAGTCCACCRCG		Kress & Erickson, 2007
rbcLajf634R	GAAACGGTCTCTCCAACGCAT		Fazekas, 2008
matK primers		**Flowering plants**	
MatK-1RKIM-f	ACCCAGTCCATCTGGAAATCTTGGTTC		Ki-Joong Kim, pers. comm.
MatK-3FKIM-r	CGTACAGTACTTTTGTGTTTACGAG		Ki-Joong Kim, pers. comm.
MatK_390f	CGATCTATTCATTCAATATTTC		Cuenoud *et al.* 2002
MatK_1326r	TCTAGCACACGAAAGTCGAAGT		Cuenoud *et al.* 2002
psbA-trnH primers		**Vascular plants**	
psbA3_f	GTTATGCATGAACGTAATGCTC		Sang *et al.* 1997
trnHf_05	CGCGCATGGTGGATTCACAATCC		Tate & Simpson, 2003
ITS2 primers		**Vascular plants**	
ITS2-S2F	ATGCGATACTTGGTGTGAAT		Chen *et al.* 2010
ITS4	TCCTCCGCTTATTGATATGC		White *et al.* 1990
ITS primers		**Fungi**	
ITS5	GGAAGTAAAAGTCGTAACAAGG		White *et al.* 1990
ITS4	TCCTCCGCTTATTGATATGC		White *et al.* 1990
ITS1	TCCGTAGGTGAACCTGCGG	internal	White *et al.* 1990
ITS2	GCTGCGTTCTTCATCGATGC	internal	White *et al.* 1990
ITS3	GCATCGATGAAGAACGCAGC	internal	White *et al.* 1990

Figure 1: Diagrammatic representation of plant barcoding genes. (Source: CCDB protocols)

that medicinal plants are generally substituted or adulterated with closely related species, as none of the samples obtained from market belonged to the authenticate species. Seventy-six per cent of the market samples belonged to other species and 24% of the samples were from other genera. Such substitutions will definitely fail to give the expected therapeutic effect and may give undesirable or sometimes lethal effects.

Rai *et al.*[93] Supported the use of ITS2 region for DNA barcoding of authentic and substitute samples of herb of the family Asparagaceae and Ascepiadaceae.

Challenges in DNA barcoding and solutions

There are several potential challenges in barcoding because of which this technique seems to be difficult for identification of the medicinal plants. For instance some challenges which could be mentioned are, presence of biologically active secondary compounds or metabolites, including tannins, alkaloids, polyphenols, flavonoids and polysaccharides, all of these can inhibit the DNA extraction and amplification by co-precipitating with or binding to DNA.[94] Isolation of pure, high molecular weight DNA from processed medicinal plant material is critical for the successful application of molecular techniques. This challenge could be overcome by either using various commercial kits or modified traditional methods for DNA extraction. Along with these Särkinen *et al.* (2012), found strong negative correlation between amplicon size and PCR success, indicating that shorter fragments are easier to amplify from herbarium DNA, for example genomic region *trnL*P6 loop (10-143 bp) is easier to amplify than amplifying *rbcL* (670 bp).

Availability of single reference sequence can create problem or can interfere in the reliability of results generated using barcoding technique. This can be avoided by including sister species and other closely related taxa to ensure species-level specificity.

Certain gene regions like, *rbcL, trnL, trnL-F* intergenic spacer, has universal primers, which work for most of the plants. Other regions like *matK* is found to be more variable and may require to custom the primer for each plant.[83,94] Along with this, use of single gene region may not give complete reproducible results. The combination of barcode regions that discriminate related species will have to be determined separately for every medicinal plant. Availability of reference sequences for the entire gene regions for each medicinal plants could lead to non-confirmation of the identification of target plant.[95]

Another important challenge in DNA barcoding of market samples is the age and condition of the plant at the time of collection, conditions of drying, processing and transporting. The quality of DNA is likely to be lower in such plant samples. Depending on the plant part collected, it may be difficult to extract DNA, especially in the case of bark or sap.

Species identification and phylogenetic analysis requires accurate multiple sequence alignment, which is time-consuming and highly complicated if multiple indels are present. This problem can be avoided if pairwise alignment software is used.[52]

Despite of all the challenges DNA barcoding has the potential to uniquely identify medicinal plants and provide quality control and standardization of the plant material supplied to the pharmaceutical industry.[94]

Data Availability

It is important and desirable to have access to a single barcode library for medicinal material used. Currently, several barcode libraries are freely accessible.[25]

1. BOLD: The Barcode of life data system – http://www.barcodinglife.com

2. CBOL: Consortium of the barcode of life – http://www.barcodeoflife.org/

3. iBOL: International Barcode of life project – http://www.ibol.org/

4. The GenBank online genetic sequence database – http://www.ncbi.nlm.nih.gov/genbank/

5. MMDBD: Medicinal Materials DNA Barcode Database – http://137.189.42.34/mherbsdb/index.php

6. The GDR: Genome Database for Resource – http://www.rosaceae.org/

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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