

CURRENT PERSPECTIVES AND EMERGING TRENDS IN PCR-BASED DNA BARCODING FOR MOLECULAR IDENTIFICATION OF PLANTS: PROGRESS, CHALLENGES, AND FUTURE PROSPECTS

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ABSTRACT

DNA barcoding is an efficient approach for categorization and identification of plant species using genomic sequences. It is straightforward, time- and cost-efficient, and accurate. The key to successful DNA barcoding is the selection of an appropriate DNA marker. Since the concept of rapid species identification was introduced in 2003, the scientific community has recognized the potential of DNA barcodes. DNA barcoding has applications in preserving natural resources, protecting endangered species, controlling agricultural pests, identifying disease vectors, monitoring water quality, authenticating natural health products, and identifying medicinal plants. Detailed analysis is performed using amplified sequences of *rbcl*, *ITS2*, *matK*, *psbA-trnH*, *rpoC1*, and other markers, with databases such as GenBank, the National Center for Biotechnology Information (NCBI), and the Barcode of Life Data System (BOLD). DNA barcoding can efficiently detect adulteration by identifying unknown species against known database sequences. Analytical techniques such as HPLC-MS, HPLC-UV, and GC can be combined with DNA barcoding to quantify key compounds in herbal medicines responsible for pharmacological effects. Different barcode approaches, including single-locus, multi-locus, metabarcoding, mini-barcoding, and bar-HRM analysis, have various strengths and limitations. DNA barcoding plays a major role in addressing herbal medicine adulteration through species identification, detection of adulterants in raw or processed herbal medicines, database development, research, and surveillance. This review highlights progress in plant taxonomy, the success of barcode loci, the emergence of super barcodes from chloroplast genomes, and the impact of next-generation sequencing technologies.

KEYWORDS: Barcoding, chloroplast genomes, sequencing, herbal medicines, database development.

INTRODUCTION

Species identification remains a challenging and time-consuming task, even for expert taxonomists. The global decline in taxonomic expertise, driven by scientific reductionism and underinvestment, underscores the need for integrative approaches to support and revitalize this critical field. The accurate identification and classification of plant species are the cornerstones of biodiversity research, ecological studies, evolutionary biology, and conservation efforts. Traditional taxonomy relying on morphological characteristics, has long been the foundation of plant systematics.^[1] Scarcity of taxonomical information creates a major blockade to biologists for determining the correct identification of a plant or animal sample in a rapid, repeatable and reliable fashion. Molecular phylogeny has clarified many uncertainties in our view of species relationship but morphological features are difficult to identify and compare. Morphological study has delivered a basic understanding of biodiversity but it cannot discover and identify species on a planetary scale.^[2]

This problem was a major reason for the development of a new method for the quick identification of any species based on extracting a DNA sequence from a tiny tissue sample of any organism. DNA barcoding will allow users to efficiently recognize known species and speed the discovery of species yet to be found in nature.^[3] It provides a central catalogue of organismal diversity which can be assessed by anyone who needs to quickly and accurately identify an organism. It also allows us to differentiate new, previously undescribed species from those which are already been observed. The basis of DNA barcoding is that, by comparison with a reference library of such DNA sections an individual sequence can be used to uniquely identify an organism to species. The same way that a supermarket scanner uses the familiar black stripes of the UPC (Universal Product Code) barcode to identify an item in its stock against its reference database. These “are sometimes used to identify unknown species parts of an organism, or simply to catalogue as many taxa as possible, or to compare with traditional taxonomy to determine species boundaries.”^[4]

The two main issues causing inaccuracy in plant identification by barcodes are pseudogenes and hybridization. Some biological phenomena that occur at different degrees, like heteronplasmmy, paternal leakage, introgression, polyploidization, recent speciation, incomplete line age sorting, polymorphism, error in specimen identification and incorrect taxonomy, also interfere with barcoding.^[5] Studies on DNA barcoding have been progressing day by day since its introduction (Fig. 1).

This review outlines the characteristics, benefits and drawbacks of the markers used to identify plant species as well as the suggested standard DNA barcodes for plant species. Also, the paper focuses on the operational procedures, process and data analysis followed in the DNA barcoding studies, in addition to its multiple positive impacts on taxonomy and biodiversity research or barcoding operational procedures and processes.

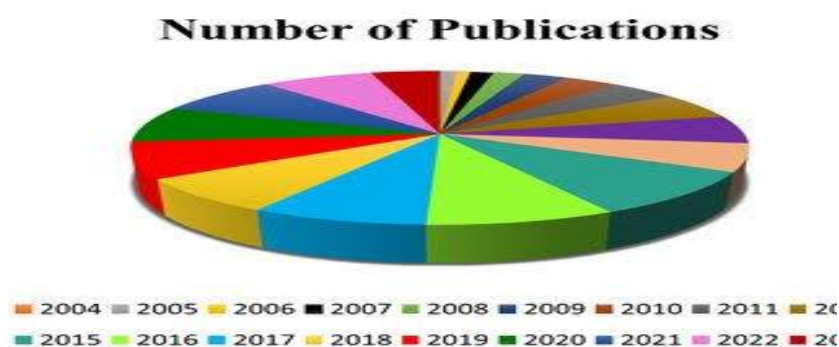


Fig. 1: Estimate on yearly increase in the published literature on DNA barcoding for last two decades. The pie diagram is based on the detailed literature searched in popular sources of scientific information viz. Pub med, google scholar, and research gate. In August 2023, data spanning from 2013 to 2023 was gathered in preparation for the drafting of this article.

TRADITIONAL APPROACHES FOR PLANT IDENTIFICATION

Expert determination: Experts prepare treatments (revisions, synopses) of the group in question, and it is probable that the more recent floras or manuals include the expert's concepts of taxa. Being reliable, this method requires valuable time of experts and creating delays for identification.^[6]

The use of keys and similar devices: Most widely used method and does not require the time, materials, or experience involved in comparison and recognition.

Comparison: Comparison of an unknown with named specimens, photographs, illustrations or descriptions.

Recognition: This is based on extensive, experience of the identifier with the plant group in question.

NOVEL APPROACHES FOR COMPUTATIONAL METHODOLOGIES

DNA Metabarcoding: The fundamental purpose of DNA metabarcoding is to identify broad groups of species in a single environmental sample at the same time. This method combines high-throughput sequencing (HTS) with a polymerase reaction based on DNA amplification to provide unbiased amplification from a variety of DNA templates. Metabarcoding uses universal primers to identify numerous plant species at the same time.^[7] It has proven feasible to detect the content of medicinal plants in processed herbal products using a metabarcoding technique.

Neural network: The neural network is a mathematical/computational representation of a biological system. Back Propagation (BP) is a supervised learning approach that may be used to train multi-layer feed-forward neural networks. The approach entails training a network using a reference dataset of sequences, then utilising that trained network to identify query sequences.

Machine learning: This method, when used in conjunction with bioinformatics, is particularly useful for detecting species with non-coding barcodes, and it outperforms their previously published BP neural network approach. By employing graphical representations of DNA sequences through a DV-Curve or FJ-Curve technique, the method overcomes the difficulty of sequence alignment. When forming conclusions, it is less reliant on assumptions.

Composition Vector (CV) method: The composition vector (CV) method is a non-alignment methodology that is particularly well suited to non-protein-coding sequences used as barcodes. The tree topologies generated using the CV approach match those found using standard methods(8). The CV technique consists of four steps:(i) calculating the frequency for every k-string in the sequence, (ii) building a CV for the sequence, (iii) computing the distance between every two CVs to generate a distance matrix, and (iv) building a phylogenetic tree.

DNA BARCODES IN PLANTS

Four plant DNA barcode region (rbcL, matK, trnH-psbA, and ITS2) in the mitochondrial, plastid, and nuclear genomes have been developed and tested (9). Plant Working Group (PWG) of the Consortium for the Barcoding of Life (CBOL) recommended the regions of two plastid genes rbcL and matK. They can be adopted as the standard plant DNA

barcodes, with the recognition that supplementary markers may be required (**Table 1**). **RbcL**: Ribulose 1,5-biphosphate carboxylase **MatK**: Maturase K. **Other Barcoding Regions of Plant**: trnH-psbA, atpF-F, psb k1, rpo C1, rpo B, rpo C2, ndh J, trn L, ycf 5, acc D

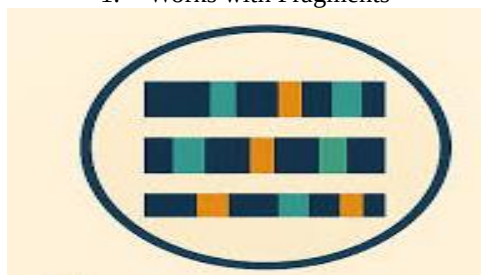
Table 1: DNA markers for plant barcoding.

Sr. No.	Genera	Barcode Markers used	Success rate of unique identification (%)
1.	Lemnaceae	atpF-atpH	92.8
2.	Asteraceae	ITS2	97.4
3.	Fabaceae	matK	96
4.	Rutaceae	ITS2	100
5.	Orchid	matK	90
6.	Hydrocotyle	trnH-psbA	100
7.	Solanum	trnS-trnG/ndhF	100
8.	Medicinal plants	ITS2	99.8

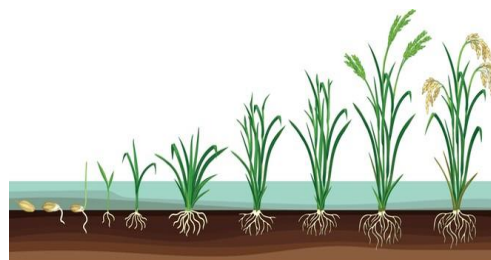
Non coding Regions, ITS (Internal Transcribed Spacer and trnH-psb A perform better, but required great manual effort. matK, ndh J shows great variation of rate among subgenera rpoB, rpo C1and acc D have very low variation for the use of single barcode.^[10]

WHY BARCODING?

1. Works with Fragments



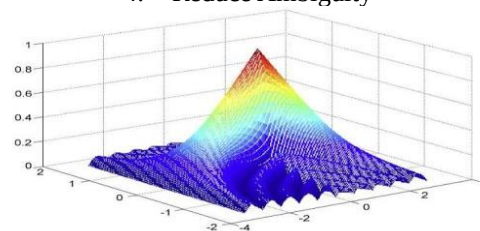
2. Works at all stages of Life



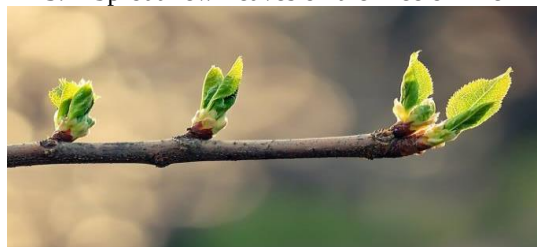
3. Unmask Look Alikes



4. Reduce Ambiguity



5. Sprout new Leaves on the Tree of Life



6. Speed writing the life of encyclopedia

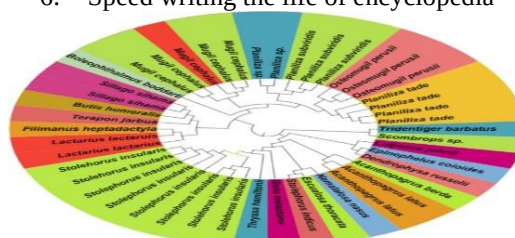


Fig. 2: Importance of Barcode Inventory System.

PROCEDURE OF DNA BARCODING

The process of DNA barcoding involves two basic steps: First step is to build a barcode library of identified species and second is matching the barcode sequence of the unknown sample with the barcode library (known as sequence alignment) for its identification. The first step requires ecologic expertise in selecting one or several individuals per species as reference samples in the barcode library.^[10] Tissue samples can be collected from live specie in field or from specimen in museum for generating. These specimens go through lab processes that are tissue sampling and DNA processing and sequencing to generate DNA barcode in form of chromatogram. Chromatogram is visual representation of DNA sequence produced by sequencer. This barcode can be stored in database for future use or can be used as query sequence to be compared with sequence already present in database (Fig. 3).

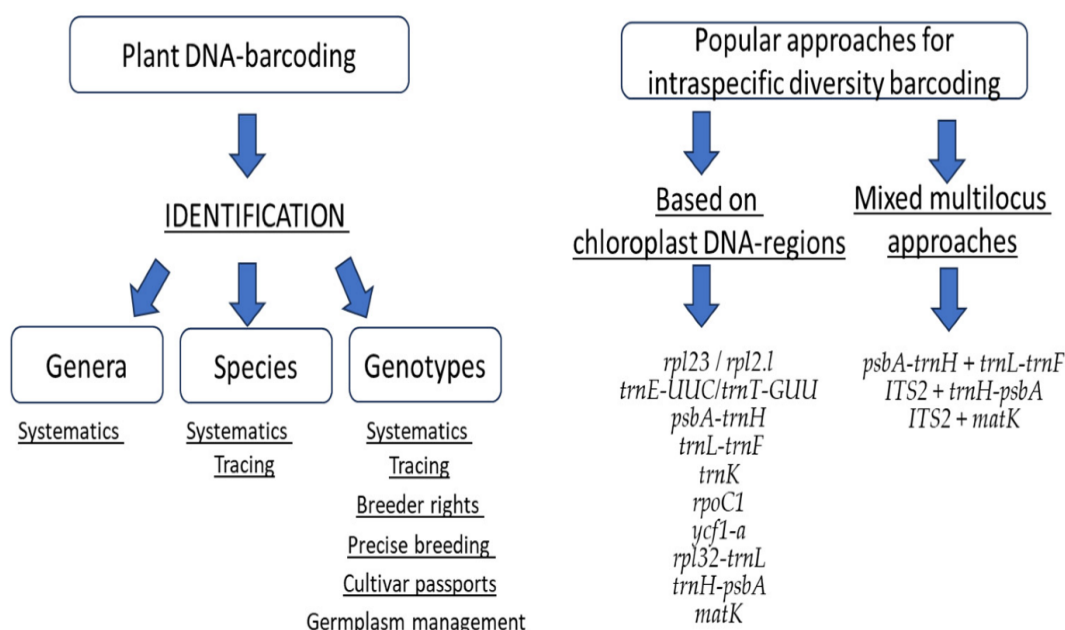


Fig. 3: A flowchart showing generalized procedures of DNA barcoding technology.

THERE ARE FOUR COMPONENTS OF BAR-CODING PROJECTS^[11]

1. The Specimen Collection (from herbaria, frozen tissue collections, seed banks, etc.)
2. The Laboratory Analysis
3. The Data Base -Two main barcode databases are- The International Nucleotide Sequence
4. Database Collaborative (INSDC) and Barcode of Life Database (BOLD).
5. The Data Analysis – Barcode of Life Data Portal offers flexible ways to store, manage, analyze and display barcode data.

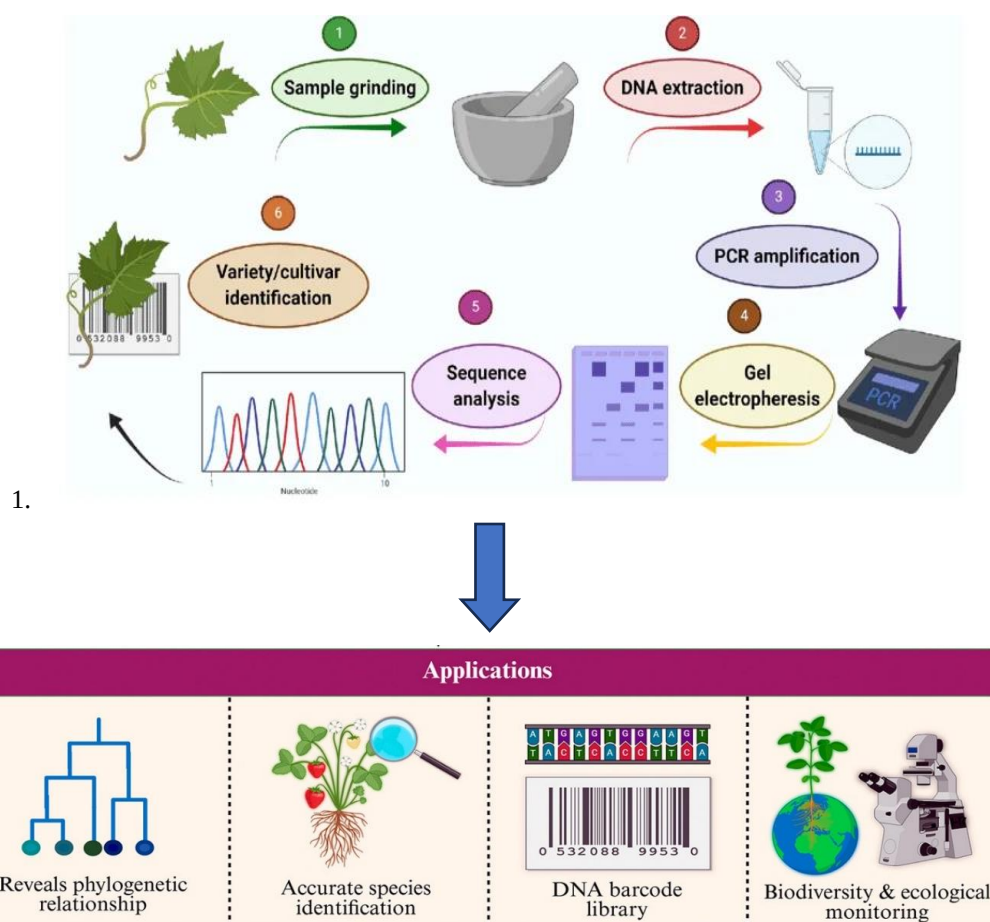


Fig. 4: Schematic diagram representing DNA barcoding steps involved in plant DNA barcoding with applications.

SOFTWARE AVAILABLE FOR BARCODING IN PLANTS.^[8]

Table 2: Tools used for plant DNA barcoding.

Sr. No.	Tool	Function
1.	Spider	Analysis of species identity and evolution, particularly DNA barcoding
2.	BRONX	DNA Barcode Sequence identification
3.	ecoPrimers	Identification of New barcode markers and their associated PCR primers
4.	OTUbase	Analysis of Operational Taxonomical Unit (OTU) data and sequence Classification
5.	TaxonGap	Visual comparison of the discriminated Poer of multiple biomarkers for a set of OTUs
6.	SAP	Statistical Assignment Package –DNA barcoding based on Bayesian phylogenetics
7.	4SALE	RNA sequence and secondary structure alignment and editing
8.	B (Barcode Quality Index)	Assessment of sequence quality and contig overlap
9.	BLOG	Barcoding with LOG ic formulas –DNA Barcoding through data mining
10.	CAOS	Software Character based DNA Barcoding
11.	CBC Analyzer	Inferring phylogenies based on Compensatory base changes in RNA secondary structures
12.	Taxi	Distance based DNA Barcoding

PCR AMPLIFICATION REQUIREMENTS

Taq polymerase, Forward and Reverse primers, (Bovine serum Albumin) PCR additive, DNA, Molecular biology grade water, PCR tubes or 96-well PCR plates, Heat-sealing PCR film, Thermocycler with 96-well plates. The following *rbcl* and *matK* primers are commonly used to amplify plant species.^[12]

Table 3: Components of PCR Required to amplify *rbcL* and *matK*.

Components	Amount needed per sample (μl)	Amount required for a 96 well plate (96+4 for pipetting errors) (μl)	Details
2xTaq-polymerase master mix (biolinebiomix)	1.0μl	100μl	Company name biolinebiomix
Forward primer	0.4μl	40μl	10μM, dilution done with the help of TE buffer
Reverse primer	0.4μl	40μl	10μM, dilution done with the help of TE buffer
BSA	0.8μl	8μl	1mgper ml solution required.
H2O	6.4μl	64μl	Use molecular biology grade water
DNA	2.0μl	-	2.0 μl per sample

CHOOSING MARKERS BY USE CASE^[7,8]

General animal identification: COI is the first choice; use mini-COI for degraded material. **Routine plant identification:** *rbcL* + *matK* as the core pair; add ITS/ITS2 for clades that need extra resolution. **Herbal authentication and botanicals:** *rbcL*/*matK* plus ITS2 captures common substitutions in closely related species. **Fungi and lichenized fungi:** ITS/ITS2 typically provide better resolution; verify reference coverage for your group. **Seafood authenticity testing:** COI or mini-COI with strong documentation; consider multiple tissue sources if processing varies. **Archived or precious specimens:** Short amplicons and low-input extraction protect material and raise success rates.

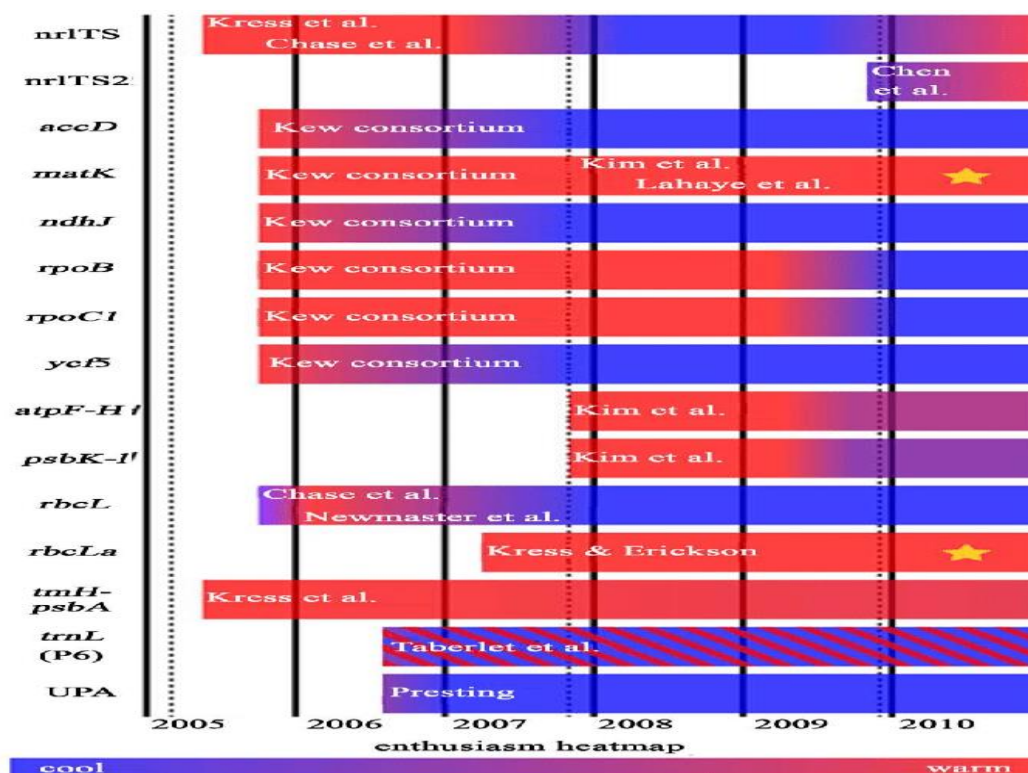


Fig. 5: Schematic timeline of different DNA barcode markers. Colours (red = warm; blue = cool) represent an informal measure of enthusiasm among DNA barcoding researchers in the systematics community for CBOL and iBOL.

WHEN NOT TO USE DNA BARCODING AND LIMITS

Barcoding is powerful, but it has boundaries. Recognising them early keeps projects on track. **Incomplete or uneven references.**^[13] Some clades are undersampled. A strong numerical match to a weak record can mislead. In such cases, add a second locus or seek expert taxonomy. **Biological edge cases.** Hybridization, introgression, and numts (nuclear mitochondrial pseudogenes) can confuse mitochondrial signals. Careful sequence inspection and confirmatory evidence reduce the risk. **Highly processed material.**^[14] Heat or chemical treatment can fragment DNA. Mini-barcodes, targeted capture, or orthogonal assays can help, but set expectations early. **Mixed-organism samples.** If your question is "who is present in this water or soil?", classic barcoding is the wrong tool. Choose metabarcoding or eDNA workflows designed for mixed DNA. Start with our comparison: DNA Barcoding vs Metabarcoding. **DNA barcoding** shines for single-specimen, species-level identification using standard gene regions.^[15] For community profiling, move to metabarcoding; for population structure, consider additional genomic methods.

APPLICATIONS OF DNA BARCODING

The advances in sequencing techniques have enhanced the applicability of DNA barcoding for various biomonitoring applications. **DNA barcoding used in the identification of substitutes/adulterants:** Due to either shortage of resources or economic benefits, some important herbals have many substitutes or adulterants. The phenomenon of adulteration in the herbal market has become increasingly common. Substitutes/adulterants may cause serious consequences such as poisoning or even death.^[16] **DNA barcoding used for the regulation of the medicinal market:** National governments have applied DNA barcoding technology in the field of the identification of herbal medicines. The World Health Organization recommended DNA barcode identification associated with chemical analyses to guarantee medicinal plants quality. **Taxonomists:** To rapidly sort specimens by highlighting divergent taxa that may represent new species.^[17] **Conservationists:** For assessment of threats requires species delimitation and recognition. **Environment managers:** For identification of harmful organism's/indicator species. **Legal:** In forensics, wildlife crime, illegal trade in biodiversity. **Health:** Identification of species with harmful attributes.^[18] **Utilization (in agriculture and medicine):** Identification of species with useful attributes. **Horticulture:** For stock identification.

FUTURE PROSPECTS FOR PLANT DNA BARCODING

DNA barcodes were first offered to the botanical community over a decade ago and have since been used in a range of inquiries in both applied and fundamental plant study.^[19] One of the primary reasons that DNA barcoding has not been widely used for species identification is because no one marker can completely distinguish between species in most taxonomic categories.^[20,22] Plant DNA barcoding will improve in two essential ways to benefit the botanical group in the future: 1. building a worldwide plant DNA barcode library for universal or worldwide usage. 2. Developing and implementing novel marker technologies, as well as implementing latest sequencing techniques.

CONCLUSION

As the advantages and limitations of barcoding become apparent, it is clear that taxonomic approaches integrating DNA sequencing, morphology and ecological studies will achieve maximum efficiency at species identification.^[20] DNA barcoding may help speed the work of taxonomists and others interested in species identification. The evidence from a number of studies largely confirms the feasibility of such a system.^[21] Despite some drawbacks of using DNA barcoding, the reported success of using the barcoding region in distinguishing species from a range of taxa and to

reveal cryptic species is remarkable. Efforts should therefore be made to develop nuclear barcodes to complement the barcoding regions that are currently in use.

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