Utility of DNA Barcoding for Plant Biodiversity Conservation

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ABSTRACT DNA barcoding is a technique that provides rapid identification of species without using morphological cues. The method employs relatively small-standardized DNA fragments as tags to define or discover species. In plants, the mitochondrial genome evolves much more slowly than in animals. There is currently no consensus on which candidate markers comprise the best plant DNA barcoding region; however, DNA barcodes such as *rbcL*, *matK*, *psbA-trnH* and ITS have been proposed for the plant kingdom. And also very recently the chloroplast intergenic spacer (IGS) like *trnE-trnT*, *trnT-psbD*, *ndhF-rpl32* and *rpl14-rpl16* were also employed for discriminating the cultivar species. The region ITS2 showed better intra-species variation, followed by *psbA-trnH*. Several analyses reveal that the ITS2 region is able to distinguish all tested species of the plant kingdom, but evaluations of DNA barcodes have to be conducted for more species covering many genera to confirm the above results. In this review we discussed the current view of DNA barcoding.

Keywords DNA barcode, PCR, *matK*, *rbcL*, *atpB*

INTRODUCTION

DNA barcoding is a technique, which provides quick identification of species without involving the morphological cues. It uses a relatively small-standardized DNA fragment as a tag, to define or discover a species. DNA barcoding uses minor differences of nucleotides in the particular gene loci of different organisms as a key to discriminate the organisms. The difference in base pairs of the gene is sequenced and deposited in the barcode database, which is termed as DNA barcodes. These genetic codes could be accessed through a digital library and used to identify the unknown species by any scientist around the world. Ideal DNA barcode should be normally a uniform short sequence of DNA (400–800 bp), able to be simply generated and characterized for all the living organisms (Savolainen *et al.* 2005). Paul Hebert's groups are the first one to design and use the short DNA sequences for biological identifications. DNA barcodes identifies the species across all forms of life

including animals, plants and microbes at a rapid and reliable rate. The idea of barcoding has first emerged to describe the microorganisms in which the morphological keys were lacking. Now it is being applied successfully to animals. A massive online digital library of barcodes will be a standard to which the DNA barcode sequence of an unknown sample can be matched for the identification. The key process in DNA barcoding is identifying novel candidate gene, which can be used universally. It should allow the users efficiently to distinguish the known species and accelerate the discovery of species. DNA barcoding uses the information of one or a few regions in the genome to recognize all the species in a genus (Lahaye *et al.* 2008). The effectiveness of DNA barcoding depends on the detection and description of new cryptic species (Handfield and Handfield 2006; Smith *et al.* 2006; Anker *et al.* 2007; Bucklin *et al.* 2007; Gomez *et al.* 2007; Pfenninger *et al.* 2007; Tavares and Baker 2008) and sibling species (Hogg and Hebert 2004; Amaral *et al.* 2007; van Velzen *et al.*

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2007).

In addition, it is important to distinguish, detect and trace the distribution of patented organisms in agro-biotechnology, to certify the source organism like truffles (Rastogi *et al.* 2007). DNA barcode has many scientific domains like ecology, biomedicine, epidemiology, evolutionary biology, biogeography, conservation biology and bio-industry. The low cost and time-effectiveness makes the process easier for enabling automated species identification in massive sampling campaigns (Rusch *et al.* 2007). Other novel molecular technologies like bioengineering (e.g. silicon-based microarrays, nylon membrane based macroarrays, etc.) are becoming cheaper and integrated into the second step of DNA barcoding (Summerbell *et al.* 2005). PCR-based techniques are highly applied in the fields of taxonomy, food and forensic (Teletchea *et al.* 2005).

DNA barcoding will open up new opportunities in DNA based investigations ranging from community phylogenetics (Webb *et al.* 2002) to ecological genomics (van Straalen and Roelofs 2012). Despite the lack of consent regarding a universal plant barcode, taxonomists, ecologists, evolutionary biologists and conservationists are already envisioning the application of genetic identifiers to a wide set of research and practical programs. The suitability of a locus for large-scale DNA barcoding can be easily investigated by comparing loci across a similar set of taxa under a selected set of reaction conditions. Thus, the statistics was taken into account between the ability to amplify a locus and the rate of divergence of that locus across a phylogenetic range of taxa. Additionally, the sequence alignment methodologies are available, which can be evaluated for the use of DNA barcodes with the following regards, the purpose of assurance limits to species assignment, the use of a part of sequences in database searches and strength of search algorithms of sequence length variation due to insertion/deletion events and the informative nature of these mutations.

DNA barcoding mirrors the distributions of intra- and inter-specific variation that are separated by a distance known as DNA barcoding gap (Hebert *et al.* 2003; Meyer and Paulay 2005). The Consortium of Barcode of Life coordinates DNA barcoding development and implementation universally. DNA barcoding is very essential for, (i) the molecular identification of already described species

(Hebert *et al.* 2003) and (ii) the discovery of undescribed species (Valentini *et al.* 2006). DNA barcoding is the conjugation of molecularization the use of the variability in molecular markers as a discriminator, computerization, the transposition of the data using informatics and standardization. The ultimate aim of DNA barcoding is to discriminate the species using an automated system so that unexplored living organisms can be named before it gets extinct. Rapid progress in DNA sequencing and computational technologies made CBOL to build a universal organization for a living beings inventory: the BOLD system (Ratnasingham and Hebert 2007).

Many of the published work used a simple distance matrix analysis, a Neighbour-Joining (NJ) algorithm with Kimura-2-parameters (K2P). The identification and characterization of molecular entities are the main goal in DNA barcoding studies. An ideal DNA barcode should possess high inter-specific, low intra-specific sequence divergence. It should undergo universal amplification with standard primers and technically simple to analyze. It should be readily recoverable from the museum or herbarium samples and other degraded samples.

Plant DNA barcoding

Plant DNA barcoding has a huge role in the conservation biology especially in assessment of biodiversity hotspots and also to monitor the international trade in rare species. Plants have not been given much importance in the early stages of DNA barcoding due to inability of cytochrome oxidase (*COX1*) to work as a barcode in plants (Cho *et al.* 2004). The contest was set among the botanists to find a more suitable marker (Pennisi 2007). Many candidate gene regions have been recommended as possible barcodes for plants, but still there is no universal barcode (Kress *et al.* 2005). The lack of consensus region in plants as in the case of *COX1* in animals as a universal barcode for plants has not been found until now, instead several groups have put forward different barcode candidates successfully for the smaller taxa. Several factors are considered in selecting a plant DNA barcode. These are (i) universal PCR condition, (ii) range of taxonomic diversity, (iii) power of species differentiation, and (iv) dry lab analysis and application. The various loci and intergenic spacers have been proposed

as plant barcodes. DNA barcoding must be realistic for a wide range of practitioners and the methodology must be accessible and easily carried out by multiple users. The strength of DNA barcoding was directly related to the data available in the barcode libraries which helps in building a very complete DNA barcoding database (Ekrem *et al.* 2007). These considerations required a standard range of PCR conditions along with a set of standard PCR primers per gene which serve as a robust barcode marker for the widest range of taxa and users. And also focused on the feasibility of barcoding plants from highly degraded samples (Taberlet *et al.* 2006) (e.g. permafrost samples) and other applied fields (e.g. processed food, customs, medicinal plants). They suggested the chloroplast *trnL* (UAA) intron or a shorter fragment of this intron (the P6 loop, 10-143 bp), in spite of relatively low resolution could be improved with highly conserved primers.

In plants, the mitochondrial genome evolves much more slowly than in animals. The mitochondrial gene *COXI* region was unsuitable for plant species distinction (Rubinoff 2006). The CBOL plant-working group (PWG) concluded that plant DNA barcoding would be multi-locus, with one 'anchor' (i.e. universal across the plant kingdom) and 'identifiers' to distinguish closely related species. Several combinations of DNA regions have been recently proposed by (Kress *et al.* 2005; Chase *et al.* 2007; Kress and Erickson 2007; Pennisi 2007). At present, there is still no consensus on which candidate markers are the best plant DNA barcoding region. The future combination will certainly contain noncoding intergenic spacers like *trnH-psbA* and plastidial coding sequences like *matK* (Chase *et al.* 2007).

Many applications can be implemented with the help of DNA barcodes like identification of different life stages thereby enabling discrimination between seed and seedlings and discovering the changes of metamorphosis. It is difficult to distinguish morphologically the young leaves, seeds and seed coats of certain species, which are related. DNA barcoding helps in distinguishing them at the molecular level. DNA barcoding can be employed in forensic analysis, helps in identifying adulterated products from original components. It performs as a biosecurity for trade in the controlled species like illegal import and export of economically valuable things. It can be employed to

assess the taxonomically diversified species both native and invaded species. The DNA barcode project aims to develop a simple diagnostic tool based on strong taxonomic data that is collated in the DNA barcode reference library (Schindel and Miller 2005).

Potential plant barcode candidates

The plant DNA barcoding studies were initially restricted to the chloroplast genome to understand the variation of its gene sequences of coding (*matK*, *rbcL*, *rpoCl* and *psbA-trnH*) and non-coding (ITS, *psbA-trnH*), which has been summarized by Chen *et al.* (2010). The studies proved that the chloroplast genome showed high degree of variation and discrimination ability (Lahaye *et al.* 2008). The slow rate of their substitution, limits the ability to identify plants among the species (Baldwin *et al.* 1995). The evidence suggests that species-lineage reconstructions using Chloroplast DNA (cpDNA) regions show significant errors, when hybridization and introgression events or linear sorting events occur (Doyle 1992).

matK

The *matK* gene of chloroplast codes for maturase like protein, which is involved in Group II intron splicing. The gene contains approximately 1500 base pairs, which was located within the intron of the *trnK*. The two exons of the *trnK* gene that flank the *matK* were lost, leaving the gene intact in the event of splicing (Wolfe *et al.* 1992). The gene contains high substitution rates within species and is emerging as one of the potential gene and it is being used in the studies of molecular systematics and evolution (Johnson and Soltis 1995). A homology search specifies 102 amino acids at the carboxyl terminus that are structurally related to portions of maturase-like polypeptide and this might be implicated in splicing of Group II introns (Mohr *et al.* 1993). The *matK* gene has been used effectively in phylogenetic studies for the families Saxifragaceae (Johnson and Soltis 1994), Polemoniaceae (Steele and Vilgalys 1994), Poaceae (Liang and Hilu 1996), Orchidaceae tribe Vandaeae (Jarrell *et al.* 1995), and Myrtaceae (Gadek *et al.* 1996). Among the available Chloroplast genes, *matK* has got high substitution rates, which makes it as one of the ideal barcode candidates. *matK* has been proven as DNA

barcode for the plant family Zingiberaceae (Selvaraj *et al.* 2008). In 2010, the Linnaean Society of London, performed large scale analysis of *matK* gene, compared the conventionally available primer to assess the existing primer match with previously generated *matK* sequences and to suggest possible modifications primarily to simplify the complications in barcoding.

rbcL

The *rbcL* gene in higher plant presents as a single copy per chloroplast genome, but many copies of the genome are present in each plastid, the actual *rbcL* copy number per chloroplast can be high. *rbcL* contains only exons, polypeptide with ~475 amino acids. Several chloroplast genes, including *rbcL*, the transcriptional (Bradley and Gatenby 1985; Gruissem and Zurawski 1985) and translational (Gatenby *et al.* 1989) identified sequences resemble as those found in prokaryotic organisms to the extent that chloroplast *rbcL* genes can be readily expressed in *Escherichia coli* (Gatenby *et al.* 1981). Transcriptional initiation rates from the *rbcL* promoter are not only influenced by the promoter sequence but also modified by the nearby *atpB* promoter. The two promoters are positioned 400 bp apart in opposite orientations, resulting in divergent transcription. They do not function independently, because RNA polymerase binding at the *rbcL* promoter interferes with binding and transcription of the *atpB* promoter, presumably by steric hindrance at the two RNA polymerase binding sites. Removals of one promoter or increasing the space between them eliminate this mutual interference (Hanley-Bowdoin and Chua 1987), which may be a control mechanism to regulate the different levels of expression in chloroplasts.

The substitutions between *rbcL* of the parent species are non-synonymous. Even single amino acid replacements in *rbcL* could result in differences in the CO₂ and O₂ specificity of ribulose 1, 5 biphosphate carboxylase/oxygenase (RuBisCO) (Galmes *et al.* 2005). The speculation is that the amino acid difference in *rbcL* plays an important role in ecological adaptation. It provides all the catalytically essential residues of RuBisCO, a significant enzyme for both the reductive and oxidative photosynthetic carbon cycles. The sequence of *rbcL* has great phylogenetic importance because of its conserved nature (Albert *et al.*

1994), although substitutions occur in sites of known functional importance (Kellogg and Juliano 1997). A contingency analysis was performed to evaluate the *rbcL*+*matK* barcode among the major taxonomic groups of specifically, non-angiosperms vs. angiosperms and mono- vs. polytypic-genera (Burgess *et al.* 2011). By comparing the two DNA barcode markers *rbcL* and *matK*, *rbcL* shows better coverage than *matK* for the native flowering plants and conifers of Welsh flora (de Vere *et al.* 2012).

psbA-trnH

Intergenic cp DNA regions contain a wealth of information useful for inquiries in population genetics and low-level systematics (McCauley 1995; Provan *et al.* 2001). Portions of these non-coding regions whose functions were unknown but much of the variation may result from the spread of mutations not restrained by selection (Olmstead *et al.* 1992; Hamilton *et al.* 2003; Monde *et al.* 2000; Manuell *et al.* 2004). Population genetic studies have revealed that the end spacer of the intergenic spacer *psbA-trnH* nearest to *psbA* was highly conserved and showed a large inversion at the end nearest to *trnH*, which appears to be more variable. The intergenic spacers have been given more importance in ongoing DNA barcoding efforts (Kress and Erickson 2007; Shaw *et al.* 2005). The structure of *psbA-trnH* have a small spacer region with a length of about 200–500 bp in angiosperm and gymnosperm species, which is amplified using the universal primers developed by (Hamilton 1999, Storchova and Olson 2007). However, the high nucleotide variation has made it difficult to identify conserved regions among the highly diverged taxonomic groups. Surprisingly, short regions (6–30 bp) were conserved across angiosperms. Unfortunately, their brevity limits their potential for phylogenetic inference across highly diverged taxa. The *psbA-trnH* intergenic region contains two parts that differ in their evolutionary conservation, *psbA* 3'UTR, which is responsible for the regulation of gene expression, and *psbA-trnH*, which is a non-transcribed intergenic spacer that has no function because of its high variability across angiosperms. The *psbA* gene encodes the D1 reaction center protein of photosystem II and its expression depends on light intensity, the plant developmental stage and the physiological state of

the plant (He *et al.* 1998). This gene accumulates to high levels in chloroplasts, but is only present at low levels in amyloplasts (Deng and Gruissem 1987). The role of chloroplast *psbA* UTRs in regulation of gene expression has been examined rigorously for more than twenty years (Zurawski *et al.* 1982). The frequent inversions in a region of *trnH-psbA* are flanked by inverted repeats. There was an inversion in *trnH-psbA* that has been noted before in various plant lineages. The *trnH-psbA* region shows several traits, it is short length (often, 500bp), suspected ubiquity in plants, high interspecific sequence divergence and universal flanking primers that allow easy amplification and sequencing from both high molecular weight and it also effective in degraded DNA which suspects desirable barcode (Chase *et al.* 2005; Kress *et al.* 2005; Shaw *et al.* 2005; Erickson *et al.* 2008). But in few plant lineages *trnH-psbA* does not show better amplification or multiple bands (Sass *et al.* 2007). It is irregularly longer than the possible barcode candidate (Shaw *et al.* 2005; Hollingsworth *et al.* 2009). It also contains mononucleotide repeats which are difficult for sequence accuracy and insertion events (Wang *et al.* 2006). Within some groups, *trnH-psbA* was not effectively variable to differentiate among the closely related species (Sass *et al.* 2007) and in others intra-specific variation was found to be high (Edwards *et al.* 2008). In the meta-analysis of plant DNA barcodes, *trnH-psbA* was significantly higher in identification than those of the other *matK+rbcL* for 18 families and 21 genera (Pang *et al.* 2012).

atpB

The *atpB* (adenosine triphosphate β subunit) gene is involved in synthesis of the β subunit of ATP synthase and its size is estimated to be about 1497 bp in spinach. The chloroplast ATPase is composed of five subunits, α , β , γ , δ , and ϵ . The structure and organization of the *atpB* gene in spinach (*Spinacia oleraceae*) was studied by Zurawski *et al.* (1982). They found out that, the gene *atpB* and *atpE* lie closer to *rbcL*. It was demonstrated that the protein can be synthesized by *in vitro* translation of chloroplast RNA that had been selected on the basis of its hybridization to specific fragments of spinach chloroplast DNA and from this, it is evident that the α and β subunit are encoded by

chloroplast DNA. Also, the coding region assigned to the β and ϵ subunit has four nucleotide in common and also the ϵ subunit translation start codon overlaps with the β subunit stop codon. The overlapped coding regions are co-transcribed into a single RNA species. The comparison of β and ϵ subunit of spinach chloroplast ATPase with those of *E. coli* showed that β subunit to be far more highly conserved than the ϵ subunit. Also it is established that *atpB* and *rbcL* are transcribed divergently in the chloroplast DNA.

ITS

The internal transcribed spacer (ITS) region belonging to the nuclear genome is a non-functional RNA sequence located between the 18S and 25S rRNA coding regions. The ITS1 is present between 18S and 5.8S rRNA, while ITS2 is between 5.8S and 25S rRNA (Gerbi 1985). The ITS was a transcriptional unit situated between the structural ribosomal RNA during rRNA maturation, the ITS spacers are excised and non-functional maturation product are rapidly degraded. Mutational studies of the ITS region of yeast (*Saccharomyces cerevesiae*) have shown that deletions of certain regions within ITS1 inhibited the production of mature small and large subunit rRNAs (Musters *et al.* 1990; van Nues *et al.* 1994), whereas certain deletions or point mutations in ITS2 prevented or reduced processing of large subunit rRNAs (van der Sande *et al.* 1992). The length of ITS1 and ITS2 of all flowering plants varies, but is less than 300 bp for ITS1 (187 to 298) and nearly 250 bp for ITS2 (187 to 252). The total length of the ITS region is around 700 bp, including the 5.8S rRNA region, which has a constant length of 163 or 164 bp (Goldman *et al.* 1983; Stewart *et al.* 1983; Gonzalez *et al.* 1990). The length of the ITS region varies due to point mutations. The percentage of mutations of ITS1 at the intra-specific level is 5.1%, while it is 8.1% at the inter-specific level. The percentage of mutations of ITS2 at the intra-specific level is 6.3%, while it is 6.7% at the inter-specific level (Baldwin 1992).

The ITS region of the nuclear DNA (nrDNA) occurs as tandem repeats at multiple chromosomal loci (Rogers and Bendich 1987; Hamby and Zimmer 1992). The high copy number of the ITS region promotes detection, amplification, cloning and sequencing of nr DNA (Baldwin *et al.* 1995). The PCR efficiency of the ITS region is high when

compared to barcode candidates therefore, it can be further subjected to restriction digestion, which generates distinctive diagnostic bands that can be used to effectively differentiate and identify plants at their species level (Manhart and McCourt 1992).

Fortunately, comparison of chloroplast DNA with that of nuclear DNA has provided an effective means of identification that can significantly improve our understanding of the origin of polyploid species (Soltis and Kuzoff 1993). For this reason, we investigated the nuclear genome under the assumption that nr DNA regions must be evolutionarily conserved, phylogenetically interpretable, easily examined in the laboratory with universal primers and potentially useful characters for phylogenetic reconstruction (Baldwin 1992). DNA barcoding research is aimed at identification of unique candidate genes for identification of all plant species using both coding and non-coding regions (Kress and Erickson 2008). In the 3rd International Barcode of Life Conference, 2009, *matK* and *rbcL* were confirmed as Universal Plant DNA Barcodes. In the 4th International Barcode of Life Conference, 2011, *matK*, *rbcL* and ITS were proposed as barcode candidates for both plant and tree species. Earlier, quantitative measurements to compare barcode candidates were based on simple statistics calculations, PCR universality and sequence divergence. These analyses should allow direct comparison of putative DNA barcode markers.

From various studies, analysis and reports, it has been confirmed that the nuclear ITS2 and chloroplast *psbA-trnH* are capable of distinguishing closely related species. ITS2 proved to be better than *psbA-trnH* for the common medicinal plant species used in Chinese pharmacopoeia. ITS2 can also be used to group the plant samples into their right genus and has a relatively high accuracy for grouping samples into their exact species. The impressive practical value for DNA barcoding is to identify the plant specimens by individuals without enough taxonomic training and also it plays an important role in evolutionary studies (Coleman 2003, 2007). The ITS2 have also been proven to discriminate the plant species of Fabaceae (Gao *et al.* 2010a). Plant samples (1410) representing 893 species in 96 diverse genera from Rosaceae were evaluated using ITS2 as a barcode gene. ITS2 showed success rates of discriminating

species of 78% and 100% at the species and genus levels, respectively (Pang *et al.* 2012). The ITS2 region is commonly used to authenticate Chinese herbal medicine (Coleman 2009; Song *et al.* 2009).

ITS2 has been shown to have a success rate of 80% for distinguishing between plant species of the Asteraceae (Gao *et al.* 2010b). ITS2 was also used to identify morphologically similar species such as *Swartzia grandifolia* and *S. longicarpa* for classification of the controversial species *Caranga rosea* and *C. sinica* of the plant Fabaceae (Gao *et al.* 2010a). Additionally, ITS2 has been shown to identify the system Eukaryota (Schultz *et al.* 2007), Pan-eukaryote (Coleman 2007) and as a universal barcode for plants and animals. There is also a report of using the chloroplast inter-generic region *psbA-trnH* to identify *Dendrobium* species and it has been recommended as an ideal DNA barcode candidate (Song *et al.* 2009). DNA barcoding was employed to identify cryptic species distributed in the Velliangiri hills of India, adding value to both traditional and scientific knowledge (Newmaster and Raghupathy 2010). The same technique was applied to identify the Indian *Berberis* species (Roy *et al.* 2010) and Indian endangered species of *Paphiopedilum* (Parveen *et al.* 2012). The ITS2 has also been used to distinguish the morphologically similar species of *Boerhavia* (Selvaraj *et al.* 2012). ITS2 in combination with *rbcL* has been used to identify contaminants in North American herbal products (Newmaster *et al.* 2013). And recently the chloroplast intergenic spacer (IGS) like *trnE-trnT*, *trnT-psbD*, *ndhF-rpl32* and *rpl14-rpl16* were applied for phylogenetic analysis and molecular evolution studies for *Panax* species (Kim *et al.* 2013). Additionally with coding regions of *rbcL*, *matK*, the IGS like *atpF-atpH*, *psbK-psbI* and *trnH-psbA* were used as DNA barcodes for discriminating the species of Korean Orchidaceae (Kim *et al.* 2013, in press).

DNA barcode candidate from the chloroplast genome like *rbcL*, *matK*, *rpoCl*, *atpB*, *psbA-trnH* (intergenic spacer) and *nrITS* (internal transcribed spacer) has been evaluated for the plant family of Apocynaceae. This study identifies the *nrITS* region to be better candidate than other regions tested. Especially the region ITS2 showed 4% intra-specific variation followed by *psbA-trnH*, 2%, whereas the other proposed candidates showed only 1% variation. The

parameters used to characterize the inter-specific divergence and intra-specific divergence based on within each genus and species. The identification efficiency was confirmed by two methods (a) BLAST1 method, and (b) Distance method at species level. ITS clearly distinguishes the 17 species of *Cynanchum* by using Minimum evolution method representing the out group *C. auriculatum_isolate* as shown in the Fig. 1. Henceforward, the present study concludes that ITS2 can be used to discriminate the plant species of Apocynaceae. However screening of more Apocynaceae species was required. Secondly the proposed DNA barcodes *rbcL*, *matK*, *psbA-trnH* and ITS2 were

evaluated for the plant species of Zingiberaceae. The region ITS2 showed 4% intra-species variation followed by *psbA-trnH* (0.8%) shown in the Table 1. The analyses revealed, ITS2 region is able to distinguish all the tested species of Zingiberaceae. As Zingiberaceae is one of the larger families in the plant kingdom, the evaluations of the DNA barcodes have to be carried out for more number of species covering many genera in order to confirm the above results. Identification methods like BLAST and Distance were used for the gene ITS2 for the nine large genera, which were applied to species of Zingiberaceae (Table 2). These proposed barcode candidates were also applied to

Fig. 1. Phylogenetic tree constructed by Minimum Evolution method for the seventeen species of *Cynanchum* using ITS gene.

Table 1. Analysis of interspecific divergence between congeneric species and intra-specific variation among the tested four loci for the plant family Zingiberaceae.

	Markers			
	<i>psbA-trnH</i>	ITS2	<i>matK</i>	<i>rbcL</i>
All inter-specific distance	0.037 ± 0.112	0.143 ± 0.431	0.0157 ± 0.0308	0.0110 ± 0.0202
Theta prime	0.019 ± 0.049	0.122 ± 0.146	0.0175 ± 0.0212	0.0018 ± 0.0116
Minimum inter-specific distance	0.013 ± 0.085	0.090 ± 0.208	0.003 ± 0.0260	0.0055 ± 0.0183
All intra-specific distance	0.012 ± 0.0812	0.088 ± 0.180	0.0164 ± 0.079	0.0029 ± 0.0057
Theta prime	0.009 ± 0.273	0.023 ± 0.098	0.010 ± 0.0352	0.002 ± 0.0037
Coalescent depth	0.081 ± 0.202	0.078 ± 1.876	0.044 ± 0.799	0.001 ± 0.0373

differentiate and authenticate the medicinally important species of *B. diffusa* from other species of *Boerhavia* (*B. repanda*, *B. erecta* and *B. verticillata*) using DNA barcodes. In this case ITS was able to distinguish *B. diffusa* from other three species as shown in the Fig. 2. Hence, this

study clearly proved that DNA barcoding could be used to authenticate the medicinal plant. More so, the information presented here is useful and applicable for identifying and discovering the new species as well as certifying the correct species of the plant lineages.

Fig. 2. Phylogenetic tree constructed by Minimum Evolution method for the fifteen species of *Boerhavia* using ITS gene (Selvaraj *et al.* 2012).

Table 2. Identification efficiency for nine large genera using ITS gene for the plant family Zingiberaceae.

Genus	Length (bp)	Number of species	% of Variation	Method	% Success Identification
<i>Afromum</i>	605	53	0.3	BLAST1	89.0
				Distance	92.2
<i>Boesenbergia</i>	577	3	0.09	BLAST1	93.0
				Distance	89.6
<i>Curcuma</i>	602	9	0.09	BLAST1	93.0
				Distance	89.6
<i>Globba</i>	572	2	0.06	BLAST1	90.0
				Distance	97.6
<i>Haniffia</i>	575	2	0.0	BLAST1	100.0
				Distance	100.0
<i>Hedychium</i>	572	4	0.03	BLAST1	98.0
				Distance	97.0
<i>Kaempferia</i>	689	3	0.1	BLAST1	89.0
				Distance	93.3
<i>Roscoea</i>	569	5	0.04	BLAST1	95.0
				Distance	98.8
<i>Zingiber</i>	450	21	0.21	BLAST1	81.2
				Distance	88.3

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