International Journal of Botany Studies
www.botanyjournals.com
ISSN: 2455-541X
Received: 10-11-2021, Accepted: 26-11-2021, Published: 11-12-2021
Volume 6, Issue 6, 2021, Page No. 993-997

16s rRNA sequencing for the medicinal plant species *Anisomeles malabarica* R.Br.ex sims and *Dendrophthoe falcata* (L.F.) ettingsh

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Abstract
The study of 16s rRNA sequencing of traditional medicinal plants *Anisomeles malabarica* and *Dendrophthoe falcata* were specially done for making the accuracy of the genotype identification. The clustal alignment of the forward and the reverse reads was performed to identify the contig sample. BLAST analysis was performed for the contig to identify the plant species. Our results revealed that the contig showed maximum coverage and sequence similarity to the *Anisomeles* and *Dendrophthoe* species. So that it is necessary to adapt this technique to conserve medicinal plants for the future generations.

Keywords: *Anisomeles malabarica, Dendrophthoe falcata,* 16s rRNA sequencing, genotype identification

Introduction

Anisomeles malabarica, more commonly known as the Malabar catmint (GRIN) is a species of herbaceous shrub in the family Lamiaceae. **s**The decoction of the leaf and essential oil are also used externally in rheumatic arthritis (Singh *et al*., 2003). There is evidence to support most of these applications, in addition to being effective for epilepsy, intestinal worms, halitosis, and gout (Annapoorani, S. 2019) [5]. *Dendrophthoe falcata* is one of the hemiparasitic plants that belong to the mistletoe family Loranthaceae. It is the most common of all the mistletoes that occur in India. *D.falcata* bears grey bark, thick coriaceous leaves variable in shape with stout flowers (Wealth of India. 2002) [12]. The whole plant is used in indigenous system of medicine as cooling, bitter, astringent, aphrodisiac, narcotic and diuretic (Alekuty *et al*., 1993) [2] and is useful in treating pulmonary tuberculosis, asthma, menstrual disorders, swelling wounds, ulcers, renal and vesical calculi and vitiated conditions of kapha and pitta (Anarthe *et al*., 2008; Sastry, 1952; Pattanayak *et al*., 2008) [4, 10]. Also, the decoction of plant used by women as an anti-fertility agent has been evidenced to possess anticancer activity (Nadkarni, 1993) [9]. In the 1960s, Dubnau *et al*., noted conservation in the 16S rRNA gene sequence relationships in *Bacillus* spp. Widespread use of this gene sequence for bacterial identification and taxonomy followed a body of pioneering work by Woese (1987) [13], who defined important properties. Foremost is the fact that it seems to behave as a molecular chronometer.

Materials and methods

Genomic DNA extraction

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

Protocol

1. **Lysis/homogenization:** Grind approx., 1g of sample to a powdered form and mixed with 450 µl of lysis buffer in a 2 ml micro centrifuge tube and lyse the cells by repeated pipetting.
2. Add 4 µl of RNAse and 250 µl of neutralization buffer.
3. Vortex the content and incubate the tubes for 30 minutes at 65˚C in water bath. To minimize shearing the DNA molecules, mix DNA solutions by inversion.
4. Centrifuge the tubes for 20 minutes at 14,000 rpm.
5. Following centrifugation, transfer the resulting viscous supernatant into a fresh 2 ml micro centrifuge tube without disturbing the pellet.
6. **Binding:** Add 600 µl of binding buffer to the content and mix thoroughly by pipetting and incubate the content at room temperature for 5 minutes.
7. Transfer 600 µl of the contents to a spin column placed in 2 ml collection tube.
8. Centrifuge for 2 minutes at 14,000 rpm and discard flow-through.
9. Reassemble the spin column and the collection tube then transfer the remaining 600µl of the lysate.
10. Centrifuge for 2 minutes at 14,000 rpm and discard flow-through.
11. **Washing:** Add 500 µL washing buffer I to the spin column. Centrifuge at 14,000 rpm for 2 mins and discard flow-through.
12. Reassemble the spin column and add 500µl washing buffer II and Centrifuge at 14,000 rpm for 2mins and discard flow-through.
13. Transfer the spin column to a sterile 1.5-ml micro centrifuge tube.
14. **Elution:** Add 100 µl of Elution buffer at the middle of spin column. Care should be taken to avoid touch with the filtrate.
15. Incubate the tubes for 5 minutes at room temperature and Centrifuge at 6000 rpm for 1 min.

16. Repeat the above-mentioned step 14 and 15 for complete elution. The buffer in the micro centrifuge tube contains the DNA.17.DNA concentrations were measured by Qubit 3.0

PCR Protocol

Polymerase Chain Reaction (PCR) is a process that uses primers to amplify specific cloned or genomic DNA sequences with the help of a very unique enzyme. PCR uses the enzyme DNA polymerase that directs the synthesis of DNA from deoxynucleotide substrates on a single-stranded DNA template. DNA polymerase adds nucleotides to the 3`end of a custom-designed oligonucleotide when it is annealed to a longer template DNA. Thus, if a synthetic oligonucleotide is annealed to a single-stranded template that contains a region complementary to the oligonucleotide, DNA polymerase can use the oligonucleotide as a primer and elongate its 3`end to generate an extended region of double stranded DNA.

Composition of the Taq Master Mix

- Taq DNA polymerase is supplied in 2X Taq buffer
- 0.4mM dNTPs,
- 3.2mM MgCl2 and
- 0.02% bromophenol blue.

Primer Name	Sequence Details	No of Base
RBCLAF	5'ATGTCACCACAAACAG AGACTAAAGC3'	26
RBCLAR	5'GTAAAATCAAGTCCAC CRCG3'	20

Add 5 µL of isolated DNA in 25 µL of PCR reaction solution (1.5 µL 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.

1. Denaturation

The DNA template is heated to 95°C. This breaks the weak hydrogen bonds that hold DNA strands together in a helix, allowing the strands to separate creating single stranded DNA.

2. Annealing

The mixture is cooled to anywhere from 55℃. This allows the primers to bind (anneal) to their complementary sequence in the template DNA.

3. Extension

The reaction is then heated to 72° 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.

PCR CONDITION

Stages	Temperature	Time	
Initial Denaturation	95˚C	2 min	25 Cycles
Denaturation	95˚C	30 sec	
Annealing	55˚C	30 sec	
Extension	72˚C	2 min	
Final extension	72˚C	10 min	
Hold	4˚C	∞	

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 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 resuspended in distilled water and subjected to electrophoresis in an ABI 3730xl sequencer (Applied Biosystems).

Bioinformatics protocol

1. The 16s rRNA 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 HKY85 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 atleast as accurate as other existing phylogeny programs using simulated data, while beingone order of magnitude faster. The program Tree Dyn 198.3 was used for tree rendering. (Dereeper *et al*., 2008) [6].

Result

***Anisomeles malabarica* Chromatogram of the forward read sequence**

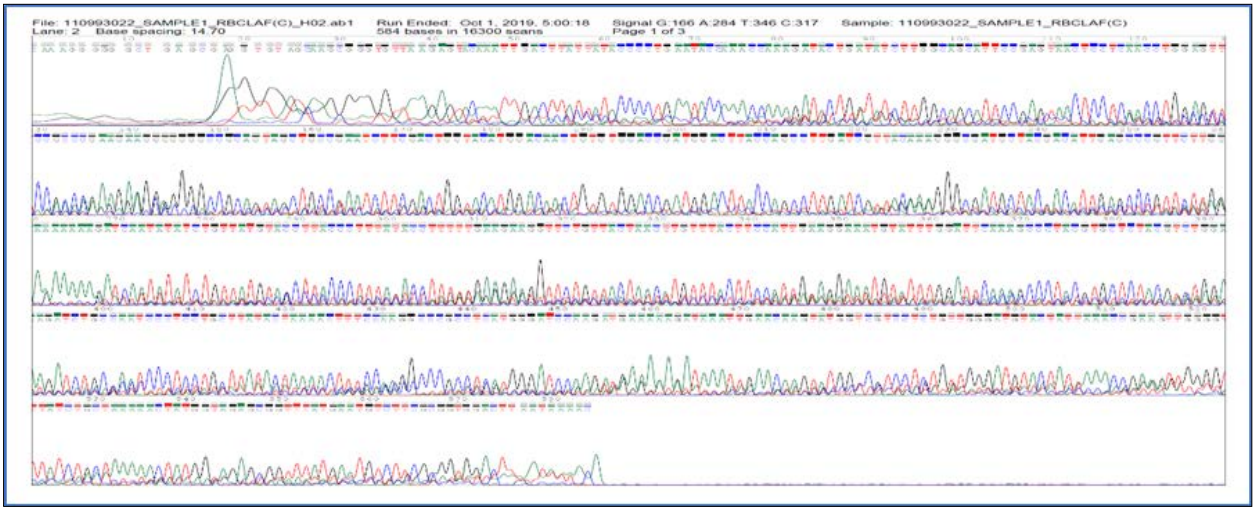

Fig 1

Forward Read Sequence

CAAAGGGGGGCTGAGCGAGGTGTAGCAAGCGGGTGTTAAGAGTACAAATTGACTTATTATACCCCTGAATACC
AAACCAAAGATACTGATATCTTGGCAGCATTCCGAGTAACTCCTCAACCTGGAGTTCCGCCCGAAGAAGGGGGG
GCCGCAGTAGCTGCCGAATCTTCGACTGGTACATGGACAACTGTGTGGACCGATGGACTTACCAGCCTTGATCG
TTACAAAGGGCGATGCTACGACATTGAGCCCGTTCTTGGAAAAAAAGATCAATATATCTGTTATGTACCTTACC
CTTTATACCTTTTTGAAGAAGGTTCTGTTACTAACTTGTTTACTTCCATTGAAGGAAATGTATTTGGATTCAAAGC
CCTACGTGCTCTACGTCTGGAAGATCTGCCAATCCCTCCTGCTTATACTAAAACTTTCCAAGGCCCGCCTCATGG
GATCCAAGATGAAAAAGATAAATTGAACAAGTATGGTCGTCCTCTGTTGGGATGTACTATTAAACCGAAGTTGG
GGTTATCTGCTAAAAACTATGGTAGAGCGGTTTATGAATGTCTTCGCGGTGGACTTAATAAAAC

Chromatogram of the reverse read sequence

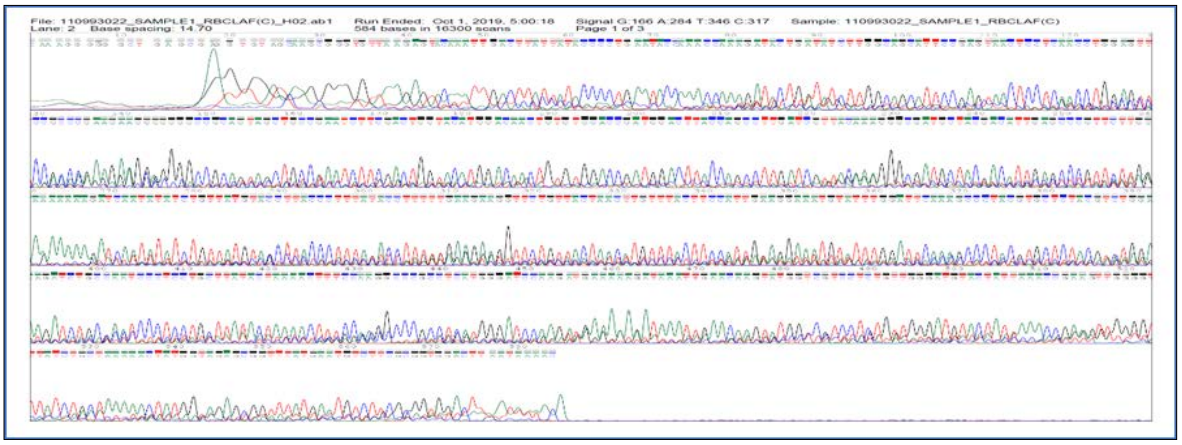

Fig 2

Reverse read sequence

CTCGAAAAACCGGCCTCTACATAGTTTTTAGCAGATAACCCCAATTTCGGTTTAATAGTACATCCCAACAGAGG
ACGACCATACTTGTTCAATTTATCTCTCTCAACTTGGATCCCATGAGGCGGGCCTTGGAAAGTTTTAGTATAAGC
AGGAGGGATTCGCAGATCTTCCAGACGTAGAGCACGTAGGGCTTTGAATCCAAATACATTTCCTACAATGGAAG
TAAACATGTTAGTAACAGAACCTTCTTCAAAAAGGTCTAAAGGGTAAGCTACATAACAGATATATTGATCTTTTT
CTCCAAGAACGGGCTCAATGTCGTAGCATCGCCCTTTGTAACGATCAAGGCTGGTAAGTCCATCGGTCCACACA
GTTGTCCATGTACCAGTCGAAGATTCGGCAGCTACTGCGGCCCCTGCTTCTTCGGGCGGAACTCCAGGTTGAGG
AGTTACTCGGAATGCTGCCAAGATATCAGTATCTTTGGTTTGGTATTCAGGGGTATAATAAGTCAATTTGTACTC
TTTAACACCCGCTTTGAATCCAACACTTGCTTTAGTCTCTGTTTGGGGGTGACATAGGG

> Contig – *Anisomeles malabarica*

TCCGAGTAACTCCTCAACCTGGAGTTCCGCCCGAAGAAGCAGGGGCCGCAGTAGCTGCCGAATCTTCGACTGGT
ACATGGACAACTGTGTGGACCGATGGACTTACCAGCCTT

GATCGTTACAAAGGGCGATGCTACGACATTGAGCCCGTTCTTGGAGAAAAAGATCAATATATCTGTTATGTAGC
TTACCCTTTAGACCTTTTTGAAGAAGGTTCTGTTACTAACATGTTTACTTCCATTGTAGGAAATGTATTTGGATTC
AAAGCCCTACGTGCTCTACGTCTGGAAGATCTGCGAATCCCTCCTGCTTATACTAAAACTTTCCAAGGCCCGCCT
CATGGGATCCAAGTTGAGAGAGATAAATTGAACAAGTATGGTCGTCCTCTGTTGGGATGTACTATTAAACCGAA
ATTGGGGTTATCTGCTAAAAACTATGGTAGAGGCCGGTTTATGAATGTCTTCGCGGTGGACT

Dendrophthoe falcate

Chromatogram of the forward read sequence

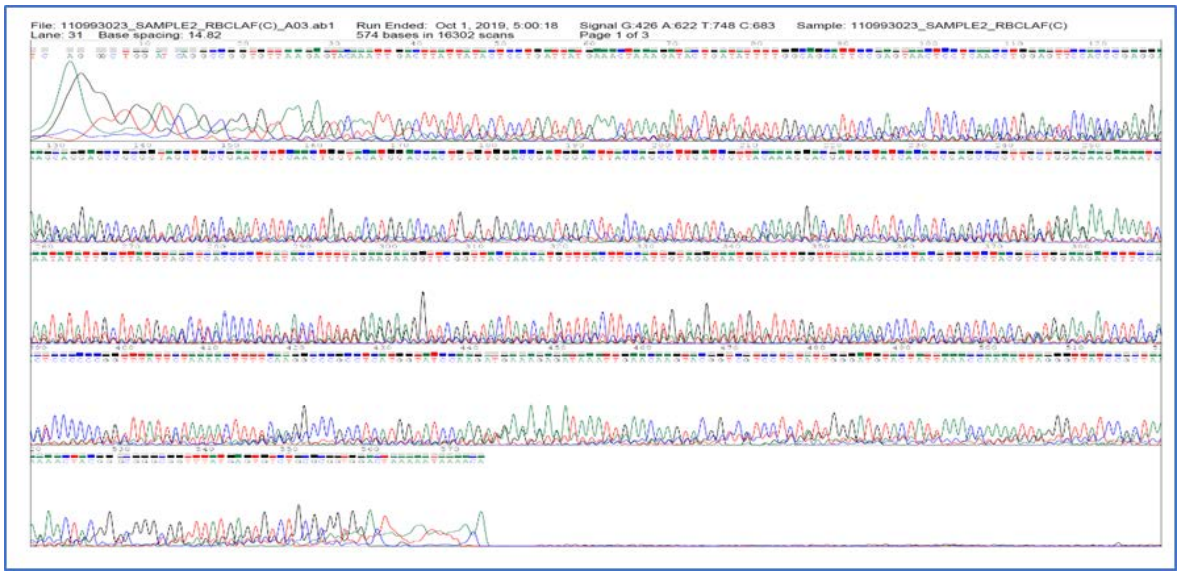

Fig 3

Forward Read Sequence

TCAGGGCTGGATCAGGCCGGTGTTAAGAGTACAAATTGACTTATTATACTCCTGATTATGAAACTAAAGATACT
GATATTTTGGCAGCATTCCGAGTAACTCCTCAACCTGGAGTTCCACCCGAGGAAGCAGGAGCCGCGGTAGCTGC
CGAATCTTCAACTGGTACATGGACCACTGTGTGGACCGATGGACTTACCAGCCTTGATCGTTACAAAGGACGAT
GCTATCACATCGAGCCCGTTGCTGGAGAAGAAAATCAATATATTGCTTATGTAGCTCACCCCTTATACCTTTTAG
AAGAAGGTTCGGTTACTAACATGTTTACTTCCATTGTAGGTAATGTATTTGGTTTTAAAGCCCTACGTGCTCTAC
GTCTGGAAGATCTTCCACTCCCCCCGGTTTATTCTAAAACTTTTCAAGGCCCGCCTCATGGTATCCAAGATGAAA
GAGATAAATTGAACAAGTACGGTCGTCCTCTATTGGGATGTACTATTAAACCAAAATTAGGGTTATCCGCTAAA
AACTACGGGCGGGCGGTTTATGAGTGTCTGCGCGGTGGACTAAAAATAAAACA

Chromatogram of the reverse read sequence

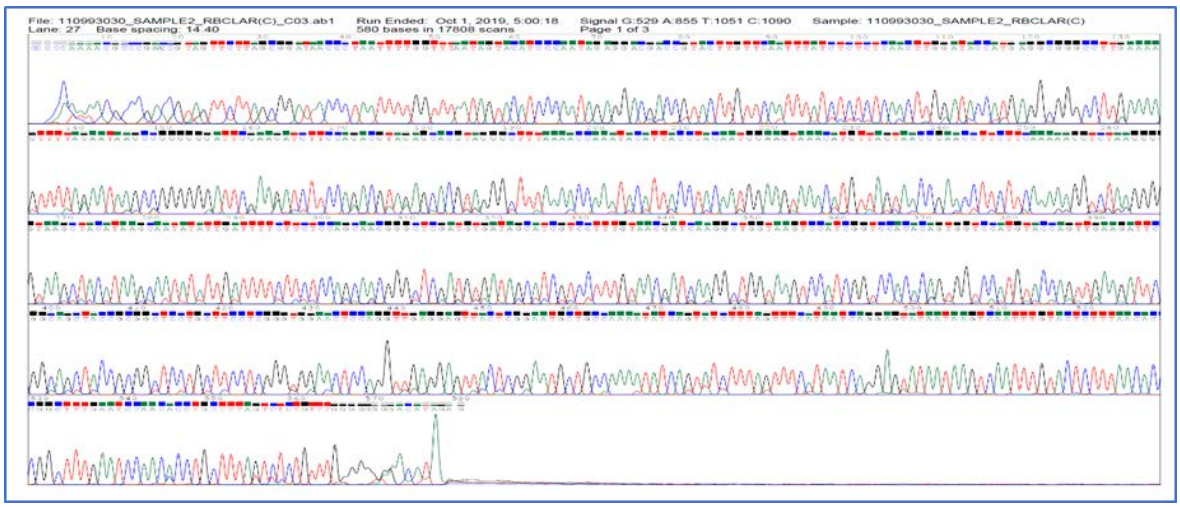

Fig 4

Reverse read sequence

CCCCCAAAACGCCCGAACCGTAGTTCTTAGCGGATAACCCTAATTTTGGTTTAATAGTACATCCCAATAGAGGA
CGACCGTACTTGTTCAATTTATCTCTCTCAACTTGGATACCATGAGGCGGGCCTTGAAAAGTTTTAGAATAAGCC
GGGGGGATTCGAAGATCTTCCAGACGTAGAGCACGTAGGGCTTTAAAACCAAATACATTACCTACAATGGAAGT
AAACATGTTAGTAACCGAACCTTCTTCAAAAAGGTCTAAGGGGTAAGCTACATAAGCAATATATTGATTTTCTTC
TCCAGCAACGGGCTCGATGTGATAGCATCGTCCTTTGTAACGATCAAGGCTGGTAAGTCCATCGGTCCACACAG
TGGTCCATGTACCAGTTGAAGATTCGGCAGCTACCGCGGCTCCTGCTTCCTCGGGTGGAACTCCAGGTTGAGGA
GTTACTCGGAATGCTGCCAAAATATCAGTATCTTTAGTTTCATAATCAGGAGTATAATAAGTCAATTTGTACTCT
TTAACACCGGCTTTGAATCCAACACCTGCTTTAGTCTCTGTTTGGGGGGGGACATAGAG

> *Contig – Dendrophthoe falcate*

AGCATTCCGAGTAACTCCTCAACCTGGAGTTCCACCCGAGGAAGCAGGAGCCGCGGTAGCTGCCGAATCTTCAA
CTGGTACATGGACCACTGTGTGGACCGATGGACTTACCAGCCTTGATCGTTACAAAGGACGATGCTATCACATC
GAGCCCGTTGCTGGAGAAGAAAATCAATATATTGCTTATGTAGCTTACCCCTTAGACCTTTTTGAAGAAGGTTCG
GTTACTAACATGTTTACTTCCATTGTAGGTAATGTATTTGGTTTTAAAGCCCTACGTGCTCTACGTCTGGAAGATC
TTCGAATCCCCCCGGCTTATTCTAAAACTTTTCAAGGCCCGCCTCATGGTATCCAAGTTGAGAGAGATAAATTGA
ACAAGTACGGTCGTCCTCTATTGGGATGTACTATTAAACCAAAATTAGGGTTATCCGCTAAGAACTACGGTTCG
GGCGGTTTATGAGTGTCTGCGCGGTGGACTAAAA

Discussion

Although our study necessarily addresses important technical details, its goal is to explore the full potential of the 16S gene for discriminating bacterial taxa rather than re-evaluate a parti-Cular Although our study necessarily addresses important technical details, its goal is to explore the full potential of the 16S gene for discriminating bacterial taxa rather than re-evaluate a parti-cular Although our study necessarily addresses important technical details, this goal is to explore the detail of 16s for discriminating bacterial taxa rather than re-evaluate a particular sequencing technology (Jethro S Johnson *et al*., 2019) [8], For plant species identification this alignment based method is also opted. Thus, contig of *Anisomeles malabarica* and *Dendrophthoe falcata will* allow plant genotype identification that is more robust, reproducible, and accurate compared to phenotype identification.

Conclusion

The technique of 16s rRNA gene sequencing play a role in the identification of genotype of traditional medicinal plants. As the extraction of bioactive compounds have antibacterial and anticancer activity, this technical study is between 16s gene based and clinical identities.

Acknowledgement

The author is very much thankful to Prof. M.Reginald Appavoo and Prof. Irene Wilsy, Research Department of Botany, Scott Christian College, Nagercoil-1, for providing necessary facilities and constant encouragement to carry out this study.

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