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Application of DNA fingerprinting tools for authentication of ayurvedic herbal medicines- A review

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Abstract

Ayurvedic herbal medicines played a pivotal role in maintaining health and treating the disease worldwide since ancient times. Due to renewed interest and popularity the global market strategy is worth about $ 60 billion annually. As commercialization and increased demand safety, quality and assurance of Ayurvedic herbal medicines is biggest lacuna. The adulteration of herbal materials usually occurs as a result of materials not having readily distinguishable morphological features, materials sharing similar common names and the substitution of economically valuable materials with inexpensive herbs. Proper authentication process is necessary to prevent the adulteration. Since the standardization of botanical preparations most of the regulatory authorities suggest macroscopic, microscopic and chemical evaluation like TLC, HPTLC and HPLC. However, these methods have limitations because the composition and relative amount of chemicals in a particular species of plant varies with growing conditions-harvesting period, post harvesting period and storage conditions. The present review mainly focuses on authentication of Ayurvedic herbal medicines by DNA based fingerprinting methods to prevent intentional and in-advertant adulteration or substitution of targeted Ayurvedic medicinal herbs.

Keywords: Adulteration, Authentication, DNA Fingerprinting, Herbal Medicine, Standardization.

Introduction

Ayurvedic herbal medicines played a pivotal role in maintaining health and treating the disease worldwide since ancient times. Due to renewed interest and popularity of Ayurveda and herbal medicine in recent times, the global market trade is worth about $ 60 billion annually[1] and annual turnover of Ayurvedic medicines in international market is 3500 crores.[2] According to WHO guidelines authenticity, purity and safety are important aspects of standardization and in evaluation of traditional medicines, the first step is authentication.[3,4] Authenticity relates to proving the material is true and corresponds to right identity. As commercialization and increased demand safety, quality and assurance of ayurvedic herbal medicines is biggest lacuna. Herbal medicinal plant material are often substituted and/or adulterated either accidentally or intentionally with herbs from closely related species which are morphologically indistinguishable or by materials from unrelated plants. The adulteration of herbal materials usually occur as a result of materials not having readily distinguishable morphological features, materials sharing similar common names and the substitution of economically valuable materials with inexpensive herbs.[5] Proper authentication process is necessary to prevent the adulteration of target plant with other plant materials .For the standardization of botanical preparations most of the regulatory authorities and pharmacopeias suggest macroscopic, microscopic and chemical evaluation. As macroscopic identity of botanical materials is based on parameters like shape, size, colour, texture, surface characteristics, fracture characteristics, odor, taste and such organoleptic properties that are compared to a standard references. Microscopy involves comparative microscopic inspection of broken or powdered crude botanical materials. Chemical profiling establishes a characteristics chemical pattern for a plant material. Chromatography tools like Thin Layer

Chromatography (TLC), High performance thin layer chromatography (HPTLC) and High-performance liquid chromatography (HPLC) are routinely used for qualitative determination of small amounts of impurities.[6] Macroscopic and microscopic examinations and chemical analysis can be used as rapid and inexpensive method for plant identification and detection of contaminants.[7] However these methods have limitations because the composition and relative amount of chemicals in a particular species of plant varies with growing condition-harvesting period, post harvesting period and storage conditions.[8] Each herb contains large number of compounds and therefore it is also not possible to analyze or trace the presence or absence of all compounds of interest either qualitatively or quantitatively. These serious difficulties in testing for active principles or chemical constituents are well known.[9]

Authentication of botanicals which are medicinally valuable is an important issue globally because of unavailability/underutilization of appropriate tools for standardization. Since DNA is more stable and does not vary seasonally and with age of the plant. DNA based fingerprinting techniques have greater role in the authentication of botanicals which are medicinally important. DNA markers are reliable for information on genetic polymorphism as the genetic composition is unique for each species irrespective of plant part used and is not affected by age, physiological condition as well as environmental factors.[10]

The present review mainly focuses on authentication of Ayurvedic herbal medicines by DNA based fingerprinting methods to prevent intentional and in-advertant adulteration or substitution of targeted Ayurvedic medicinal herbs.

DNA Fingerprinting

DNA fingerprints are a bar-code like patterns generated by amplification of chromosomal DNA of an individual which can distinguish the uniqueness of this individual from another.[11] Also called DNA typing, genetic fingerprinting, DNA profiling and DNA typing.[12] DNA fingerprinting in plants can be applied to a number of applications and uses.[13] DNA based marker analysis has been proven as an important tool in herbal drug standardization. DNA is the basic component of all living cells. The characteristics, traits and morphological features of plants are determined by the specific arrangement of DNA base pair sequences in their cell. DNA in cell is made of nucleotides i.e., Adenine, Guanine, Thymine and Cytosine and pentose sugar joined by phosphate bonds. These regulate the production of specific metabolites like enzymes and proteins.[14] DNA fingerprinting is based on the identity of an organism at molecular level i.e., genetic characteristics. The basic technique of DNA Fingerprinting was discovered by Great Britain geneticist Alec J. Jeffrey in 1984. DNA profiling is primarily used in botanicals for protection of biodiversity, identifying markers for traits, identification of gene diversity and variation etc.[15]

DNA markers are used in molecular biology and biotechnology experiments where they are used to identify a particular sequence of DNA. As the DNA sequences are very highly specific, they can be identified with the help of the known molecular markers, which can find out a particular sequence of DNA from a group of unknown.[16]

DNA profiling of plants can be used in solving disputes over the identity of commercially important cultivars.[17]

Types of DNA Based Markers

Various types of DNA based techniques are available to evaluate DNA polymorphism. These are hybridization based methods, polymerase chain reaction (PCR) based methods and sequencing based methods.[18]

Generally accepted classification of markers is as follows:[19,20]

Hybridization based

- Restriction Fragment Length Polymorphism (RFLP)
- Variable Number Tandem Repeat (VNTR)
- Probe hybdriduzation with Micro and Minisatellite
- Random Genomic Clone
- cDNA Clone

PCR based

- Inter Simple Sequence Repeat (ISSR)
- Random Amplification Polymorphic DNA (RAPD)/Arbitrary Primed PCR
- Amplified Fragment Length Polymorphism (AFLP)
- DNA Amplification Fingerprinting (DAF)

Sequence based

- Simple Sequence Repeats (SSR)
- Sequence Characterized Amplified Region (SCAR)
- Cleaved Amplified Polymorphic Sequence (CAPS)
- Single Nucleotide Polymorphism (SNP).

Methodology of DNA fingerprinting

The basic methodology of DNA profiling in plants involve first the isolation of DNA from plant cells, quantification and quality assessment of isolation. The important steps involved in DNA fingerprinting are shown in Fig no 1.

The further steps are of two types,

1) PCR based. Eg- RAPD, ISSR, SSR
2) Non PCR based. Eg – RFLP.

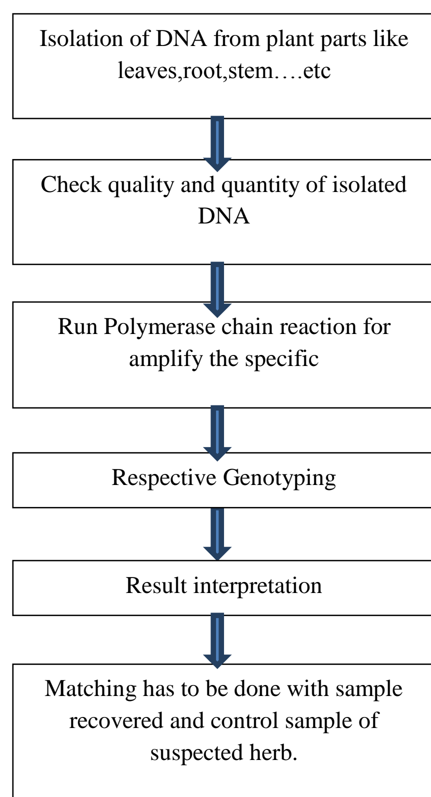

Figure 1: Flow Chart of DNA Profiling

DNA Isolation

DNA from plant tissue is isolated by removal of cell wall and nuclear membrane around the DNA and the separation of DNA from other cell components such as cell debris, proteins, lipids or RNA without affecting the integrity of the DNA. The most commonly preferred method is CTAB method. The DNA is isolated from tissues of plants, generally fresh leaves are preferred.[21]

DNA Quantification and Quality assessment

DNA quantification and quality assessment is done by using UV-VIS spectrophotometry. Normally quality check is performed through the A260/A280 ratio that is 1.8 value shows the highest purity, if more than 1.8 shows the presence of RNA contamination and less than that indicates protein contamination.[22]

Polymerase Chain Reaction (PCR)

The DNA amplification by thermal cycling called Polymerase Chain Reaction is *in vitro* method that can be used to amplify a specific DNA segment from small amounts of DNA template or duplex into millions of copies.

Steps involved in PCR are:

- Heat Denaturation.
- Annealing.
- Primer Extension.

Heat denaturation: This temperature denatures the double stranded DNA into two individual stands. Denaturation temperature is 95° C for 30 seconds or is 97° C for 15 seconds, however higher temperature may be appropriate, especially for Guanine and Cytosine rich nucleotides.

Annealing: During this time one primer binds with the 5'end of one DNA strand and the other primer binds with 3'end of its complementary strand. Annealing is hybridization of primers to single stranded DNA and the length of time required for primer annealing depends on the base composition, length and concentration of primers.

Primer Extension: This temperature varies for Taq DNA polymerase which adds complementary nucleotides one by one to the 3' OH group of the primer. Estimates for the rate of nucleotide incorporation at 72° C vary from 35-100 nucleotides per second depending upon the buffer, pH salt concentration and nature of DNA template.[21]

Then Application of different genotyping methods like RAPD, AFLP, RFLP, ISSR are done. Some of them are discussed here.

Simple sequence repeats (SSRs): Microsatellites are simple sequence repeats (SSRs), 1 to 6 nucleotides in length, which show a high degree of polymorphism. Specific microsatellites can be isolated using hybridized probes followed by their sequencing. Like any DNA fragment, SSRs can be detected by specific dyes or by radio-labeling using gel electrophoresis.[23]

*Restriction fragment length polymorphisms (RFLP)***:** RFLPs are unequal lengths of DNA fragments obtained by cutting Variable Number of Tandem Repeat (VNTRs) sequences up to 30 sequences long with restriction enzymes at specific sites. VNTRs vary between plant species, as do the number and location of restriction enzyme-recognition sites. On an agarose gel, RFLPs can be visualized using radiolabeled complementary DNA sequences. There is no need for PCR amplification of DNA in this method.[23]

Amplified fragment length polymorphism (AFLP): AFLP is a PCR-based derivative method of RFLP in which sequences are selectively amplified using primers.[23] AFLP uses restriction enzymes to cut genomic DNA, followed by ligation of adaptors to the sticky ends of the restriction fragments. A subset of the restriction fragments are then amplified using primers complementary to the adaptor and part of the restriction site fragments. The amplified fragments are visualized on denaturing polyacrylamide gels either through auto-radiography or fluorescence methodologies.[24]

Random amplified polymorphic DNA (RAPD): RAPD is one of the most commonly used primary assays for screening the differences in DNA sequences of two species /individuals of plants. RAPD consists of fishing/searching for the sequence using random amplification. Here, plant genomic DNA is cut and amplified using short single primers at low annealing

temperatures, resulting in amplification at multiple loci. By running a 2-dimensional electrophoresis gel, it is possible to determine the change in sequence pattern by superimposing the 2 gels. Once the band of interest is identified, the gel is cut, and the DNA is isolated and sequenced.[23]

Inter Simple Sequence Repeat (ISSR): ISSR is a general term for a genome region between microsatellite loci. The complementary sequences to two neighboring microsatellites are used as PCR primers. The variable region between them gets amplified. The limited length of amplification cycles during PCR prevents excessive replication of overly long contiguous DNA sequences, so the result will be a mix of a variety of amplified DNA strands which are generally short but vary much in length.[24]

Utility of DNA Based Markers

DNA fingerprinting is a specific technique, which makes possible to read a plants individual genetic makeup and have been widely used for authentication of plant species of medicinal importance. This is especially useful in case of those that are frequently substituted or adulterated with other species or varieties that are morphologically and/or phytochemically indistinguishable.[25]

It resolves adulteration related problems faced by majority by the ayurvedic herbal pharmaceutical industries and practitioners.

The advantage of DNA fingerprinting (Table 1) is it can be done in processed commercial samples because genomic DNA of plant is available even after they are processed. Adulterants can be distinguished even in processed samples and finished products, enabling the authentication of the drug.[2]

Table 1: Various DNA Fingerprinting Techniques Advantages & Disadvantages

Marker	Advantages	Disadvantages
RFLP	• Unlimited number of loci • Can be converted to SCARs • Robust in usage • Detects in related genomes • No sequence information required	• Fairly expensive • Large quantity of DNA needed • Often very low levels of polymorphism • Needs considerable degree of skill
RAPD	• Results obtained quickly &Fairly cheap • No sequence information required • Relatively small DNA quantities required • High genomic abundance	• Highly sensitive to laboratory changes • Low reproducibility within and between laboratories • Cannot be used across • Populations nor across species
ISSR	• Highly polymorphic • Robust in usage	• Species-specific
SSR	• Fast and Robust • Highly polymorphic • Can be automated • Only very small DNA	• High developmental and Start up costs • Species-specific • Sometimes difficult interpretation because of stuttering
AFLP	• Small DNA quantities required • No sequence information required • Can be automated • Can be adapted for different uses	• Marker clustering • Can be technically challenging • Evaluation of up to 100 loci

DNA fingerprinting is an efficient, precise and sensitive method for identifying components for Churnas that has been established and will contribute significantly in quality control.[27] As the DNA sequences are very highly specific, they can be identified with the help of the known molecular markers, which can find out a particular sequence of DNA from a group of unknown.

Various DNA based methods are used for authentication and adulteration detection in medicinal plants that have been explained in Ayurveda are published some of them are as follows.

For Identification & Authentication

Swertia species known as *Kiratatikata in Ayurveda* are authenticated using AFLP and SCAR markers and identified 483 bp amplicon primer which is specific to *Swertia chirayita*.[28,29]

ISSR markers for authentication of *Withania somnifera (Ashwagandha)* are used.[30] AFLP markers for identification of *Zingiber officinale (Ardraka*) from its various species is done.[31]

For Adulteration Detection

RAPD markers used for authentication and identification of genuine and adulterant samples of *Emblica officinalis (Amalaki)*[32],*Diascoria bulbifera (Varaahikanda)*[33],*Andrographis paniculata (Bhunimba)*[34],*Glycyrrhiza glabra* L.(*Yashtimadhu*) from its adulterant *Abrus precatorius* L.(*Gunja*)[35]and *Terminalia arjuna(Arjuna*), *Terminalia bellerica(Vibhitaki*) and *Terminalia chebula(Haritaki*)[36] are done.

RAPD-based SCAR marker was developed to identify *Bacopa monnieri (Brahmi)* from its adulterants *Centella asiatica (Mandukaparni*), and *Malva rotundifolia (Suvarchala*). A putative 589 bp marker specific to *Bacopa monnieri* was identified.[37]

RAPD technique was employed for authentication of dried stems as well as seeds of *Cuscuta reflexa (Akasha valli)* a parasitic climber and its adulterant *Cuscuta chinensis*[38] and *Piper nigrum (Maricha)* from its adulterant *Carica papaya*[39]And many others are discussed in table 2.

Table 2: Authentication of Various Ayurvedic Herbs by DNA Fingerprinting

S. No	Plant Name	Ayurvedic Name	Technique used for Authentication	Reference
1.	*Saussurea lappa* C.B Clarke.	*Kushta*	Sequencing Based Markers	Chen .F *et al* [45]
2.	*Embelia ribes* Burm.F.	*Vidanga*	SCAR	Devaiah K.M *et al* [46]
3.	*Pueraria tuberose* Roxb.ex Willd.	*Vidari kanda*	SCAR	Devaiah K.M *et al* [47]
4.	*Cinnamomum zeylanicum*	*Ela*	Sequencing	Kojoma.M. *et al*[48]
5.	*Aloe arborescens* Miller.	*Kumara*	RAPD	Shioda.H *et al*[49]
6.	*Apium graveolens* L.	*Yavani*	Real time PCR	Mustrop.S *et al*[50]
7.	*Lycium barbarum* L.		SCAR	Sze.S.C *et al*[51]

For Species Differentiation

319 bp unique primers is Species-specific SCAR marker for identification of *Phyllanthus urinaria (Bhumi amalaki*) from its various species.[40] Identification *clematis (Moorva*), *Indigofera (Neeli)* and various species of *Acorus (Vacha*) were done from their substitutes and adulterants.[41]

Identification and differentiation of the *Taxus baccata* aerial parts from its species *using* technology of Single Nucleotide Polymorphism (SNP).[42]

For Identification in Compound Formulations

RAPD technique was also employed for determination of the components in processed Ayurvedic herbal prescription, *Rasayana* Churna.Contains dried stem of *Tinospora cordifolia(Guduchi)*, dried fruit of *Emblica officinalis(Amalaki*) and dried fruit of *Tribulus terestris(Gokshura*), identification and quantification of *T. cordifolia, E. officinalis* and *T. Terestris* in *Rasayana* churna. Primer OPC-6 clearly differentiates all components of *Rasayana*churna.[43]

DNA Fingerprinting of *Tinospora Cordifolia (Guduchi*) Using RAPD Analysis, Four Primers Op A-16, Op C-7, Op C-13 And Op G -5, it can be applied to detect the presence of the drug in formulations.[44]

Conclusion

DNA fingerprinting has a wide range of utility and used to authenticate medicinal plants. DNA fingerprinting can differentiate between individuals, species and populations and has been proved as method for the Authentication and Identification of different adulterants. DNA-based tools for authentication of medicinal plants is an evolving new pharmacognostic measure aimed at quality control and quality assurance in medicinal plant research as well as in clinical usage.These markers have most effective utility in quality control of commercially important medicinal herbs which are adulterated. And these tools are also utilised in any form of the drug i.e., processed or unprocessed.

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