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DNA BARCODE AND MOLECULAR CONSERVATION OF SELECTED MEDICINAL PLANT SPECIES FROM KANNIYAKUMARI DISTRICT, TAMILNADU.

Dr. J. L Lekshmi[1], V. Sundara Prabha[2], Sreeja[3]

[1,2]Asst. Professor, Department of Botany, S.T Hindu College, Nagercoil, [3]PG student, Department of Botany, S.T Hindu College

Abstract

DNA barcoding has proven to be one of the successful methods for the rapid identification of species, and thus conserving them. This method has been widely used in the different fields including Boral exploration. In spite of debates for using suitable gene for plant species, matK and rbcL are selected as the core barcodes for plants. The availability of molecular data as well as modern technologies in DNA sequencing have made DNA barcoding a popular process in many taxonomic studies. DNA barcoding is not a replacement to the traditional taxonomic classification but seen as a complement to traditional taxonomy and to accelerate the identification process.

This study was carried out with the aim to generate DNA barcodes, from *Calophylum inophylum* and *Couroupita guianesis.* Flower specimens were collected for the DNA analysis. For the extraction of DNA barcodes, all laboratory procedures have been done in TICEL bio park phase - Ill, Yaazh Xenomics, Coimbatore. Sequence editing was carefully done using MUSCLE 3.7 software to each successfully generated barcode. After the editing of the sequences, the molecular identification and phylogenetic trees were constructed. Using rbct, matK and combination of both markers, two phylogenetic trees were constructed in this study using two different methods (Neighbor Joining and Maximum Likelihood). For the construction of phylogenetic trees, sequences obtained from the leaves samples and sequences downloaded from NCBI were used. The rbcL, matK sequences from collected samples correctly clustered together with the Genbank sequences representing the same genera. However, only very few of sequences clustered together with the Genbank sequences representing same species.

Keywords: matK, DNA barcoding, Sequence and Medicinal plants

INTRODUCTION

Canadian taxonomist Paul Hebert first proposed the concept of DNA barcoding in 2003 (Hebert *et al*. 2003a). It involves using one or several standard and universal DNA fragments of the genome to identify species. Because of its rapid, simple, and accurate features, DNA barcoding has been adopted worldwide to facilitate DNA recognition and species identification (Hebert *et al*. 2003b; Kress *et al*. 2005; Hollingsworth *et al*. 2011, Miller, 2011). DNA barcodes can identify unknown samples by matching a specific genetic marker to a reference sequence library (Begerow *et al*. 2010; Seifert *et al*. 2008; Hebert *et al*. 2003). Short gene sequences can easily be taken from the vouchered specimen and have successfully been used to distinguish species as well as populations (Craft and Philbrick, 2016). Methods that are applied in DNA-based identification systems are based on standard molecular biology techniques. The laboratory method includes extraction of DNA, PCR amplification and identification by DNA sequencing following sample selection and documentation. At the same time, data management involves sequence alignment and assignment of barcode IDs to sequence for further identification.

The alignment is performed using different software such as BLAST and Clustal W, which precisely distinguish species by comparing their DNA sequences to those of known sequences as presented in reference libraries. (Hebert *et al*. 2003; Letourneau *et al*. 2010; Zheng *et al*. 2014). In addition, this method can effectively identify unknown species or species having complex morphometric behaviour. This method is also used to study both interspecific and intraspecific variations (Nater *et al*. 2017). DNA barcoding is a robust emerging technique that confirms species boundaries from a small plant tissue using a short DNA section from a specific gene or genes (Hartvig *et al*. 2015). The CBOL- Plant working group, suggested a combination of plastid (mutk/Kim matK/rbcL) and nuclear region (TTS) as the efficient barcode tool to investigate plant species discrimination (Han *et al*. 2021).

Some controversy exists over the value of DNA barcoding (Holmes,2004), largely because of the perception that this new identification method would diminish rather than enhance traditional morphology-based taxonomy, that species determinations based solely on the amount of genetic divergence could result in incorrect species recognition, and that DNA barcoding is a means to reconstruct phylogenies when it is actually a tool to be used largely for identification purposes (Scotland *et al*.2003; Seberg *et al*. 2003; Will and Rubinoff, 2004).

Our objective in this paper is not to debate the validity of using barcodes for palm identification, but rather to determine appropriate DNA regions for use in flowering plants. Species identification is the fundamental step for measuring biodiversity and developing our primary understanding of the biological world. The traditional morphometric taxonomic study is time-consuming as it is dependent on the growth stage of the organism such studies are also laborious that are dependent on pre-determined classifications and expertise (Costion *et al*. 2011, Huang *et al*. 2015). DNA barcoding is a modem marker-based approach in molecular systematics research that

aims to establish a shared community resource of DNA sequences for rapid identification, effective discrimination between taxa, and authenticated classification based on molecular data (Bandyopadhyaya *et al*. 2013).

Objectives:

The general objectives is to conserve the plant species in a molecular and morphological variation by using a DNA barcode. To achieve the objectives, the following tasks have been completed.

1. DNA isolation from plants samples were done using the plant DNA isolation kit.
2. Evaluate DNA barcoding performance in species identification.
3. Construct phylogenetic relationships to compare molecular and morphological identification.

MATERIALS AND METHODS

Calophyllum species are evergreen trees or shrubs, bark smooth, with longitudinal or diamond shaped fissures, inner bark reddish with usually with resinous milky or yellow latex. The species under the Calophyllum genus range from very high trees to shrubs. However, most of the species are medium-sized trees. The habitat of the species ranges from wet tropical rainforest of the lowlands to drier areas at higher altitudes. Some of the species are also found in flooded areas. The genus has several distinguishing taxonomical characteristics like red-coloured outer bark with diamond-shaped fissures and presence of opposite leaves with closely and alternating parallel veins. *Couroupita guianensis*, known by a variety of common names including cannon ball tree, is a deciduous tree in the flowering plant family Lecythidaceae. It is native to the tropical forests of Central and South America, and it is cultivated in many other tropical areas throughout the world because of its beautiful, fragrant flowers and large, interesting fruits. Parts of the plant have been used in traditional medicine. It has been used to treat hypertension, tumours, pain, and inflammation, the common cold, stomach ache, skin conditions and wounds, malaria, and toothache, although data on its efficacy are lacking.

METHODOLOGY

RBCLA Gene Sequencing Protocol

Genomic DNA isolated by:

DNA isolation from Plant samples were done using the EXpure Plant DNA isolation kit developed by Bogar Bio Bee stores Pvt Ltd.,

PCR Protocol

Polymerase Chain Reaction (PCR) is a process that uses primers amplify specific cloned or genomic DNA sequences with the help of a unique enzyme. PCR uses the enzyme DNA polymerase that directs the synthesis of

DNA from deoxynucleotide substrates on a singlestranded DNA template. DNA polymerase adds nucleotides to the 3' end of a custom-designed oligonucleotide when it is annealed to a longer template DNA.

Composition of the Taq Master Mix

- Taq DNA polymerase is supplied in 2X Taq buffer
- 0.4mM dNTPs,
- 3.2mM MgC12 and
- 0.02% bromophenol blue.

Primer details of the selected plants

Primer Name	Sequence Details	Number of Base
RBCLAF	5'ATGTCACCACAAACAGAGACTA	26
RBCLAR	5'GTAAAATCAAGTCCACCRCG3'	20

Add 5 µL of isolated DNA in 25 µl- of PCR reaction solution (1.5 PL of Forward

Primer and Reverse Primer, 5 µL of deionized water, and 12 µL of Taq Master Mix).

Perform PCR using the following thermal cycling conditions such as Denaturation, Annealing, Extension.

PCR condition of *Calophylum inophylum* and Couroupita guianensis

Stages	Temperature	Time	Cycles
Initial Denaturation	95 ℃	2 min	
Denaturation	95 ℃	30 sec	25 cycles
Annealing	55 ℃	30 sec	
Extension	72 ℃	2 min	
Final extension	72 ℃	10 min	
Hold	40 ℃	∞	

Purification of PCR Production

Removed unincorporated PCR primers and dNTPs from PCR products by using Montage PCR Clean up kit (Millipore). The PCR product was sequenced using the primers. Sequencing reactions were performed using

a ABI PRISM@ BigDyeTM Terminator Cycle Sequencing Kits with AmpliTaq@ DNA polymerase (FS enzyme) (Applied Biosystems).

Sequencing protocol

Single-pass sequencing was performed on each template using below 16s rRNA universal primers. The fluorescent-labeled fragments were purified from the unincorporated terminators with an ethanol precipitation protocol. The samples were suspended in distilled water and subjected to electrophoresis in an ABI 3730xl sequencer (Applied Biosystems).

Bioinformatics protocol:

1. The sequence was blast using NCBI blast similarity search tool. The phylogeny analysis of query sequence with the closely related sequence of blast results was performed followed by multiple sequence alignment.
2. The program MUSCLE 3.7 was used for multiple alignments of sequences (Edgar 2004). The resulting aligned sequences were cured using the program Gblocks 0. 91b. This Gblocks eliminates poorly aligned positions and divergent regions (removes alignment noise) (Talavera and Castresana 2007). Finally, the program PhyML 3.0 aLRT was used for phylogeny analysis and 1--1KY 85 as Substitution model.
3. PhyML was shown to be at least as accurate as other existing phylogeny programs using simulated data, while being one order of magnitude faster. PhyML was shown to be at least as accurate as other existing phylogeny programs using simulated data, while being one order of magnitude faster. The program Tree Dyn 198.3 was used for tree rendering (Dereeper *et al*. 2008).

RESULTS AND DISCUSSION

The study revealed to plants species *Calophyllum inophyllum* and *Couroupita guianensis* belonging to the family Calophyllaceae and Lecythidaceae. Polymerase Chain Reaction (PCR) is a process that uses primers to amplify specific cloned or genomic DNA sequences with the help of a unique enzyme. PCR uses the enzyme DNA polymerase. DNA polymerase adds nucleotides to the 3' end of a custom-designed oligonucleotide when it is annealed to a longer template DNA. DNA polymerase can use the oligonucleotide as a primer and elongate its 3' end to generate an extended region of double stranded DNA.

There are two primers used in which RBCLAF it shows the sequence details are 5'ATGTCACCACAAACAGAGACTAAAGC3' and the number of bases is 26. The other primer RBCLAR shows the sequence details are5'GTAAAATCAAGTCCACCRCG3'and the number of bases is 20. The DNA template is heated to 95 ^{0}C. This breaks the weak hydrogen bonds that hold DNA strands together in a helix, allowing the strands to separate creating single stranded DNA. The mixture is cooled to anywhere from 55 ^{0}C. This allows the

primers to bind (anneal) to their complementary sequence in the template DNA. The reaction is then heated to 72 ^{0} C, the optimal temperature for DNA polymerase to act. DNA polymerase extends the primers, adding nucleotides onto the primer in a sequential manner, using the target DNA as a template.

The sequence from collected samples are as Molecular similarities of *Calophylum inophyllum* (sample id — MN099226.1), *C. brasilines* (MG718278.1), *C. verticillatum,* (HQ332023.1), *C.polyanthum* (KR528884.1), *C .vexans* (HQ33204.l) shows that the molecular similarity was 99.79% and the evolutionary distances is 0.002. The molecular variability from the Calophyllum species shows *Calophyllum inophyllum* (BOT-C12) molecular variability is 99.79% C. Soulattri (MC 068749.1) the molecular variability is 99.58%. Mesua ferrea (CMS-BOT-MF) the molecular variability is 99.37% *Caraipa heterocarpa* (PEROI 1) the molecular variability is 99.16%. C.calaba (KYUM<JPN>:39) the molecular variability is 100%.

Molecular variability of Calophyllum species

Plants Names	Molecular variability
Calophyllum inophyllum	99.79%
Calophyllum soulattri	99.58%
Mesua ferrea	99.37%
Caraip heterocarpa	99.16%
Calophyllum calaba	100%

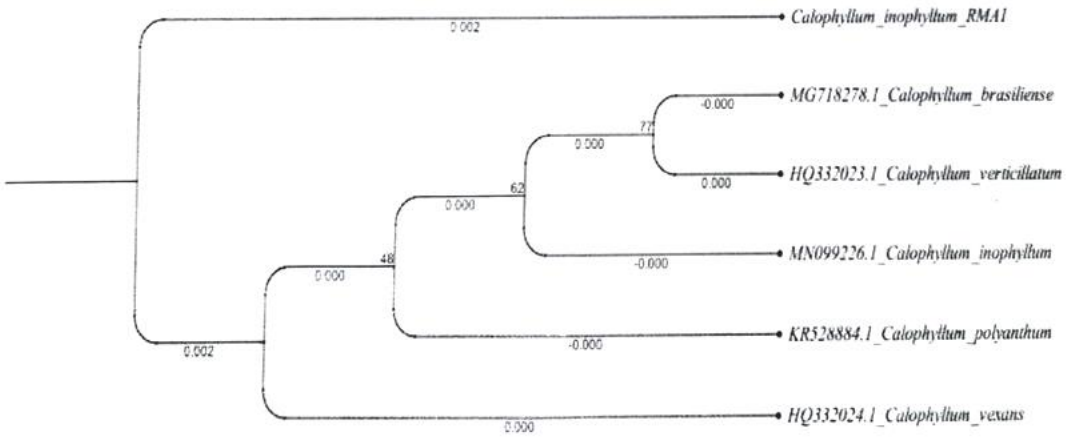
The molecular variability from the Couroupita Species shows Molecular *Courtari guianensis* (P01860327) Variability is 99.57%. *Lecythis congestiflora* (T 10076) the molecular Variability is 99.36% variability is *C. legalis* (rbcL gene) the molecular variability is 99.14% *C. tauari* (recl gene) the molecular variability is 98.93% from the *Couroupita guianensis* species.

Molecular variability of Couroupita species

Name of the plants	Molecular variability
Couroupita guianensis	100.00%
Couratari guianensis	99.57%
Lecythis congestiflora	99.36%
Couroupita legalis	99.14%
Couroupita tauari	99.93%

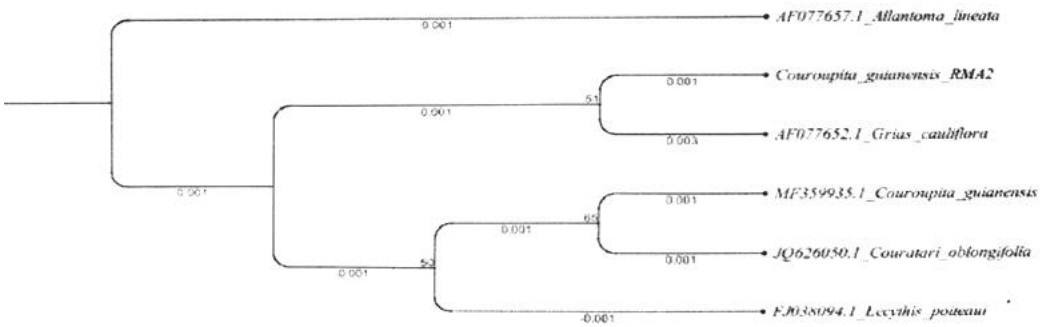
PHYLOGENETIC TREE OF *CALOPHYLUM* SPECIES

(FIGURE-1)



PHYLOGENETIC TREE OF *COUROUPITA* SPECIES

(FIGURE-2)



Research Through Innovation

SUMMARY AND CONCLUSION

DNA barcoding combined with Next Generation Sequencing (NGS) will lead to tremendous growth (i.e., in light of the available sequence data) to improve taxonomy and classification. The DNA barcodes are at the beginning of its application in species discovery, and inferring ecological and evolutionary relationships between species is anticipated to turn into a standard identification protocol for different living organisms.

The selected plants *Calophylum inophylum* and *Couroupita guianesis* shows molecular and morphological varience respectively,the sequence details shows number of base is 26 and 20.

The blast results of query length *Calophylum inophylum* shows 474 nm.

The blast results of query length *Couroupita guianesis* shows 467 nm.

Calophylum inophylum shows 99.79% similarity and *Couroupita guianesis* shows 100% similarity.

Respectively, the selected plants *Calophylum inophylum* and *Couroupita guianesis* shows molecular and morphological variance.

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