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DNA Barcoding of Endangered Sacred Plant (Stereospermum suavelons*)* in Cuddalore District, Tamil Nadu, India

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Abstract

In the present investigation, DNA barcodings were made for the sacred trees of Main (Big) Temples in Cuddalore district, namely*, Stereospermum suavelons* to document their reliable identification, conservation, discrimination, similarities and evolutionary trend among them and with their related taxa for future use. DNA isolation from leaf samples of present study species was carried out by using a modified CTAB method and good isolation was got for the species studied.Gradient PCR amplification was performed for the isolated DNA using matK gene and the primers matK472F & matK1248R. The amplification success was 90-95%. PCR amplification was tested with 1 % agarose gel electrophoresis using ethidium bromide and the products were confirmed. The PCR products were sent to Xcelris Labs Ltd. Sydney House, Premchand Nagar Road, Bodakdev, Ahmedabad 380054, India, for DNA sequencing and sequences were got for the species with the success rate of 95 %. Pairwise sequence alignments were made with BLAST and multiple sequence alignments are made with ClustalW, and based on the sequence alignments, dentrograms were constructed using software Mega 5 and Neighbor joining method to study the phylogenetic aspects of the species studied and with their related taxa.

Keywords: DNA barcoding, matK gene, matK472F, matK1248R, sacred plants, *Stereospermum suavelons*, Phylogenetic tree, identification, conservation and documentation.

Introduction

DNA Barcoding is a new tool for the science of taxonomy at genotypic level. DNA barcoding as a scientific idea, initiated by Paul Herbert in 2003 has been attracting international attention for its significance in advancing the taxonomy of life forms. In other words, DNA barcoding is a taxonomic method that uses a short genetic marker in an organism's DNA to identify it as belonging to a particular species. It differs from molecular phylogeny in that the main goal is not to determine classification but to identify an unknown sample in terms of a known classification. Although barcodes are sometimes used in an effort to identify unknown species or assess whether species should be combined or separated, such usage, if possible at all, pushes the limits of what barcodes are capable of plants.

Although a few million species have been discovered and identified, many millions more, including micro – organisms, remain to be discovered and documented. Whether a species is endemic to a geographical region or is widely distributed, barcoding of life is truly international in scope.

In addition, DNA sequences have potential for routine plant identification in conjunction with a high throughput and bioinformatics system and optimal for the taxonomically wide plant identification with high relatedness within taxon variability and lack of confident assignment of orthology to overcome these hurdles (Blaxter, 2004). Some DNA regions are sufficiently conserved to permit primer sets for PCR amplification and contain sufficient informative sequence variation to discriminate up to the species level. For plants, many genes have been proposed as DNA barcodes till now. Applications include, for example, identifying plant leaves even when flowers or fruit are not available, identifying the diet of an animal based on stomach contents or feces, and identifying products in commerce (e.g. Herbal supplements or wood).

As barcode has multiple applications, has been used for ecological surveys (Dick & Kress, 2009), cryptic taxon identification (Lahaye et al., 2008), and confirmation of medicinal plant samples (Xue & Li, 2011). The DNA Barcoding has inspired a global initiative dedicated to create a library of new knowledge about species diversity, making that knowledge accessible and applying that knowledge to create tangible benefits. The uses and global benefits of DNA barcoding include i) Controlling agricultural pest, ii) identifying disease vectors, iii) Sustaining natural resources, iv) protecting endangered species and v) Monitoring water quality.

Universal matK primers for DNA barcoding

The chloroplast maturase K gene (*matK*) formerly known as *orf*K is one of the most variable coding genes of angiosperms and has been suggested to be a "barcode" for land plants. However, *matK* exhibits low amplification and sequencing rates due to low universality of currently available primers and mononucleotide repeats. To resolve these technical problems, the entire *matK* region has been evaluated to find a region of 600–800 bp that is highly variable, represents the best of all *matK* regions with priming sites conservative enough to design universal primers, and avoids the mononucleotide repeats. The following are the important universal matK primers used widely. 1. matK-2.1aF ATCCATCTGGAAATCTTAGTTC Royal Botanic Gardens, Kew (2007) 2. matK-5R GTTCTAGCACAAGAAAGTCG Royal Botanic Gardens, Kew (2007) 3.matK-1018R GTACYACYGAAKRATYBAGYCSCAC Zhang *et al.*(2006) 4.matK-390F CGATCTATTCATTCAATATTTC

Cuénoud *et al.* (2002) 5.matK-1326R TCTAGCACACGAAAGTCGAAGT Cuénoud *et al.* (2002) 6. matK472F 5'-CCCRTYCATCTGGAAATCTTGGTTC-3' Yu et al. (2011) and 7. matK1248R 5'-GCTRTRATAATGAGAAAGATTTCTGC-3' Yu et al. (2011).

Sacred trees

Sacred Trees (Sthalavrikshas) have a unique place in the fabric of Indian heritage. Trees symbolize knowledge and spirituality. In ancient times, the trees were worshipped along with Gods and established in the temples as Sthalavrikshas, i.e., trees associated with the deity in the temple and became an inseparable part of the faith. Such trees were guarded and their saplings from mother plants were given to other temples.

Sacred trees are important not only for their botanical, economical, medicinal, environmental, religious and mythical values but also form an important biological heritage of our nation that plays role in the conservation of environment and biodiversity.

The Sthalavrikshas constitute a part of genetic resources for the conservation of species diversity. Propagation of Sthalavrikshas in temples contributes to the conservation of floral diversity. Some trees are important for their economic role in ship building or in the timber industry, some for providing homes for various animals, birds and others for their medicinal and air purifying qualities.

Stereospermum suavelons (Bignoniaceae) is a medicinal tree species native to India (Troup R.S., 1986). The Bignoniaceae having about 100 genera with 800 species, are known for their antimicrobial, antiprotozoal, and anti-inflammatory properties (Binutu O.A., et al., 1996; Onegi B, et al., 2002). Both the timber (Sandermanns W. 1957) and the root heartwood (Joshi k.c. et al., 1977) of *S. suaveolens* were found to contain lapachol, elicitor of contact dermatitis (Schulz K.H. et al., 1977)

Moreover, barks, flowers, roots and leaves of *S. suaveolens* are used by traditional healers, rural communities and pharmaceutical companies for remedies of diseases like heating, vomiting, eructation, piles, acidity, diarrhoea, gonorrhoea, loss of taste, malaria and other fevers (Troup R. S., 1986).

Materials and Methods

Sample collection

The materials used and the methods followed in the present investigation are described below. In Cuddalore district, young leaf samples of sacred trees, namely, *Stereospermum suavelons* DC. Pathiri (Bignoniaceae) were collected from Padaleshwarar Temple, Cuddalore for the isolation of genomic DNA.

DNA isolation, PCR amplification, DNA sequencing

DNA isolation was carried out by using modified CTAB (Cetyl Trimethyl Ammonium Bromide) method (Sambrook, J., and Russell, D. W. 2001), which was designed for plants producing large amounts of secondary metabolites, exudates, milky or latex and oil substances. As the chloroplast maturase K gene (matK gene) is one of the most variable coding genes of angiosperms and suggested to be a "barcode" for land plants, in the present study, the same was used.

Gradient PCR was performed using isolated genomic DNA of this study to determine the optimum annealing temperatures of the Primers used, namely *matK*472F (5′-CCC RTY CAT CTG GAA ATC TTG GTT C-3′) and *matK*1248R (5′-GCT RTR ATA ATG AGA AAG ATT TCT GC-3′). This primer was obtained from Genei, Bangalore. The PCR reaction mixture was consisted of 10x Taq buffer, dNTP mix 100 mM, Taq polymerase (3U / µl) and 5 – 50 ng of template DNA. Thermal cycling conditions for gradient PCR were as follows: Initial DNA denaturation at 94°C for 3 minutes, followed by 40 cycles of final DNA denaturation at 94°C for 30 second, primer annealing temperature at 48-52°C for 40 second, DNA strand extension at 72°C for 1 minute, and final extension at 72°C for 10 minutes. The PCR products were verified by electrophoresis in 1 % agarose gel stained with ethidium bromide.

The PCR products were sent to the Xcelris Labs Ltd. Sydney House, Premchand Nagar Road, Bodakdev, Ahmedabad 380054, and India for DNA sequencing and the sequences were obtained. All the obtained sequences were submitted to gene bank. (Note: In 10 µl sequencing reactions, using 3 µl sample and 0.25 µl BigDye Terminator V 3.1, methods ABI – 3730 XL sequencer gave good amplification and sequences).

Sequence Analysis

Each obtained sequence was entered into Gene Bank's BLAST search function (http://blast.ncbi.nlm.nih.gov/Blast.cgi), using Megablast parameter to assess the similarities and differences between the coded sequences of the sacred plants of the present study and with other related taxa. The similarity was also examined for the closest matches among them.

Phylogenetic analysis

All the sequences were aligned using ClustalW and the genetic distances were computed using MEGA 5 software, and the phylogenetic tree was built using NJ method. The bootstrap test with 1000 replicates was applied to assess the reliability of phylogenetic trees.

Results

DNA isolation

For the isolation of genomic DNA of the species studied, the modified protocol of Cetyl Trimethyl Amonium Bromide method (CTAB method) was used and good isolation was obtained for the sample.

PCR amplification

The Gradiant PCR amplification was performed for the isolated genomic DNA of five species of sacred trees mentioned earlier by using matK gene and its primers, namely matK472F and matK 1248R, and the amplifications were obtained for the species studied.

DNA Sequence

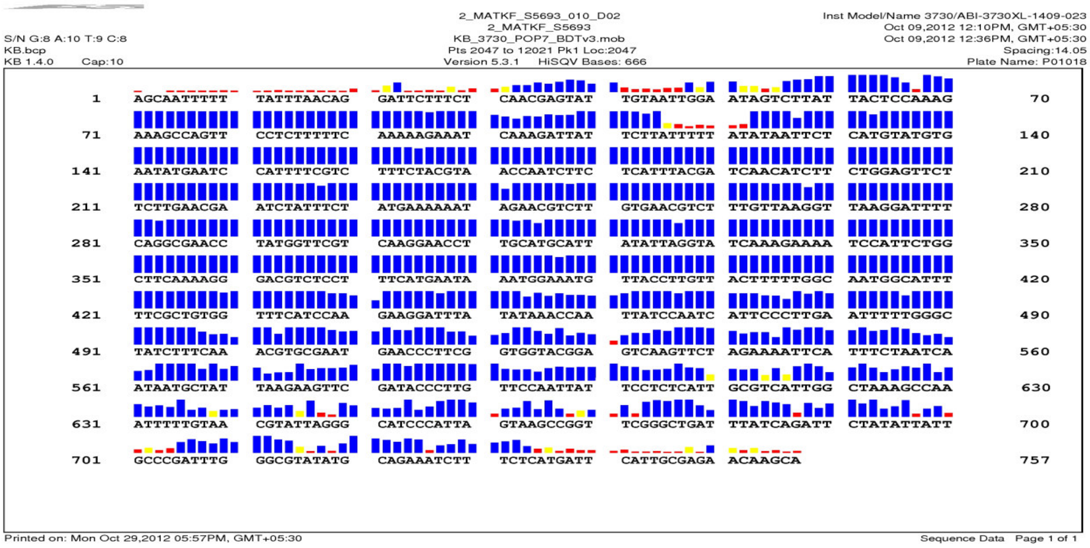
The amplification of Gradient PCR product was strong enough for isolation of bands or direct sequencing and in the present study, the DNA sequences were done at Xcelris Labs Ltd**.** Sydney House, Premchand Nagar Road, Bodakdev, Ahmadabad 380054, India. The methods of ABI – 3730 Xl sequencer gave a success rate of 90-95 % and read length of 700 bases or more.

Stereospermum suavelons **DC.**

AGCAATTTTTATTTAACAGGATTCTTTCTCAACGAGTATTGTAATTGGAATAGTCTTATTACTCCA
AAGAAAGCCAGTTCCTCTTTTTCAAAAAGAAATCAAAGATTATTCTTATTTTTATATAATTCTCATG
TATGTGAATATGAATCCATTTTCGTCTTTCTACGTAACCAATCTTCTCATTTACGATCAACATCTTCT
GGAGTTCTTCTTGAACGAATCTATTTCTATGAAAAAATAGAACGTCTTGTGAACGTCTTTGTTAAGG
TTAAGGATTTTCAGGCGAACCTATGGTTCGTCAAGGAACCTTGCATGCATTATATTAGGTATCAAA
GAAAATCCATTCTGGCTTCAAAAGGGACGTCTCCTTTCATGAATAAATGGAAATGTTACCTTGTTAC
TTTTTGGCAATGGCATTTTTCGCTGTGGTTTCATCCAAGAAGGATTTATATAAACCAATTATCCAAT
CATTCCCTTGAATTTTTGGGCTATCTTTCAAACGTGCGAATGAACCCTTCGGTGGTACGGAGTCAAG
TTCTAGAAAATTCATTTCTAATCAATAATGCTATTAAGAAGTTCGATACCCTTGTTCCAATTATTCC
TCTCATTGCGTCATTGGCTAAAGCCAAATTTTTGTAACGTATTAGGGCATCCCATTAGTAAGCCGGT
TCGGGCTGATTTATCAGATTCTATATTATTGCCCGATTTGGGCGTATATGCAGAAATCTTTCTCATG
ATTCATTGCGAGAACAAGCA

Fig. 1 - Padaleshwarar Temple (Cuddalore)

Fig. 2 – Sthalavriksham – *Stereospermum suavelons* DC



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                                  2_MATKF_S5693                    Oct 09,2012 12:10PM, GMT+05:30
S/N G:8 A:10 T:9 C:8           KB_3730_POP7_BDTv3.mob              Oct 09,2012 12:36PM, GMT+05:30
KB.bcp                       Pts 2047 to 12021 Pk1 Loc:2047                          Spacing:14.05
KB 1.4.0      Cap:10         Version 5.3.1    HiSQV Bases: 666                  Plate Name: P01018

  1   AGCAATTTTT  TATTTAACAG  GATTCTTTCT  CAACGAGTAT  TGTAATTGGA  ATAGTCTTAT  TACTCCAAAG   70
 71   AAAGCCAGTT  CCTCTTTTTC  AAAAAGAAAT  CAAAGATTAT  TCTTATTTTT  ATATAATTCT  CATGTATGTG  140
141   AATATGAATC  CATTTTCGTC  TTTCTACGTA  ACCAATCTTC  TCATTTACGA  TCAACATCTT  CTGGAGTTCT  210
211   TCTTGAACGA  ATCTATTTCT  ATGAAAAAAT  AGAACGTCTT  GTGAACGTCT  TTGTTAAGGT  TAAGGATTTT  280
281   CAGGCGAACC  TATGGTTCGT  CAAGGAACCT  TGCATGCATT  ATATTAGGTA  TCAAAGAAAA  TCCATTCTGG  350
351   CTTCAAAAGG  GACGTCTCCT  TTCATGAATA  AATGGAAATG  TTACCTTGTT  ACTTTTTGGC  AATGGCATTT  420
421   TTCGCTGTGG  TTTCATCCAA  GAAGGATTTA  TATAAACCAA  TTATCCAATC  ATTCCCTTGA  ATTTTTGGGC  490
491   TATCTTTCAA  ACGTGCGAAT  GAACCCTTCG  GTGGTACGGA  GTCAAGTTCT  AGAAAATTCA  TTTCTAATCA  560
561   ATAATGCTAT  TAAGAAGTTC  GATACCCTTG  TTCCAATTAT  TCCTCTCATT  GCGTCATTGG  CTAAAGCCAA  630
631   ATTTTTGTAA  CGTATTAGGG  CATCCCATTA  GTAAGCCGGT  TCGGGCTGAT  TTATCAGATT  CTATATTATT  700
701   GCCCGATTTG  GGCGTATATG  CAGAAATCTT  TCTCATGATT  CATTGCGAGA  ACAAGCA                757

Printed on: Mon Oct 29,2012 05:57PM, GMT+05:30                           Sequence Data   Page 1 of 1
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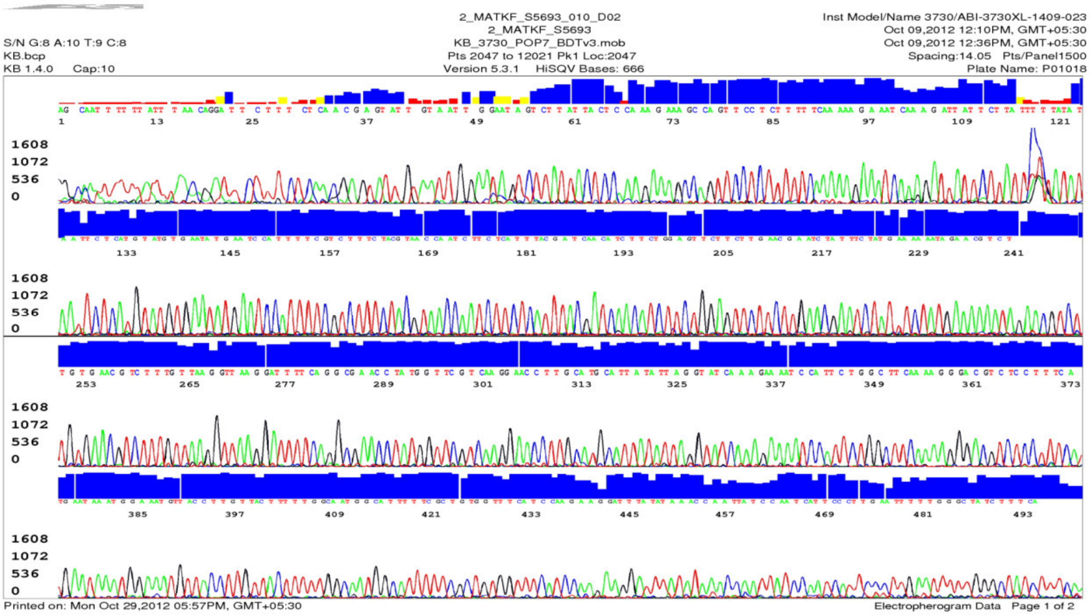
Fig. 3a. DNA Sequences of *Stereospermum suavelons* DC



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                              2_MATKF_S5693_010_D02          Inst Model/Name 3730/ABI-3730XL-1409-023
                                  2_MATKF_S5693                    Oct 09,2012 12:10PM, GMT+05:30
S/N G:8 A:10 T:9 C:8           KB_3730_POP7_BDTv3.mob              Oct 09,2012 12:36PM, GMT+05:30
KB.bcp                       Pts 2047 to 12021 Pk1 Loc:2047            Spacing:14.05   Pts/Panel1500
KB 1.4.0      Cap:10         Version 5.3.1    HiSQV Bases: 666                  Plate Name: P01018

Printed on: Mon Oct 29,2012 05:57PM, GMT+05:30                  Electropherogram Data   Page 1 of 2
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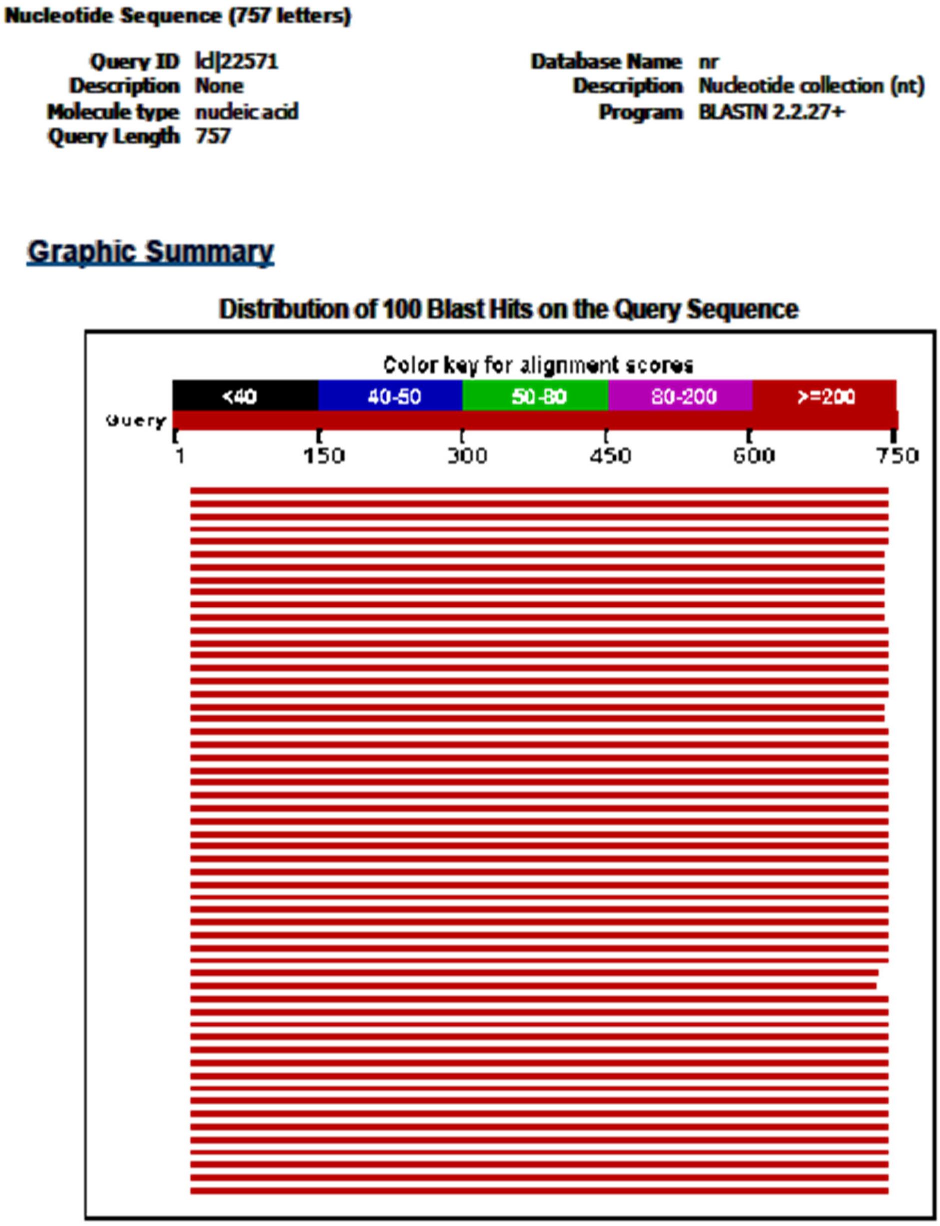
Fig.3b. Electropherogram of *Stereospermum suavelons* DC

Basic Local Alignment Search Tool



Nucleotide Sequence (757 letters)

Query ID lcl|22571
Description None
Molecule type nucleic acid
Query Length 757

Database Name nr
Description Nucleotide collection (nt)
Program BLASTN 2.2.27+

Graphic Summary

Distribution of 100 Blast Hits on the Query Sequence

Fig 4: BLAST Formatting Results of *Stereospermum suavelons* DC

MULTIPLE SEQUENCE ALIGNMENT

```
HQ384523        TTTCTCAACGAGTATTGTAATTGGAATAGTCTTATTACTCCAAAGAAAGCCAGTTCCTCT
HQ384519        TTTCTCAACGAGTATTGTAATTGGAATAGTCTTATTACTCCAAAGAAAGCCAGTTCCTCT
JF270862        TTTCTCAACGAGTATTGTAATTGGAATAGTCTTATTACTCCAAAGAAAGCCAGTTCCTCT
JF270837        TTTCTCAACGAGTATTGTAATTGGAATAGTCTTATTACTCCAAAGAAAGGCAGTTCCTCT
sample          TTTCTCAACGAGTATTGTAATTGGAATAGTCTTATTACTCCAAAGAAAGCCAGTTCCTCT
                ************************************************* **********

HQ384523        TTTTCAAAAAGAAATCAAAGATTATTCTTATTCTTATATAATTCTCATGTATGTGAATAT
HQ384519        TTTTCAAAAAGAAATCAAAGATTCTTCTTATTCTTATATAATTCTCATGTATGTGAATAT
JF270862        TTTTCAAAAAGAAATCAAAGATTATTCTTATTCTTATATAATTCTCATGTATGTGAATAT
JF270837        TTTTCAAAAAGAAATCAAAGATTATTCTTATTCTTATATAATTCTCATGTATGTGAATAT
sample          TTTTCAAAAAGAAATCAAAGATTATTCTTATTTTTATATAATTCTCATGTATGTGAATAT
                *********************** ******** ***************************

HQ384523        GAATCCATTTTCGTCTTTCTACGTAACCAATCTTCTCATTTACGATCAACATCTTCTGGA
HQ384519        GAATCCATTTTCGTCTTTCTATGTAACGAATCTTCTCATTTACGATCAACATCTTCTGGA
JF270862        GAATCCATTTTCGTCTTTCTACGTAACCAATCTTCTCATTTACGATCAACATCTTCTGGA
JF270837        GAATCCATTTTCGTCTTTCTACGTAACCAATCTTCTCATTTACGATCAACATCTTCTGGA
sample          GAATCCATTTTCGTCTTTCTACGTAACCAATCTTCTCATTTACGATCAACATCTTCTGGA
                ********************* ***** ********************************

HQ384523        GTTCTTCTTGAACGAATCTATTTCTATGGAAAAATAGAACGTCTTGTGAACGTCTTTGTT
HQ384519        GTTCTTCTTGAACGAATCTATTTCTATGGAAAAATAGAACGTCTTGTGAACGTCTTTGTT
JF270862        GTTCTTCTTGAACGAATCTATTTCTATGAAAAAATAGAACGTCTTGTGAACGTCTTTGTT
JF270837        GTTCTTCTTGAACGAATCTATTTCTATGAAAAAATAGAACGTCTTGTGAACGTCTTTGTT
sample          GTTCTTCTTGAACGAATCTATTTCTATGAAAAAATAGAACGTCTTGTGAACGTCTTTGTT
                **************************** *******************************

HQ384523        AAGGTTAAGGATTTTCAGGGGAACCTATGGTTGGTCAAGGAACCTTGCATGCATTATATT
HQ384519        AAGGTTAAGGATTTTCAGGCGAACCTATGGTTGGTCAAGGAACCTTGCATGCATTATATT
JF270862        AAGGTTAAGGATTTTCAGGCGAACCTATGGTTCGTCAAGGAACCTTGCATGCATTATATT
JF270837        AAGGTTAAGGATTTTCAGGCGAACCTATGGTTCGTCAAGGAACCTTGCATGCATTATATT
sample          AAGGTTAAGGATTTTCAGGCGAACCTATGGTTCGTCAAGGAACCTTGCATGCATTATATT
                ******************* ************ ***************************
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Fig. 5: Multiple Sequence Alignment of *Stereospermum suavelons* DC using clustalW method.

Table 1: BLAST identification of the sample (*Stereospermum suavelons*)

Accession No.	Accession Description	E value	Max ident
JF270837	*Kigelia Africana* voucher OM217 maturase K(matK) gene, partial cds	0.0	98 %
JF270862	*Markhamia zanzibarica* voucher OM629 maturase K (matK) gene, partial cds	0.0	98 %
HQ384519	*Catalpa aff. speciosa* Olmstead 88-003 trnK gene, partial sequence; and maturase (matK)gene, partial cds; chloroplast	0.0	97 %
HQ384523	*Eccremocarpus scaber* trnK gene, partial sequence; and maturase (matK) gene, partial cds; chloroplast	0.0	98 %

Table 2: The sequences retrieved from NCBI – gene bank for the study of the sample has given in the table.

S. NO.	ACCESSION NO.	SPECIES	AUTHORS
1	JF270837	*Kigelia africana*	Yessoufou et al., 2012
2	JF270862	*Markhamia zanzibarica*	Yessoufou et al., 2012
3	HQ384519	*Catalpa aff. speciosa Olmstead*	Refulio-Rodriguez,N.F. and Olmstead,R.G. 2012
4	HQ384523	*Eccremocarpus scaber*	Refulio-Rodriguez,N.F. and Olmstead,R.G. 2012

Stereospermum suavelons

Morphologically it was identified that the sample 2 is *Stereospermum suavelons.* The matK gene sequence for *Stereospermum suavelons* was not available in gene bank and the present study sequence showed more similarity to the *Kigelia africana* species, belonged to Bignoniaceae family. The *Stereospermum suavelons* also coming under the same family. The other species used in the present study were *Markhamia zanzibarica, Catalpa aff. speciosa Olmstead, Eccremocarpus scaber* and *Kigelia Africana*. All these species were belonging to Bignoniaceae family (Fig.7). The Boot strap value 76 – 93 % was shown in the tree. (Fig. 6)

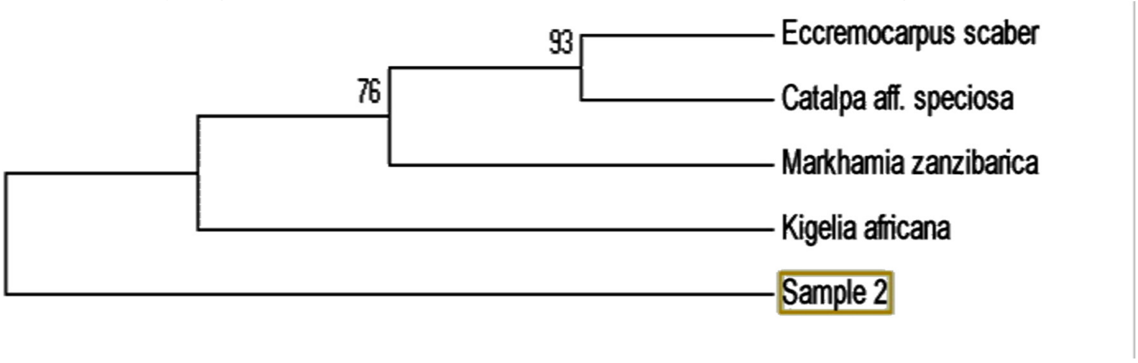

Fig. 6: Phylogenic tree represents the evolutionary relationship between the plant species of *Stereospermum suavelons* DC.

Discussion

DNA barcoding

DNA barcoding is a molecular-based identification system, recently introduced in the scientific community. The method is not completely new to science, but the real innovation is not in the discrimination system itself.

DNA barcoding is promising in providing a practical, standardized, species-level identification tool that can be used for different study including forensic analysis (Lahaye et al. 2008) and animal diet determination when the food is not identifiable by morphological criteria.

Given the pace of advancement in technology, it is not unrealistic that in the span of a few years we may be using a barcoding tool for routine identifications, discovering new species, solving ecological puzzles, controlling the pathways of invasive species, and for quality control in the food and herbal industries.

The characterization of nucleotide and amino acid substitution along the gene may also provide information on site-dependent probabilities of nucleotide substitutions. Such information could provide a guide to the regions to be used in phylogenetic analysis since methods of phylogenetic inference assume the probabilities of replacements are independent of site (Clegg et al., 1994).

Sacred trees

In India, communities have been involved as custodians or stewards in conserving germplasm of crop plants, horticulture, medicinal plants and some species of animals *in situ* over the years. For example, some species are protected by the communities in the form of sacred groves, which may contain endangered species. Communities do not use plant species or parts of the species in the sacred groves. Some species of plants have religious significance among Hindus. For example, *Aegle marmelos*, popularly called Bilva tree, is conserved by planting it in the premises of Hindu temples. Another example: *Ocimum tenuiflorum*, popularly called Tulsi, has some medicinal properties. It is protected by planting it in the backyards of households to have ready access to the plant for medicinal purposes. In fact, Hindu accords a sacred status to the plant and worships it so that it is not neglected.

Scientists recognize the need to describe and document the wide-ranging biodiversity that remains to be explored in India. They stated that DNA barcoding is a valuable technology for this purpose. In India, as in many other countries, there is a perception that taxonomy is a less attractive specialty (Prathapan et al., 2006). Perhaps one of the reasons is that scientists seem to attach more ‘glamour’ to a research career in molecular biology (Haribabu 2000). Research relating to DNA barcoding may change the situation and fill the shortfall in the number of taxonomists. This calls for focused training programs that attract young scientists.

Scientists mentioned that a democratic decision has to be taken regarding which species in the country have to be barcoded, given the fact that there are endemic, endangered and commercially significant species. Since the legislation treats all bio-resources as national resources, accessing the species for barcoding has to be based on formal approvals at different levels. The scientists are of the view that the barcoded information has to be kept in the public domain except in the case of some endemic species which have commercial applications. In this connection, they strongly argue that in any international collaboration Indian scientists should not part with samples of species to be barcoded to collaborating scientists in foreign countries. The samples should be kept in a national repository, or bio – bank and the sequence information may be shared with scientists in other countries.

Based on the vital reasons discussed above, it is necessary to take DNA barcode studies on sacred trees to document their reliable discrimination and identification for future use as they are socially, economically, culturally and religiously important one, and many of them are in the state of endemic and endangered. The Sacred Trees preserved through millennia by our ancestors as potential bio resources should be respected and conserved for the future generations. Hence, the present study was undertaken.

DNA Extraction

Generally, DNA extraction is performed using mainly fresh young leaves. The lower amplification and sequencing success in a study could be explained by the fact that the quality of DNA extracted from young leaves might be influenced by several factors, such as drying and storage conditions and preservation procedures used in herbaria (Erkens et al. 2008).

One of the early studies showed that leaflets were clipped from live plants, dried in silica gel, and then stored at - 80ºC. Whole genomic DNA was extracted using DNeasy Plant Mini Kits (Qiagen, Valencia, CA) or a modified CTAB method (Doyle JJ, Doyle JL. 1987) from fresh or frozen tissue.

Today, as literatures revealed, a modified Cetyl Trimethyl Amonium Bromide method (CTAB method) is followed in majority of the DNA barcode studies to isolate DNA from plant samples and in this study also a modified CTAB method suggested by Sambrook and Russal (2001) was followed to isolate DNA from fresh leaf samples of six sacred trees studied.

PCR amplification

The Gradiant PCR amplification was performed for the isolated genomic DNA of the present study using the matK primers, namely matK472F and matK 1248R and the Gradiant PCR was performed to determine the optimum annealing temperatures of the primer *matK*472F (5′-CCC RTY CAT CTG GAA ATC TTG GTT C-3′) and *matK*1248R (5′-GCT RTR ATA ATG AGA AAG ATT TCT GC-3′). In the early studies, the matK primers 472F and 1248R gave 94% and 96% amplification success rates respectively. In this study also it was confirmed as they gave 95% of amplification rate (Yu et al., 2011). In the present study, the PCR reaction mixture consisted of 10x Taq buffer, dNTP mix 100 mM, Taq polymerase (3U / µl) and 5 – 50 ng of template DNA and the thermal cycling conditions for gradient PCR were as follows: Initial DNA denaturation at 94°C for 3 minutes, followed by 40 cycles of final DNA denaturation at 94°C for 30 second, primer annealing temperature at 48-52°C for 40 second, DNA strand extension at 72°C for 1 minute, and final extension at 72°C for 10 minutes. The PCR products were verified by electrophoresis in 1 % agarose gel stained with ethidium bromide. In the early studies, depending upon the plant materials, there were slight changes in the temperature as well as time duration for denaturation, annealing and extension and they were as follows. Initial DNA denaturation ranged from 93 to 95°C for 1 to 4 minutes and 35 to 40 cycles. Final denaturation ranged from 93 to 95°C for 30 seconds. For primer annealing, it was between 48 to 55°C for 30 to 40 seconds. For DNA extension, it was seen as 72°C for 45 seconds to 1 minute and for final extension it was noted as 72°C for 5 to 10 minutes (Kevin et al., 2011; Xiaorong Guo et al., 2011; Jing YU et al., 2011).

Important criteria for evaluating the suitability of DNA barcode are amplification and sequencing success (CBOL Plant Working Group 2009). In this regard, the coding matK gene region had the best performance in this study with 90 - 95% success in sequencing rate and this result is in consistent with the previous studies on land plants that have reported rates from 78.5 % to 99 % (Kress & Erickson 2007; Fazekas et al. 2008; Gonzalez et al. 2009; Hollingsworth et al. 2009; Kress et al. 2009).

Barcode gene(s) and primer(s)

DNA barcoding, a concept that has recently become popular, is characterized by using one or a few DNA fragments to identify different species (Kress et al., 2005). The mitochondrial cytochrome *c* oxidase subunit 1 (*CO1*) gene was selected to be a DNA barcode for animal species (Hebert et al., 2003). However, consensus is still to be reached regarding gene fragments for a plant DNA barcode, although the Consortium for the Barcode of Life (CBOL) Plant Working Group (2009) has suggested *matK* + *rbcL*. The difficulty in selecting specific gene(s) to be a plant barcode is due to the imperfection of any gene from either the chloroplast, mitochondrial, or nuclear genomes. The plant mitochondrial genes evolve slowly, and therefore, are ineffective for distinguishing between different plant species. Plant nuclear genes often occur in multiple copies and are highly variable, making the design of universal primers difficult. The search for a plant DNA barcode has focused on genes of the chloroplast genome and several candidates, such as *acc*D, *atpF–atpH*, *matK, nhdJ*, *psbK– psbI*, *rbcL*, *rpoB*, *rpo*C1, and *trnH–psbA* have been proposed (Chase et al., 2005; Kress et al., 2005; Newmaster et al., 2006; Yoo et al., 2006). Unfortunately, very few of these loci are variable enough to identify or distinguish between plant species when used alone, leading to the use of several combinations of loci, such as *rpoC1* + *rpoB* + *matK*, *rpoC1* + *matK* + *trnH–psbA*, *rbcL* + *trnH–psbA*, *matK* + *atpF-H* + *psbK-I*, and *matK* + *atpF-H* + *trnH–psbA* (Yoo et al., 2006; Chase et al., 2007; Kress & Erickson, 2007; CBOL Plant Working Group, 2009).

***mat*K gene**

The matK gene with its underlying features represents a molecule that has strong potential in providing insight into evolutionary and systematic problems at various levels. The 1500 – bp size and the high rate of substitutions make the gene a valuable source of information for addressing systematic and evolutionary questions at various taxonomic levels. The presence of a relatively conserved 3′ region and a less conserved 5′ region provide two sets of characters from the matK that can be used at a different taxonomic hierarchy from the tribal to the kingdom level.

As matK is one of the most rapidly evolving plastid coding regions and it consistently shows high levels of discrimination among angiosperm species and suggested as the best plastid option for a DNA barcode sequence that has good priming sites length and interspecific variation, in the present study it was used to barcode the species studied.

matK primer

A desirable DNA barcode must simultaneously possess enough sequence variations for species identification and adequate conserved flanking sites for the designation of the universal primers (Stoechle 2003). In systematics, the full-length *matK* sequence is amplified and sequenced with primers designed within the *trnK* region (Wang et al., 2006; Li & Zhou, 2007); however, for DNA barcoding, a fragment of 600–800 bp is usually sufficient.

The success rate of amplifying the matK regions with the universal primer pairs in higher plants were the highest among all the candidate DNA barcodes (Kress et al., 2005; CBOL, 2009).

In the present study, the matK primer pairs, namely *matK*472F, *matK*1248R were used along with primer 390F/1326R as a control to get good amplification and sequencing of matK region of the samples studied and good amplification and sequence were got for the species.

Although *matK*472F + *matK*1248R worked reasonably well for the samples tested in this study, its usefulness for all angiosperms needs to be evaluated. To avoid using too many degenerate bases, one has to ignore rare base substitutions. More specific primers can be designed by modifying *matK*472F and *matK*1248R according to the desired sequence.

DNA sequence

DNA sequencing has emerged as one of the most utilized molecular approaches for inferring phylogenies because of the direct comparison of the nucleotide sequences and the relative ease of interpreting the sequence information. The cycle sequencing method used in this study directly sequenced the DNA by the PCR method.

Sequence analysis

Currently, there is no standardize method for comparing unknown sequences to reference sequences. The generally used BLAST algorithm (Altschul *et al.* 1997) is not specifically designed for barcoding, but it has repeatedly been used for this purpose in recent years (e.g. Ford *et al.* 2009), and comparisons based on test datasets show that it does not perform worse than other methods (Little & Stevenson, 2007). A disadvantage of BLAST is that there are no statistical methods that can give a measure of the accuracy of identifications (Munch *et al.* 2008). However, the E-value and maximum identity are two statistics that can be used as an informal measure of the likeliness of an identification being correct. In general, one can assume that the closer a hit approaches 100% in sequence identity (and an E-value of 0), the more likely it is to have been correctly identified to species as well. However, there is a possibility that hits scoring 100% in sequence identity may be incorrect, if there are closely related species in the target geographical area that were not included in the reference database. Based on the reasons, in the present study too, the BLAST algorithm was used to identify, compare and discriminate the species studied and it showed fare results.

Sequence retrieval

Sequence retrieval leads to the question of what quality of match is required to use barcodes for identification. A match of 100 % between a query sequence and a reference sequence is unambiguous at one level – each base pair is exactly matched. However, if the query sequence is 150 base pairs long, and the reference sequence is 2000 base pairs long, the 100 % match might not be a meaningful one. The match might be along a part of the gene region that is highly conserved, with little to no variation among many species.

Phylogenetic tree

In the present investigation, the DNA sequences were analyzed using BLAST for pairwise sequence alignment and using clustalW for the multiple sequence alignment. The BLAST also used for identification of species using matK gene. The matK had greater resolving power of single region barcode, dominated under NJ method. In this, the basic sequence statistics, including nucleotide frequencies, transition/transversion (ns/nv) ratio and variability in different regions of the sequences were computed by MEGA, which is an integrated tool for automatic and manual sequence alignment to construct phylogenetic tree, to estimate the rates of molecular evolution and to test evolutionary hypothesis.

In the present study, the phylogenetic trees (Dendrograms) were constructed for the species studied and their related taxa by using Neighbor joining (NJ) method to study the identification, discrimination, closeness and the evolutionary trend among them and the constructed trees fulfil the needs of this study.

Conclusion

DNA Barcoding is an effective taxonomic tool for the identification of sacred trees of *Stereospermum suavelons*. In the present investigation, DNA barcodings were made for the sacred trees of Main (Big) Temples in Cuddalore district, namely *Stereospermum suavelons* to document their reliable identification, discrimination, similarities and evolutionary trend among them and with their related taxa for future use.

Fig. 7: Ovarall Phylogenic tree represents the evolutionary relationship between the plant species of *Stereospermum suavelons* DC.

Reference

Altschul, S.F., Madden, T.L., Schäffer, A.A., Zhang, J., Zhang, Z., Miller, W. & Lipman, D. J. 1997. Gapped

BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Research 25: 3389-3402.

Bezeng, B.S., Savolainen,V., Maurin,O., Yessoufou, K., Papadopulos, A. and Van der Bank, M. What drives plant invasiveness: A phylogenetic approach on Robben Island. PLN 16, 2013.

Binutu OA, Adesogan KE, Okogun JI. Antibacterial and antifungal compounds from Kigeliapinnata. Planta Med, 1996, 62(4):352–353.

Blaxter, M. 2004. The Promise of DNA taxonomy. Philos. Trans. R. Soc. Lond. B Biol. Sci. 359:669 – 679. PMID: 15253352.

CBOL Plant Working Group. 2009. A DNA barcode for land plants. Proceedings of the National Academy of Sciences USA 106: 12794–12797.

Chase MW, Cowan RS, Hollingsworth PM, Petersen G, Seberg O, Jorgsensen T, Cameron KM, Carine M. 2007. A proposal for a standardized protocol to barcode all land plants. Taxon 56: 295–299.

Chase MW, Salamin N, Wilkinson M, Dunwell JM, Kesanakurthi RP, Haidar N, Savolainen V. 2005. Land plants and DNA barcodes: short-term and long-term goals. Philosophical Transactions of the Royal Society Series B Biological Sciences 360: 1889–1895.

Clegg J S, Jackson S A and Warner A H 1994 Extensive intracellular translocations of a major protein accompany anoxia in embryos of *Artemia franciscana*; *Exp. Cell Res.* 212 77– 83

Cuenoud P, Savolainen V, Chatrou LW, Powell M, Grayer RJ, Chase MW. 2002. Molecular phylogenetics of Caryophyllales based on nuclear 18S rDNA and plastid *rbcL*, *atpB*, and *matK* DNA sequences. American Journal of Botany 89: 132–144.

Dick, C.W. and W.J. Kress. 2009. Dissecting tropical plant diversity with forest plots and a molecular tool kit *Bio Science,* 59: 745 – 755.

Doyle JJ, Doyle JL. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochemical Bulletin 19: 11 – 15.

Erkens RHJ, Cross H, Mass JW, Hoenselaar K, Chatrou LW (2008) Assessment of age and greenness of herbarium specimens as predictors for successful extraction and amplification of DNA. *Blumea*, 53, 407 – 428.

Fazekas AJ, Burgess KS, Kesanakurti PR, Graham SW, Newmaster SG, Husband BC, Percy DM, Hajibabaei M, Barrett SC (2008) Multiple multilocus DNA barcodes from the plastid genome discriminate plant species equally well. PLoS ONE 3:e2802.

Ford, C.S., Ayres, K.L., Toomey, N., Haider, N., Van Alphen Stahl, J., Kelly, L.J., Wikström, N., Hollingsworth, P.M., Duff, R.J., Hoot, S.B., Cowan, R.S., Chase, M.W. & Wilkinson, M.J. 2009. Selection of candidate coding DNA barcoding regions for use on land plants. Botanical Journal of the Linnean Society 159: 1-11.

Gonzalez MA, Baraloto C, Engel J et al. (2009) Identification of Amazonian trees with DNA barcodes. *PLoS ONE*, 4, e7483.

Haribabu, E. (2000) 'Cognitive empathy in inter-disciplinary research: the contrasting attitudes of plant breeders and molecular biologists towards rice'*, Journal of Biosciences (J. Biosci.),* Vol. 25, No. 4, pp. 323 – 330.

Hebert PDN, Cywinska A, Ball SL, DeWaard JR. 2003. Biological identifications through DNA barcodes. Proceedings of the Royal Society B: Biological Sciences 270: 313–321.

Hollingsworth, P.M., L.L. Forrest, J.L. Spouge, M. Hajibabaei, S. Ratnasingham, M. van der Bank, M.W. Chase, R.S. Cowan, D.L. Erickson, A.J. Fazedas, S.W. Graham, K.E. James, K.J. Kim, W.J. Kress, H. Schneider, J. van AlphenStahl, S.C.H. Barrett, C. van den Berg, D. Bogarin, KS. Burgess, K.M. Cameron, M. Carine, J. Chacon. A. Clark, J.J. Clarkson, F. Conrad, D.S. Devey, C.S. Ford, T.A.J. Hedderson, M.L. Hollingsworth, B.C. Husband, L.J. Kelly, P.R. Kesanakurti, J.S. Kim, Y.-D. Kim, R. Layahe, H.-L. Lee, D.G. Long, S. Madrifian. O. Maurin, I. Meusnier, S.G. Newmaster, C.-W. Park, D.M. Percy, G. Petersen, J.E. Richardson, G.A. Salazar, V. Savolainen, O. Seberg, M.J. Wilkinson, D.-K.Yi and D.P. Little. 2009. A DNA barcode for land plants. *Proc. Nat. Acad. Sci.,* 106(31): 12794 – 12797.

Jing YU, Jian-Hua XUE, Shi-Liang ZHOU. New universal matK primers for DNA barcoding angiosperms, Journal of Systematics and Evolution 49 (3): 176 – 181 (2011).

Joshi KC, Bansal RK, Patni R., Chemical examination of the roots of *Stereospermum suaveolens* DC. J Indian ChemSoc, 1977, 54:648–649.

Kevin S, Burgess, Aron J, Fazekas, Prasad R, Kesanakurti, Sean W, Graham, Brian C, Husband, Steven G, Newmaster, Diana M, Percy, Mehrdad Hajibabaei and Spencer C. H. Barrett. Discriminating plant species in a local temperate flora using the rbcL + matK DNA barcode. Methods in Ecology and Evolution doi: 10. 1111/j.2041-210X.2011.00092.x

Kress WJ, Erickson DL, Jones FA et al. (2009) Plant DNA barcodes and a community phylogeny of a tropical forest dynamics plot in Panama. Proceedings of the National Academy of Sciences of the United States of America, 106, 18621 – 18626.

Kress WJ, Erickson DL. 2007. A two-locus global DNA barcode for land plants: the coding *rbcL* gene complements the non coding *trnH-psbA* spacer region. PLOS one 2: e508.

Kress, W.J., Wurdack, K.J., Zimmer, E.A., Weigt, L.A., and Janzen, D.H. 2005. Use of DNA barcodes to identify flowering plants. Proc. Natl. Acad. Sci. USA, 102: 8369 – 8374. Doi: 10.1073/pnas.0503123102. PMID: 15928076.

Lahaye, R., M. Van der Bank, D. Bogarin, J. Warner, F. Pupulin, G. Gigot, O. Maurin, S. Duthoit, T.G. Barraclough and V. Savolainen. 2008. DNA barcoding the floras of biodiversity hotspots. *Proc. Nat. Acad. Sci.,* 105(8): 2923 – 2928.

Li X-X, Zhou Z-K. 2007. The higher-level phylogeny of monocots based on *matK*, *rbcL* and 18S rDNA sequences. Acta Phytotaxonomica Sinica 45: 113–133.

Li, H. Q., Chen, J.Y., Wang, S. and Xiong, S.Z. Evaluation of six candidate DNA barcoding loci in Ficus (Moraceae) of China. Mol Ecol Resour 12 (5), 783-790 (2012)

Little, D.P. & Stevenson, D.W. 2007. A comparison of algorithms for the identification of specimens using DNA barcodes: examples from gymnosperms. Cladistics 23: 1–21.

Munch, K., Boomsma, W., Huelsenbeck, J.P., Willerslev, E. & Nielsen, R. 2008. Statistical Assignment of DNA Sequences Using Bayesian Phylogenetics. Systematic Biology 57: 750- 757.

Newmaster SG, Fazekas AJ, Ragupathy S. 2006. DNA barcoding in land plants: evaluation of *rbcL* in a multigene tiered approach. Botany 84: 335–341.

Onegi B, Kraft C, Ko¨hler I, Freund M, Jenett-Siems K, Siems K, Beyer G, Melzig MF, Bienzle U, Eich E. Antiplasmodial activity of naphthoquinones and one anthraquinone from Stereospermum kunthianum. Phytochemistry, 2002, 60(1):39–44.

Prathapan, K D et al (2006): "Biological Diversity Act, 2002: Shadow of Permit-Raj over Research", Current Science, 91(8), 1006-07, viewed on 19 June 2010.

Refulio-Rodriguez,N.F. and Olmstead,R.G. Phylogeny of Lamiidae. PLN 08, 2012.

Roy, S., A. Tyagi, V. Shukla, A. Kumar, U.M. Singh, L.B. Chaudhary, B. Datt, S.K. Bag, P.K. Singh, N.K. Nair, T. Husain and R. Tuli. 2010. Universal plant DNA barcode loci may not work in complex groups: a case study with Indian *Berberis* species. *PLoS ONE,* 5 (10): e13674.

Royal Botanic Gardens, Kew, 2007 onwards. DNA Barcoding. http://www.kew.org/barcoding/protocols.html. Accessed July 16, 2009.

Sambrook, J., and Russell, D. W. (2001). ‘‘Molecular Cloning: A Laboratory Manual.’’ Old Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Sandermann W, Dietrichs HH Untersuchungenu¨bertermitenresistente Ho¨lzer.HolzalsRoh-und Werkstoff, 1957, 15(7):281–297.

Schulz KH, Garbe I, Hausen BM, Simatupang MH, The sensitizing capacity of naturally occurring quinones. I. Naphtho quinones and related compounds. Arch Dermatol Forsch, 1977, 258:41–52.

Stoeckle, M. 2003. Taxonomy, DNA, and the bar code of life Bioscience, 53: 796 – 797.

Troup R.S. Silviculture of Indian Trees Volume 2: Leguminosae (Caesalpinieae) to Verbenaceae. International Book Distributors, Dehradun, India,1986.

Wang Y-L, Li Y, Zhang S-Z, Yu X-S. 2006. The utility of *matK* gene in the phylogenetic analysis of the genus Magnolia. Acta Phytotaxonomica Sinica 44: 135–147.

Xiaorong Guo, Xiaoguo Wang, Wenhua SU, Guangfei Zhang, and Rui Zhou. DNA Barcodes for Discriminating the Medicinal Plant *Scutellaria baicalensis* (Lamiaceae) and Its Adulterants. Biol. Pharm. Bull. 34 (8) 1198 – 1203 (2011).

Xue, C.Y. and D.Z. Li. 2011.Use of DNA barcode *sensulato* to identify traditional Tibetan medicinal plant *Gentianopsis paludosa* (Gentianaceae).*J. Sys Evol.,* 49 (3): 267 – 270.

Yessoufou K., Maurin O., Davies J., Kuzmina M., Schaefer H., Powell M., Van der bank M. and Savolainen V. Role of mega herbivores in community phylogenetic structure in Kruger National Park. PLN 17, 2012.

Yoo HS, Eah J, Kim JS, Kim Y, Min M, Paek WK, Lee H, Kim C. 2006. DNA barcoding Korean birds. Molecules and Cells 22: 323–327.

Yu et al (2011) Journal of Systematics and Evolution 49, 1-6.

Zhang, L., Simmons, M.P., Kocyan, A. & Renner, S.S. 2006. Phylogeny of the Cucurbitales based on DNA sequences of nine loci from three genomes: Implications for morphological and sexual system evolution. Molecular Phylogenetics and Evolution 39: 305-322.