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REVIEW

DNA barcoding: a genomic-based tool for authentication of phytomedicinals and its products

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Abstract: DNA barcoding helps to identify the plant materials based on short, standardized gene sequences in a rapid, accurate, and cost-effective manner. Recent reports reveal that DNA barcoding can be used for the assignment of unknown specimens to a taxonomic group, authentic identification of phytomedicinals, and in plant biodiversity conservation. Research indicates that there is no single universal barcode candidate for identification of all plant groups. Hence, comparative analysis of plant barcode loci is essential for choosing a best candidate for authenticating particular medicinal plant genus/families. Currently, both chloroplast/nuclear regions are used as universal barcodes for the authentication of phytomedicinals. A recent advance in genomics has further enhanced the progress in DNA barcoding of plants by the introduction of high-throughput techniques like next generation sequencing, which has paved the way for complete plastome sequencing that is now termed as super-barcodes. These approaches could improve the traditional ethno-botanical and scientific knowledge of phytomedicinals and their safe use. Hence, current focus is on the investigation of phytomedicinals and herbal product integrity and authenticity through DNA barcoding with the goal of protecting consumers from potential health risks associated with product substitution and contamination.

Keywords: phytomedicinals, DNA barcoding, NGS, super-barcodes, authentication, ethnogenetics

Introduction

DNA barcoding is a technique that is used to identify the species based on species-specific differences in short regions of their DNA.[1] DNA barcoding uses state-of-the-art biotechnology to identify plant species in a rapid, accurate, and cost-effective manner. This technique is not restricted by morphological characteristics, physiological conditions, and allows species identification without specialist taxonomic knowledge. This has made researchers to use DNA barcoding technique to evaluate the herbal product authenticity.

DNA barcoding uses the data of one or a couple of regions in the genome to recognize all the species in a particular class.[2] This technique opened up new doors for DNA-based examinations extending from group phylogenetics to environmental genomics.[3,4] It has been reported that DNA barcoding is not only used to build phylogenetic trees, but to provide rapid and accurate identification of unknown organisms. Ideally, a DNA barcode should allow unambiguous species identification by having sufficient sequence variation between the species. This is an efficient tool to identify the species because levels of divergence among individuals of the same species are usually much lower than the closely-related species that exhibit a "barcoding gap" between inter- versus intraspecific divergences.[5]

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Although the traditional DNA barcoding techniques remain an effective method for identification of medicinal plants, the more advanced and newly developed high-throughput sequencing techniques like next generation sequencing (NGS) technologies are potentially revolutionizing this process.[6] DNA barcoding usually targets short regions of DNA molecule within the genome and does not require full genome-scale data. DNA barcode-based NGS identifies the multiple plant species present in herbal product admixtures, which has further modernized the method through multiplexing of different plant composite samples in a single step amplicon sequencing.[7] Hence, the NGS approach has been introduced recently in DNA barcoding research.

Molecular art behind DNA barcoding

The identification of the biological specimens using short DNA sequences from either nuclear or organelle genomes is called DNA barcoding. The term "DNA barcode" as a taxon identifier was first proposed by Paul Hebert of University of Guelph.[1] Barcoding works by matching sequence data from a query sample (an unknown specimen) to a reference sequence (from a voucher specimen).

Initial in silico and laboratory-based assessment of different loci from chloroplast (cp) and nuclear genomes led to the conclusion that no single locus universal plant DNA barcode exists, and soon it was realized that multilocus DNA barcodes are required for plants.[8] The Consortium for the Barcode of Life (CBOL), Plant Working Group (2009) evaluated seven cp genomic regions across the plant kingdom and proposed a combination of maturase K (*mat*K) and ribulose 1, 5-bisphosphate carboxylase/oxygenase large subunit (*rbc*L) as plant barcodes. High universality and less species resolution are provided by *rbc*L, whereas *mat*K affords high resolution and less universality. Hence, a combination of these two can help to achieve maximum species discrimination. However, in closely-related species, the discriminating ability of these two markers has been found to be very low.[9] Therefore, the addition of nuclear internal transcribed spacer (*ITS*) to the *matK* + *rbcL* combination was proposed as plant DNA barcode in order to achieve maximum identification efficiency even between the closely-related species.[10]

At the Fourth International Barcode of Life Conference (http://www.dnabarcodes2011.org/) held in Adelaide, Australia, the option of a three-locus barcode (*matK* + *rbcL* + *psbA-trnH*) versus a two-locus barcode was discussed. The two-locus barcode was preferred to avoid the increased costs of sequencing, so the combination of *rbcL* + *matK* became the preferred choice and core barcodes.[11] At the same time, the spacer between tRNA-His and photosystem II protein D1 (*psbA-trnH* spacer) and second internal transcribed spacer (*ITS2*) were also widely used in Vitaceae and Asteraceae families.[12–14]

Implication of genomic tools in DNA barcoding technique

In recent years, genomic approaches have been introduced in DNA barcoding technology. Many recent papers on DNA barcoding in plants have been published.[7,8] Due to these advances in DNA barcoding technique, multiple applications like ecological surveys, cryptic taxon identification, authenticaion of phytomedicinals, and herbal drugs have been employed.[17,18] These applications can be extended further if new genomic technologies are introduced into this technique.

Next generation DNA barcoding in phytomedicinals

Rapid advances in NGS technology are driving down the cost of sequencing and bringing large-scale sequencing projects feasible by the individual investigators.[19] Sample preparation has been regarded as the key factor in multiplex sequencing (sequencing of multiple tagged samples together in one lane) of plastid genomes. The application of NGS approach in DNA barcoding of plants has been extensively reviewed.[20]

It has been recently reported that NGS provides useful and efficient workflow to identify pollen at the genus and species levels without requiring specialized palynological expert knowledge.[21] Another report on biological ingredient analysis of traditional Chinese medicine through high-throughput sequencing and metagenomic analysis revealed the use of potential candidates like *ITS2* and *trnL* for the reliable identification of phytomedicinals and their contaminants.[22]

Super-barcoding: a recent genomic tool for plant discrimination

Recent reports reveal that the complete cp-genome contained as much variation as the CO1 locus in animals and may be used as a plant barcode.[23,24] Currently, DNA barcoding technology relies heavily on cp loci because of their relatively low evolutionary rates as compared to the nuclear loci.[25] These cp regions are potentially used in phylogenetic analyses. The use of cp-genome as a marker helps to overcome disputes with gene deletion, low polymerase chain reaction (PCR) efficiency,[26] and also used efficiently to distinguish between

the closely-related species.[27] The cp region provides a barcode that can be effectively implemented to study the associations in specific plant groups.[28,29]

As sequencing technology and bioinformatics continue to advance, complete plastome sequencing has revolutionized the technique of barcoding, which is termed as "Super-barcodes".[23] These plastid-genome-based species classification and identification have been progressively accepted by taxonomists.[28,29] The analysis of this super-barcode also resolved the problems of sequence retrieval usually encountered in traditional barcoding studies. Compared with the nuclear genome, the cp-genome is small in size and has a higher interspecific and lower intraspecific divergence, which makes it more suitable as a genome-based barcode.[23]

Application of DNA barcoding for the analysis of phytomedicinals

People have developed trust on the use of natural components as a therapeutic agent due to fewer side effects. Hugely popular Indian traditional medicinal systems like Ayurveda, Unani, and Homeopathy uses one or the other part of medicinal plants that have been recognized and accepted all over the world. The major problems to deal with phytomedicinals are its correct identification and substitution of rare, expensive medicinal plants with the cheaper and easily available ones.[30] Hence, there is a need for the tool that gives correct identification of plants at the molecular level. DNA barcoding has been employed effectively as an ethno-genomics tool to identify the cryptic species, phytomedicinals, and biological authentication of materials that add value to both traditional ethno-botanical and scientific knowledge.[31]

The *ITS* barcode region has been used for the identification of medicinal plants at the molecular level including medicinal plants of Selaginellaceae.[12,32] DNA barcoding mainly depends on the genetic variation, the time requirement for analysis, the cost/effectiveness ratio, and the technical expertise. Four DNA barcodes (*rbcL*, *matK*, *psbA-trnH*, and *ITS2*) have been successfully used in the identification of many different species of phytomedicinals.[33,34] Plant DNA barcodes have been used efficiently to identify species richness in unknown floras,[35] identify traditional Tibetan medicinal plant *Gentianopsis paludosa*,[16] and discover complex plant groups of Indian *Berberis* species.[36] DNA barcoding system has many prospective uses not only in the identification but also in forensic science, verification of herbal medicines, and foodstuffs, resolving ambiguity of species in plant systematics.[37]

Authentication of phytomedicinal admixtures

Recent reports prove that herbal plants can be authenticated using the barcode candidates in order to identify the contaminants among them. An extensive study has been conducted to analyze >6,600 plant samples belonging to 4,800 species from 753 distinct genera using the genomic regions *psbA-trnH*, *matK*, *rbcL*, *rpoC1*, *ycf5*, *ITS*, and *ITS2*. The results suggested that the *ITS2* of nuclear ribosomal DNA represents the most suitable region for DNA barcoding applications in these plant samples.[12] A recent review has revealed that there are 17 potential barcode regions (*matK*, *rbcL*, *ITS*, *ITS2*, *psbA-trnH*, *atpF-atpH*, *ycf5*, *psbKI*, *psbM-trnD*, *coxI*, *nad1*, *trnL-F*, *rpoB*, *rpoC1*, *atpF-atpH*, and *rps16*) that aid in the authentication and identification of phytomedicinals.[7]

Another report proved that DNA barcoding can efficiently authenticate the wholesomeness of medicinal plants that are often used in Chinese herbal medicine. A total of 37 plants from 28 families were identified using *rbcL* as candidate gene.[38] DNA barcoding technique exhibits advantages over chemical profiling because of its universal application including the identification of unknown plant species. Recently, a study was conducted to identify and authenticate selected phytomedicinals commercially sold in Manila for predicting the most effective DNA barcodes using three cp markers (*psbA-trnH*, *matK*, and *rbcL*) and a nuclear marker (*ITS*). This study revealed that *matK* and *psbA-trnH* could be the potential barcodes for authenticating commercially sold medicinal plants, where morphological details are inadequate.[39] The list of commonly used DNA barcoding candidates for authentication of phytomedicinals has been summarized in Table 1.

Herbal drug authentication by DNA barcoding

Herbal medicines have a widespread and well-documented history of use in the prevention and treatment of various diseases, which continue to gain global influence in modern medical and health services. The international trade in herbal products is a major force in the global economy and the demand is increasing in both developing and developed nations.[34] Recent reports indicate that herbal products available to consumers in the market place may be contaminated or substituted with alternative plant species and ingredient substitutions that are not listed on the labels.[17,54] A number of safety-related issues have emerged globally because of the inaccurate identification of herbal materials. According to the World Health Organization, adulteration of herbal products is causing an enormous threat to consumer safety.

Table 1 Various DNA barcoding candidates used in the authentication of phytomedicinals

Medicinal plants	Different barcode candidates analyzed	Potential barcode identified for species authenticity	Ref
Scutellaria baicalensis (Lamiaceae)	*matK, rbcL,* and *psbA-trnH*	*psbA-trnH*	40
Datura metel	*ITS2, psbA-trnH, matK,* and *rbcL*	*ITS2* or *psbA-trnH*	41
Datura innoxia			
Datura stramonium			
Brugmansia arborea (Solanaceae)			
Boerhavia diffusa (Nyctaginaceae)	*ITS, ITS1, ITS2,* and *psbA-trnH*	*ITS* and *ITS1*	42
Colchicum autumnale	*rpoC1, psbA-trnH, matK,* and *ITS*	*rpoC1, psbA-trnH,* and *ITS*	43
Arlina brachylepis			
Arundo donax (Colchicaceae, Armadillidiidae, and Poaceae)			
Asparagus racemosus	*ITS2, matK,* and *rpoC1*	*ITS2*	44
Hemidesmus indicus (Asparagaceae Apocynaceae)			
Catharanthus roseus	*matK* and *psbA-trnH*	*matK*	45
Alstonia scholaris			
Thevetia peruviana			
Allamanda cathartica			
Tabernaemontana divaricata			
Calotropis gigantea (Rauvolfioideae)			
Gentiana scabra	*rbcL, matK, ITS,* and *5S rRNA*	*5S rRNA* and *trnL-F intergenic spacers*	46
Gentiana triflora	*intergenic spacer, chloroplast psbA-trnH , trnL-F* and *rpl36-rps8*		
Gentiana manshurica and Gentiana rigescens (Gentianaceae)			
Peucedanum praeruptorum (Apiaceae)	No comparative analysis done	*ITS*	47
Crocus sativus (Iridaceae)	No comparative analysis done	*psbA-trnH*	48
Tulipa edulis (Liliaceae)	*matK, psbA-trnH,* and *rbcL*	*matK*	49
Ferula sinkiangensis	No comparative analysis done	*ITS2*	51
Ferula fukangensis (Apiaceae)			
Thunbergia laurifolia Crotalaria spectabilis (Acanthaceae and Fabaceae)	*matK, rbcL, rpoC,* and *trnL*	High resolution melting analysis using *rpoC*	52
Isatis tinctoria	*matK, rbcL, psbA-trnH,* and *ITS2*	*ITS2*	53
Polygonum tinctorium			
Strobilanthes cusia (Brassicaceae, Polygonaceae, and Acanthaceae)			

Abbreviations: *ITS*, internal transcribed spacer; *ITS2*, second internal transcribed spacer; *mat*K, maturase K; *rbc*L, ribulose 1, 5-bisphosphate carboxylase/oxygenase large subunit.

There are many reports on the ill-effects of herbal material due to the incorrect identification and adulteration of plant material.[34,55–59]

Herbal product substitution has been reported for many individual plant species due to their morphological similarity like *Echinacea* sp., *Chamomilla* tincture,[60] *Phyllanthus*,[61,62] tea,[63] and nutraceuticals.[64,65] Although there is limited research available, the frequency of product mislabeling in herbal drugs has been estimated to be in the range from 14% to 33%.[54,66] There are legitimate health concerns for consumers due to lack of confidence in the availability of safe and high quality herbal products. The immediate attention for achieving consumer confidence is driving the demand for research and market testing on herbal product authentication. Traditional methods used to authenticate herbal materials primarily include morphological, microscopic, and chemical identification.[67] However, the ability to distinguish the medicinal plants from their close relatives, inferior substitutes, adulterants, and counterfeits presents a challenge to the large number and variety of medicinal plants species, which threatens patient safety and herbal efficacy.[40]

More recently, a universal publically available DNA barcoding system for identifying herbal materials has been established based on the *ITS2*, *rbcL*, *matK*, and *psbA–trnH* barcodes.[17,43,68–71] There are only limited studies on the application of DNA barcoding to test natural products, which provide authentic assessment of commercial herbal products within the marketplace. This includes reports on detecting adulteration through DNA barcoding for commercial tea samples,[63] black cohosh herbal dietary supplements,[63] natural health products,[54] North American herbal drugs,[17] and adulterants in commercialized medicinal plant samples.[72] Recently, NGS-based approach using *ITS2* and *trnL* barcodes have been attempted to identify biological ingredients from herbal drugs of Chinese traditional medicine (Liuwei Dihuang Wan) containing mixture of phytomedicinals.[22]

DNA barcode identification can be applied to a wide range of herbal materials from the field, commercial trade,

and hospital pharmacy for authentication.[33] DNA barcoding technology has a wide-reaching potential in the phytomedicinal industry, especially to help ensure that herbal ingredients are correct and not contaminated or substituted. Accurate species identification, resource monitoring, and quality control using DNA barcoding will soon become an integral part of quality control workflow in herbal industries.[7] If the DNA barcoding result indicates that the species is different from the intended plant prescribed on the label, then the use of this sample (and probably entire batch) should be avoided by which potential adverse reactions could be prevented.[17]

Similarly, patented herbal products are increasingly popular worldwide. However, they are prone to herbal substitution and/or contamination because of the usage of conventional methods like phytochemical analysis, in which environmental factors can change the plant chemical profile drastically.[54] In this situation, DNA barcoding could play an important role in the authentication of the herbal products. This would significantly decrease bio-safety issues and minimize the trade of fake or incorrect herbs, which will ensure consumer confidence.[66]

Currently, DNA barcode-based standard reference material library and standard testing procedures are being developed for commercial herbal species that could be integrated into cost-effective "best practices" in the manufacturing of herbal products.[7,17] A recent review on the authentication of herbal drug provides clear insight on DNA barcoding as a standard method in herbal pharmacovigilance research.[18] Another report suggests that DNA barcoding must be used in conjunction with metabolomics and need-based transcriptomics and proteomics for resolving authentication challenges associated with the phytomedicinals in herbal drugs.[73] Hence, the DNA barcoding approach can support immensely the herbal product authentication.

Sequence repositories and consortia involved in plant DNA barcoding

There are currently three barcode databases available for plants that encompass the barcode sequences.

GenBank, USA

The International Nucleotide Sequence Database work together with GenBank in USA, European Molecular Biology Laboratory in Germany, and DNA Data Bank of Japan for DNA sequences. These databases regularly exchange the DNA barcode sequence information with the dedicated DNA barcode CBOL database. The National Center for Biotechnology Information web-based megablast algorithm with default settings are used to identify the query sequences. Each identification could be made manually by considering E-value, maximum identity, and number of closely-related species represented in the database.[43]

BOLD, Canada

In order to make DNA barcoding information universally and publically accessible, new databases have been made available online. Rapid progress in DNA sequencing and computational technologies made CBOL to build a universal organization for living beings inventory: the Barcode of Life Database (BOLD) system. BOLD is created and maintained by the University of Guelph in Ontario, Canada (http://www.boldsystems.org).[11] It facilitates researchers to collect, manage, and analyze DNA barcode data. BOLD will provide a DNA barcode to clearly identify the unknown specimens by facilitating accurate query assignments and by comparing the data that are obtained from geographically dispersed institutions. BOLD could serve as the universal starting point for species identification, which would convey to the users to refer the specialized databases (eg, pathogenic strains, disease vector species, endangered species).[74]

Medicinal Materials DNA Barcode database, People's Republic of China

A dedicated DNA barcoding database was developed only for medicinal plant materials (http://www.cuhk.edu.hk/icm/mmdbd.htm), which accepts all plastid DNA regions and nuclear *ITS* results. Medicinal Materials DNA Barcode (http://137.189.42.34/mherbsdb/index.php) is a website that contains DNA sequences, their information, and important references of medicinal records of the pharmacopoeia of the People's Republic of China, American Herbal Pharmacopoeia, and other related references. Medicinal Materials DNA Barcode database gives information on distinguishing medicinal materials (plant, animal, and fungi) from their common substitutes and adulterants.[75]

Consortia for plant DNA barcoding

CBOL

CBOL is an international initiative dedicated to developing DNA barcoding as a global standard for the identification of biological species.[74] It was established in 2004 through support from the Alfred P Sloan Foundation, USA. CBOL promotes barcoding through working groups, networks, workshops, conferences, outreach, and training. CBOL has 200 member organizations from 50 countries and promotes the exploration

and development of DNA barcoding for species identification. It facilitates the rapid compilation of high quality DNA barcode records in a public library of DNA sequences.

International Barcode of Life

The International Barcode of Life project is the largest biodiversity genomics initiative project with hundreds of biodiversity scientists, genomics specialists, technologists, and ethicists from 25 nations to construct a richly parameterized DNA barcode reference library (http://ibol.org/). This library will be the foundation for a DNA-based identification system for all multicellular life. There are 20 working groups operating in five theme areas, which include DNA Barcode library, Methods, Informatics, Technologies, and Administration.

China Plant Barcode of Life Group

The online DNA barcoding databases for herbal materials are being constructed by this group (http://www.tcmbarcode.cn), which provides a species identification module for herbal materials. In this database, *ITS2* and *psbA-trnH* are chosen as the core and supplementary DNA barcodes candidates for medicinal herbs. This database also contains barcoding data for their adulterants, substitutes, and closely-related species in order to correctly distinguish the actual medicinal species. This group provides access to online database and evaluates the potential benefits of supplementing the core barcode for land plants.[8]

Limitations in DNA barcoding

DNA barcoding in plants using combining multiple loci approach has always been followed successfully. However, CBOL has focused on the identification of a universally informative plant DNA barcode for the last several years, which could not be attained due to the complexity of plant genome. The PCR success rate in barcoding regions is often inhibited by the presence of secondary metabolites in plants. These problems can be overcome by the modifications in DNA extraction methods, primer sequences, and the use of an engineered polymerase enzyme.[76] DNA barcoding can be a significant tool for authentication of raw herbs, but its application for finished herbal/botanical dietary supplements is limited due to the low quality DNA obtained from these products.[77]

There are also reports which suggest the usage of a relatively longer genetic region in plant genome (ranging from ~500 to 1,000 bases), but it is difficult to amplify using universal barcodes that are not intact in most botanical extracts, ancient samples, and old herbarium DNA samples.[78] As a result, methods to sequence and analyze such material typically would lead to false negative results. Hence, "specific DNA authentication" methods are adopted recently using a mini-barcode strategy, which targets small barcode regions of fragmented DNA (~100–200 bases in length) for botanical extracts, oil, and tinctures.[79,80] Some reports also reveal that DNA barcoding method is not capable of determining the chemical constituents or plant parts of processed products and also for quantifying the amount of plant material used in the product. Therefore, the use of additional methods (ie, microscopic and chemical) is necessary to verify the label claims ensuring the safety and efficacy of the product. Hence, the use of multiple methods on processed materials is necessary to increase the level of confidence in the identity and quality of the material.[81]

Future prospects

DNA barcoding is being viewed as an integrated approach with classical taxonomy for species identification and authentication in the postgenomics era.[30,82] DNA barcoding has been employed effectively to identify the cryptic species, medicinal plants, species and biological authentication of materials, and plant biodiversity conservation that adds value to both traditional and scientific knowledge.[31,83] Contemporary approaches like ecological genomics along with the use of NGS could exploit and advance DNA barcoding research to the next level. The barcoding movement along with NGS approach could help to speed up the authentication of voucher specimens and herbal drugs.

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Disclosure

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