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Chapter- 11

DNA barcoding and medicinal plants

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Abstract:

Medicinal plants are used in several societies. It has been practiced worldwide for centuries to use herbs for maintaining a healthy life, especially for chronic diseases. Now a days, people from developed countries are also increasingly using traditional medicine as an alternative or alongside modern medicines. The medicinal plants to be used must be correctly identified for our safety. For a long time, expert botanists have identified medicinal plants based on morphological characteristics, and analytical techniques have been used to determine their quality. But neither morphological features nor previous analytical methods can easily identify closely related species and in cases involving powders or processed products obtained from plants, it is difficult to identify adulteration. DNA barcoding is an emerging molecular identification and classification technology that has been applied to medicinal plants since 2008. The application of this technique has greatly ensured the safety and effectiveness of medicinal materials. From single locus-based DNA barcodes to combined markers to genome-scale levels, DNA barcodes contribute more and more genetic information. At the same time, other technologies, such as high-resolution melting (HRM), have been combined with DNA barcoding. With the development of next-generation sequencing (NGS), metabarcoding technology has also been shown to successfully identify species in mixed samples. As a widely used and effective tool, DNA barcoding will become more useful over time in the field of medicinal plants.

Introduction:

Medicinal plants and herbal supplements have played an important role in the health of human populations for thousands of years (Maiti et al., 2010, 2013; Banerjee et al., 2014; Ghosh et al., 2022; Acharya et al., 2022a,b; 2023; Dey-Ray et al., 2024). Even today, the global market for these plant species and their products continues to grow. Globally the authentication and verification of correct plant products has become increasingly important issue over time. DNA barcode technologies have evolved from single genes to combined genes, to genomes, and most recently, metabarcoding. All these techniques are now routinely applied to track medicinal plants' use, commercialization, and authentication globally. DNA Barcode technologies have been especially useful in the ethnobotany of herbal medicines which is crucial for human health.

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Medicinal Plants

The term "medicinal plants" refers to all the plants that can be used as medicines or food supplements, playing important roles in human health (Sarkar et al., 2016; Sarkar et al., 2021; Biswas et al., 2023; Pawar et al., 2023; Rai & Sharma, 2023; Roy et al., 2023; Sarkar et al., 2023; Sarkar et al., 2024). The definitions of "medicinal plants" in different countries are different. According to Yu et al. (2021) the types of medicinal plants are grouped into five pharmacopoeias worldwide. Information for a total of 1133 medicinal plant species had been collected, covering 184 families and 656 genera. The Pharmacopoeia of China (2015) contains 610 species, belonging to 381 genera and 136 families, and Pharmacopoeia of India contains 396 species, belonging to 325 genera and 119 families (Sur et al., 2023; Swaminathan, 2024; Sarkar et al., 2024). The Japanese Pharmacopoeia contains 222 species, belonging to 151 genera and 74 families. Korean Pharmacopoeia contains 219 species belonging to 141 genera and 72 families; United States Pharmacopoeia contains 141 species belonging to 103 genera and 47 families. Only 4 species (*Curcuma longa*, *Glycyrrhiza glabra*, *Illicium verum*, *Zingiber officinale*) are common to all five Pharmacopoeias. Further analysis showed that among 1133 medicinal plants, the top 10 families were Asteraceae, Fabaceae, Lamiaceae, Ranunculaceae, Rosaceae, Apiaceae, Apocynaceae, Euphorbiaceae, Rutaceae and Solanaceae, while the top 10 genera were *Prunus*, *Clematis*, *Euphorbia*, *Solanum*, *Artemisia*, *Dioscorea*, *Acacia*, *Citrus*, *Ficus*, *Aconitum*.

DNA barcoding

A DNA barcode is a relatively short gene sequence in the genome that is unique to a species (Jian, 2014). DNA barcoding is a helpful tool for taxonomic classification and species identification by sequencing a concise, standardized DNA sequence in a well-defined gene. The species are identified by amplifying highly variable regions (DNA barcode region of the nuclear, chloroplast or mitochondrial genome) using Polymerase Chain Reaction (PCR). Regions widely used for DNA barcoding include nuclear DNA (e.g., ITS), chloroplast DNA (e.g. rbcL, trnL-F, matK, psbA, trnH, psbK) and mitochondrial DNA (e.g., COI). DNA barcoding is used for a wide range of purposes, such as the authenticity of labelling by confirming identity or purity to support ownership or intellectual property rights to reveal cryptic species, in forensics to link biological samples to crime scenes, to support food safety and in ecological and environmental genomic studies. Paul Herbert and colleagues in 2003 first proposed the use of short DNA sequences as a method of identifying species, with the aim of rapid species-level identifications across all life forms. Hebert et al. (2003), proposed to use the mitochondrial gene Cytochrome c oxidase subunit l gene (CO1) as the standard barcode for all animals. The scientific community readily adopted this and CO1 can be used to distinguish over 90% of species in most animal groups. In recent years, the barcoding movement has grown substantially, and worldwide efforts coordinated by CBOL (the Consortium for the Barcode of Life) are now being put into retrieving barcode sequences from all organisms

(CBOL, 2009). The power of DNA barcoding to identify species has been demonstrated in several studies. Research shows that DNA barcoding can rapidly identify poisonous plant materials. Mati et al. (2011) have demonstrated that a DNA barcoding approach can identify species in processed plant materials of commercial kitchen spices. DNA barcoding has been used to identify ingredients in commercial plant mixtures. studies such as Newmaster et al. 2013, showed the successful approach of DNA barcoding for species adulteration of natural health products and medicinal plant raw drugs.

DNA barcoding for identification of medicinal plants

A gene region must satisfy three criteria to be practical as a DNA barcode (Yu et al., 2021). The gene region should contain a sufficient variation to discriminate between species, consist of conserved flanks to develop universal PCR primers and have a short sequence length to facilitate current DNA extraction and amplification capabilities. A single barcoding locus combining these traits has not been found, and a combination of two or more is required to approach the level of species discrimination and universality for plants (Jian, 2014). The reliable candidates for plant DNA barcodes are several chloroplast gene regions nucleotide coding loci or noncoding spacers and coding genes. The Chloroplast genome is appropriate due to the high copy number, conserved structure, and diversity of substitution rates across genes, introns, and intergenic spacers. Examples of them are the arc ribosomal RNA maturase K (matK) coding gene and Ribulose 1, 5- bisphosphate carboxylase/oxygenase large subunit coding region (rbcL) considered core barcodes.

The CBOL recently recommended the two-locus combination of matK+rbcL as the best plant barcode with a discriminatory efficiency of only 72% (CBOL, 2009). Taxonomists have suggested that a multi-locus method may be necessary to discriminate plant species. The combination of the spacer region tRNA-His and photosystem II protein D1 (psbA-trnH spacer) and ITS is also used as a plant barcode for the majority of plant families in addition to core barcode markers (Chen et al., 2010). According to CBOL, matK and rbcL genes need to be supplemented by additional loci to discriminate among closely related species. The other plastid loci sequenced in plant systematics for phylogenetic purposes are trnL-F, rpoCl, rpoB2, 911, etc., with different success degrees (Kress et al., 2005).

Types of DNA barcode markers used for identification of medicinal plants

Single-locus DNA barcode markers

It was announced in 2009 at the 3rd World DNA Barcode Conference that the matK and rbcL markers are the core sequences of plant DNA barcodes, with ITS and trnH-psbA as complementary sequences (Group, 2009). After extensive experiments and verification, Chen and colleagues proposed the ITS2 region as the primary DNA barcode and trnH-psbA as a complementary sequence for identifying medicinal plant species (Chen et al., 2010). Since then, many plant scientists have used other several markers to evaluate the efficiency of ITS2 and trnH-psbA by identifying the species in different families or genera: atpF-atpH (Ran et al.,

2010), rpoB (Al-Qurainy et al., 2011), atpB-rbcL, trnH-psbA, trnL-F, trnS-G, atpF-H, rbcL, matK, rpoB, rpoC1, nad1 (Quan and Zhou, 2011), rbcL, matK, psbA-trnH, ITS2, ITS, trnL intron, and trnL-F (Sun et al., 2011), trnL and rpoC1 (Madesis et al., 2012), rpoC1 (L-Qurainy et al., 2014), ndhJ (He et al., 2014), matK, rbcL, atpH-atpI, rpl32-trnL (UAG), rps18-clpp, trnL-trnF, trnL-ndhJ, trnS-trnfM (Mao et al., 2014), rbcL and trnL (Buddhachat et al., 2015), rbcL, psbA-trnH and petA-psbJ (Deng et al., 2015), matK, rbcL, trnH-psbA, ITS, trnL-F, 5S-rRNA and 18S-rRNA(Mishra et al., 2016), rps16, and trnT-F (Mishra et al., 2016), trnL (Suesatpanit et al., 2017). The ITS2 secondary structures have also been used to identify the species in different genera, such as Akebia (Zhang et al., 2015), Glehnia (Zhu et al., 2015), Physalis (Feng et al., 2016), and Smithia (Umdale et al., 2017).

Multiple-locus DNA barcode markers

As single-locus marker sequence cannot always provide enough information for low level identification, some scientists used a combination of markers to identify medicinal plants. The most common combinations of DNA markers exist between matK, rbcL, trnH-psbA, and ITS sequences (Newmaster et al., 2013; Fu et al., 2011; Purushothaman et al., 2014). Some other combinations, such as atpB-rbcL+trnL-F and atpB-rbcL+ -atpF-H, have been used in identifying the species of Prunus L., which can resolve all five species (Quan and Zhou, 2011). Parveen compared the combinations of rbcL, rpoB, rpoC1, matK, and ITS in Orchidaceae, with the maximum species resolution provided by ITS+matK (Parveen et al., 2017).

Genome-based DNA barcode markers

The chloroplast genome contains all the DNA sequences in a plastid, which contains more genetic information for species identification than any commonly used single-locus marker. By 27 October 2019, 3452 plants' chloroplast genome was published on NCBI. Govindaraghavan and Li proposed that the entire plastid genome be used in the field of DNA barcoding (Govindaraghavan et al., 2012; Li et al., 2015). Sucher suggested that genomic fingerprinting can differentiate between individuals, species and populations and is useful for the detection of the homogeneity of the samples and presence of adulterants in herbal supplements (Sucher and Carles, 2008). Yang used the chloroplast genome to identify the species of *Datura stramonium* (Yang et al., 2014). He sequenced and analyzed the complete chloroplast genome of Lonicera japonica. Zhou et al. analyzed the complete chloroplast genomes of Papaver rhoeas and Papaver orientale. They concluded that the chloroplast genome could be used as a powerful tool to resolve medicinal plants' phylogenetic positions and relationships (Zhou et al., 2018).

Most popular marker sequences for plant DNA barcoding

The matK region

Ribosomal RNA maturase K (matk) is a rapidly evolving coding gene. Such a region with 800-1500 base pairs (bp) is located within the intron of the chloroplast gene trnK (Figure). According to Windelspecht (2007), MatK has a high evolutionary rate, suitable length, obvious

interspecific divergence, and a low transition/ transversion rate. But matK is difficult to amplify universally using currently available primer sets. The CBOL Plant Working Group (2009) revealed a nearly 90% success rate in amplifying angiosperm DNA using a single primer pair. However, the success was limited in gymnosperms (83%) and much worse in cryptogams (10%) even with multiple primer sets. Different primer pairs were necessary for different taxonomic groups (Chase et al., 2007). Lahaye et al. (2008) used specific primers to amplify the matK gene of 1667 angiosperm plant samples and achieved a success rate of 100%. Matk could discriminate more than 90% of species in the Orchidaceae (Kress & Erickson, 2007) but less than 49% in the nutmeg family. Many research findings suggests that the matK barcode alone is not a suitable universal barcode.

The rbcL region

Ribulose 1, 5- bisphosphate carboxylase/ oxygenase large subunit coding region (rbcL) which is located in the plastid genome, has a length of 600-750 bp. The rbcl region is a candidate for plant barcoding as it can detect generic-level evolutionary relationships (Windelspecht 2007).

The rbcL can be easy to amplify and sequenced and aligned in most land plants. Thus, it is a good DNA barcoding region for plants at both the family and genus levels. Kress et al., (2005) showed that since rbcL sequences evolve slowly, it has a low divergence of plastid genes in flowering plants. Hence it is not very suitable for discriminating at the species level. Moreover, the length is also a drawback where four primers are required for its double-stranded sequencing. However, despite its limitation, rbcL is still being wildly used for plant barcoding due to a large amount of easily accessible data and straightforward recovery of the entire gene sequence.

The trnH-psbA region

The trnH-psbA non-coding spacer, located between tRNA-His and photosystem II protein D 1, shows many features considered as desirable in a barcode. The trnH-psbA gene with 800-1500 base pairs (bp) is located within the intron of the chloroplast DNA. Since the region has a highly conserved coding sequence, it enables the designing feasible universal primers, where a single primer can amplify almost all the angiosperms. The noncoding intergenic region has a high rate of insertion and deletion, making trnHpsbA a suitable candidate for plant barcoding (Shaw et al., 2007).

However, trnH-psbA may undergo frequent inversion in certain plant linkages that may cause incorrect phylogenetic assignment. It some cases where taxon-specific internal primers are not available, the mononucleotide repeats can have premature termination of sequencing (Lee, 2016).

The ITS region

ITS (Internal transcribed spacer) region is located between the 16S and 23S rRNA genes. It is recommended only as a supplementary locus by CBOL. ITS region is not permitted to being

a core barcode marker. Firstly the ITS region is different to amplify and sequence. Secondly, it has an incomplete concerted evolution. Thirdly, the ITS region is not specific for plants as it is also present in fungus (fungal contamination), thus, ITS sequence amplification can be confused with fungi sequence (Mishra et al., 2015).

Metabarcoding

With the development of DNA high-throughput sequencing technology, DNA barcodes have been transformed into DNA metabarcoding, which can simultaneously acquire DNA barcode sequences for mixed multi-species samples. This technology uses high-throughput sequencing technology to obtain the barcoded amplicon sequences and uses bioinformatic methods to identify species diversity and composition within a sample. This technology has now been applied to the field of medicinal plants. Dietary analysis by metabarcoding was proposed at the 6th International Barcode of Life Conference (Adamowicz, 2015). De Boer used nrITS1 and nrITS2 DNA metabarcoding to identify orchids and other plant species present in 55 commercial products (De Boer et al., 2017). Arulanhu developed a multi-locus DNA metabarcoding method to identify endangered plant (including *Echinocactus*, *Euphorbia*, *Aloe variegate*, *Dendrobium*, *Cycas revolute*, *Lactuca sativa*) and animal species in complex samples (Arulandhu et al., 2017). Omelchenko improved protocols of ITS1-based metabarcoding and analyzed 39 plant-containing products (Omelchenko et al., 2019). Raclariu uses DNA metabarcoding coupled with chromatography technologies to authenticate 16 *Veronica officinalis* herbal products (Raclariu et al., 2017a), 78 *Hypericum perforatum* herbal products (Raclariu et al., 2017b), and 53 Echinacea herbal products (Raclariu et al., 2018a, 2018b). DNA metabarcoding combines other sequencing techniques can determine both prescribed and contaminated species in Traditional Chinese Medicine (TCM) preparations, such as Liuwei Dihuang Wan (Cheng et al., 2014), Jiuwei Qianghuo Wan (Xin et al., 2018).

Raclariu compared the benefits and limitations of DNA Barcoding versus Metabarcoding in herbal product authentication and concluded that both techniques have potential in the context of quality control of both well- and poorly-regulated supply systems (Raclariu et al., 2018a, 2018b). However, the accurate determination of species or herbal products by DNA metabarcoding is dependent on a comprehensive and accurate reference library of DNA sequences of a standard genetic marker.

Conclusion

Universal PCR amplification has the highest relative rate of recovery of a barcode region (Erickson et al., 2008). There are several aspects of species differentiation. A fraction of species groups cannot be resolved with any suggested DNA barcode marker, but recovery can be improved through marker selection. The complementation of loci is useful for this purpose (Kress and Erickson, 2007). Combined barcodes are recommended for numerous beneficial outcomes but opposite arguments on its usage are also being raised. Chen et al. (2010) stated that the combined barcode causes increased analytical difficulty compared to the single-locus

marker. Moreover, CBOL also demonstrated in one study that the use of seven different candidate loci combinations did not improve species-level discriminatory ability compared to rbcl+ matK. Some authors stated inconsistency between the plastid gene tree and species boundaries, resulting failure of multiple-locus barcodes. Multiple-locus barcodes cannot eliminate the inherent deficiencies of the current DNA barcoding of plants (Dubey et al., 2016).

Globally, medicinal plants have been recorded in 219 different families (52.1%). Among them, 142 families, including 832 genera, have DNA barcodes revealed (64.8%). The remaining 77 families of medicinal plants have no published record of DNA barcode sequence data (Yu et al., 2021). After more than 10 years of development, DNA barcoding with standardized genetic markers has made tremendous progress. Today, barcode markers can be combined with other biotechnologies, such as molecular, chromatographic, and spectrum technologies to obtain better identification results. These other molecular technologies include SNP (Single Nucleotide Polymorphism), HRM (High Resolution Melting), and RFLP (Restriction Fragment Length Polymorphism). Chromatography technologies include LC-MS (Liquid Chromatography -Mass Spectrometry), HPLC (High Performance Liquid Chromatography), TLC (Thin Layer chromatography). Xiao demonstrated that the chemical profiles determined by LC-MS and DNA profiles in ITS spacer domains could serve as barcode markers for the quality control of Radix *Astragali* (Xiao et al., 2011). Zhang showed that DNA barcoding is more powerful than HPLC fingerprint for the identification of *Phellodendri* Cortex and its related species (Zhang et al., 2016).

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