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# Use of Noncoding Plastid Marker *trn*L-F as DNA Barcode for Identification of True Mangrove Genus Rhizophora.

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## ABSTRACT

DNA barcoding should provide rapid, accurate identifications by using a standardized DNA region. The ability to discriminate all species is the ultimate target in barcoding. The barcoding method has been extremely useful in species identification, cryptic species identification, biodiversity studies, forensic analysis and phylogenetics. Application of DNA barcoding to plants has primarily focused on evaluating the success of candidate barcodes across a broad spectrum of evolutionary divergence. In addition to accurately identifying query sequences, results showed that DNA barcoding is useful for detecting taxonomic uncertainty, determining whether erroneous taxonomy. The magnitude of differentiation within and among the *Rhizophora* species sampled suggests that our results inform how DNA barcoding will perform among closely related species in genera.

**Keywords:** DNA barcoding, *trn*L-F , mangrove, *Rhizophora*.

## I. INTRODUCTION

The morphological characters have long been used in many studies of phylogenetic but complicated the continuity of morphological characters in defining the evolutionary relationships. The utilization of the molecular approaches based on DNA sequences is more informative to support and strengthen the morphological character data. The development of molecular biology techniques in this century is to facilitate the assessment of genetically relationship in taxa.[1]

Some phylogeny studies of plants based on cp DNA markers were reported, such as in *Morus* and *Cucumis*.[2] The cp DNA markers provided data for reconstructing phylogeny among families of flowering plants.[3] The sequences of *trn*L-F region of cp DNA are also frequently used in the phylogenetic studies at generic and specific levels.[4,5] Therefore, this platform is commonly used in the phylogenetic studies because they are easily isolated, purified, characterized and cloned. The *trn*L-F region of cp DNA is naturally conservative with a low rate evolution.[6] This region is more varied than the sequences of the coding region.[7]

## II. MATERIALS AND METHODS

Plant samples were collected from kumbalam ($9^0$ 5' N: $76^0$ 12' E) of Ernakulum district in Kerala state. The plant materials were authenticated from Botanical Survey of India and deposited in Calicut University Herbarium. The sequences obtained using barcode markers: *rbcL* and *matK* were submitted to the NCBI GenBank. (Voucher and accession numbers indicated in Table 1).

**DNA isolation using NucleoSpin Plant II Kit (Macherey-Nagel)**

About 100 mg of the tissue is homogenized using liquid nitrogen and the powdered tissue is transferred to a microcentrifuge tube. Four hundred microlitres of buffer PL1 is added and vortexed for 1 minute. Ten microlitres of RNase A solution is added and inverted to mix. The homogenate is incubated at $65^oC$ for 10 minutes. The lysate is transferred to a Nucleospin filter and centrifuged at 11000 x g for 2 minutes. The flow through liquid is collected and the filter is discarded. Four hundred and fifty microlitres of buffer PC is added and mixed well. The solution is transferred to a Nucleospin Plant II column, centrifuged for 1 minute and the flow through liquid is discarded. Four

hundredmicrolitre buffer PW1 is added to the column, centrifuged at 11000 x g for 1 minute and flow though liquid is discarded. Then 700 µl PW2 isadded, centrifuged at 11000 x g and flow through liquid is discarded. Finally 200 µl of PW2 is added and centrifuged at 11000 x g for 2 minutes to dry the silica membrane. The column is transferred to a new 1.7 ml tube and 50 µl of buffer PE is added and incubated at 65$^{o}$C for 5 minutes. The column is then centrifuged at 11000 x g for 1 minute to elute the DNA. The eluted DNA was stored at 4$^{o}$C.

**Agarose Gel Electrophoresis for DNA Quality check**

The quality of the DNA isolated was checked using agarose gel electrophoresis. 1µl of 6X gel-loading buffer (0.25% bromophenol blue, 30% sucrose in TE buffer pH-8.0) was added to 5µl of DNA. The samples were loaded to 0.8% agarose gel prepared in 0.5X TBE (Tris-Borate-EDTA) buffer containing 0.5 µg/ml ethidium bromide. Electrophoresis was performed with 0.5X TBE as electrophoresis buffer at 75 Vuntilbromophenol dye front has migrated to the bottom of the gel. The gels were visualized in a UV transilluminator (Genei) and the image was captured under UV light using Gel documentation system (Bio-Rad).

**PCR Analysis**

PCR amplification reactions were carried out in a 20 µl reaction volume which contained 1X Phire PCR buffer (contains 1.5 mM MgCl2), 0.2mM each dNTPs (dATP, dGTP, dCTP and dTTP), 1 µl DNA, 0.2 µlPhireHotstart II DNA polymerase enzyme, 0.1 mg/ml BSA and 3% DMSO, 0.5M Betaine, 5pM of forward and reverse primers. (Table-2).

The PCR amplification was carried out in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems). (PCR amplification profile;–Table-3).

**Agarose Gel electrophoresis of PCR products**

The PCR products were checked in 1.2% agarose gels prepared in 0.5X TBE buffer containing 0.5 µg/ml ethidiumbromide. 1 µl of 6X loading dye was mixed with 5 µl of PCR products and was loaded and electrophoresis was performed at 75V power supply with 0.5X TBE as electrophoresis buffer for about 1-2 hours, until the bromophenol blue front had migrated to almost the bottom of the gel. The molecular standard used was 2-log DNA ladder (NEB). The gels were visualized in a UV transilluminator (Genei) and the image was captured under UV light using Gel documentation system (Bio-Rad).

**ExoSAP-IT Treatment**

ExoSAP-IT (GE Healthcare) consists of two hydrolytic enzymes, Exonuclease I and Shrimp Alkaline Phosphatase (SAP), in a specially formulated buffer for the removal of unwanted primers and dNTPs from a PCR product mixture with no interference in downstream applications.

Five micro litres of PCR product is mixed with 2µl of ExoSAP-IT and incubated at 37oC for 15 minutes followed by enzyme inactivation at 80oC for 15 minutes.

**Sequencing using BigDye Terminator v3.1**

Sequencing reaction was done in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems) using the BigDye Terminator v3.1 Cycle sequencing Kit (Applied Biosystems, USA) following manufactures protocol.

The sequencing PCR temperature profile consisted of a 1st cycle at 96$^{o}$C for 2 minutes followed by 30 cycles at 96$^{o}$C for 30 sec, 50$^{o}$C for 40 sec and 60$^{o}$C for 4 minutes for all the primers.

**Sequence Analysis**

The sequence quality was checked using Sequence Scanner Software v1 (Applied Biosystems). Sequence alignment and required editing of the obtained sequences were carried out using Geneious Pro v5.1

## III. RESULT AND DISCUSSION

In *R. apiculata* and *R. mucronata***,** *trn*L-F showing full sequence variation, indicate that gene locus were conservative with a low rate of evolution (Table4, 5). The variation of nucleotide bases obtained by the gene sequences of *trn*L-F intergenic spacer of cp DNA was quite high. This means that the gene sequences of cp DNA *trn*L-F is very proper to be used in distinguishing the phylogenetic relationship at the level of species and infraspecies. The pattern that emerges from cp DNA markers was not always associated with the pattern created by morphological markers and vice versa. Chloroplast was inherited uniparentally, or passed down from the female parent. While the morphology characters were inherited from two parents through the recombination process and influenced by the environment. Therefore, this was a reason there were

differences in morphology and cp DNA grouping. Differences in nucleotide sequence variations indicate the evolutionary processes caused by mutations (changes in the nucleotide sequence). Mutations cause differences in phenotypic characters encoded by genes as a form of adaptation to different environments.[8] Diversity indicated by cp DNA markers diversity was relatively different from indicated by morphological markers.[9] The pattern that emerges from cp DNA markers was not always associated with the resulting pattern of morphological markers and vice versa.

This was possible because the expression at the level of morphology was a result of the recombination of two parents and the environmental factors. Additionally, the gene sequences located on the DNA chloroplast experienced a lower rate of evolution than on DNA core.[10] A non-coding area had a high mutation rate, so it had more variations and more informative than the coding area.[11]

*Trn*L-F intergenic spacer region as non-coding sequences (intron) has more variation and higher mutation rate than the coding region. It is also clear that non-coding region (intron) plays a role in the regulation of gene expression that can be affected by the environment or habitat niches, which expressed in phenotype characters.[8]

## IV. CONCLUSION

DNA barcoding has been proposed as a useful technique within many disciplines for determining the taxonomic identity of a sample based on nucleotide similarity to samples of known taxonomy. The rate of evolution can take place faster/slower depending on the mechanism of adaptation and the environmental circumstance habitat.

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**Table-1.** Details of the mangrove species used in the present study with family, status, life form, Gen Bank accession numbers and voucher number.

| No | Specimen | Family | Status | form | Ac.No. | Vou. No |
|---|---|---|---|---|---|---|
| 1 | *R. apiculata* | Rhizophoraceae | TM | Tree | KX231364 | 6927 |
| 2 | *R.mucronata* | Rhizophoraceae | TM | Tree | KX231365 | 6928 |

TM-True mangrove, Ac.No.-Accession number, Vou. No-Voucher number.

**Table-2.** Primers used.

| | | | |
|---|---|---|---|
| *trnL* | *trn*L-F | Forward | CGAAATCGGTAGACGCTACG |
| | *trn*L-R | Reverse | ATTTGAACTGGTGACACGAG |

**Table-3.** PCR amplification profile.

| step | Time (sec). | | Cycles | |
|---|---|---|---|---|
| Gene | *trn* L-F | | | |
| Initial denature | 98 | 30 | 1 | 1 |
| Denature | 98 | 5 | 40 | 40 |
| Annealing | 58 | 10 | 40 | 40 |
| Extention | 72 | 15 | 40 | 40 |
| Final Extention | 72 | 60 | 1 | 1 |
| Hold | 4 | ∞ | _ | _ |

**Table-4.** Consensus sequences of *trn* L-F gene in *Rhizophora apiculate* (904bp).

| Consensus sequences | bp |
|---|---|
| CTATCCCGACCATTCCCAACGTGTCATCTTTTTTATTTTACTCAAGGACT<br>TGGGTCTATGTCAATTAAAAAAAAAAAGACGACAAAGTTTTCTAAAAT<br>CCTATATTCAGAACTTGGAATGTTTTCTATCTTTTGTTTTGCTCAAAAAT<br>GTTTATTTGTATGTGTCTCATATCTATCACAAGACTTGTGAAAATAAAA<br>AAAAAAAAATGAAGCCCGGATATGTTTGTGAAAGAATCGAATGGATG<br>AGAAAAGAAAGATAACGAATTGTGAACCATTAACGAAAAGAGAAAAT<br>ATAATAAGAAATTTAGGGAGTCGAACGAGCCCCTTTTTTGTTTTGGGG<br>ATAGAGGGACTTGAACCCTCACGATTTCAAAAGTCGACGGATTTTCCT<br>CTTACTATCAATTTCCTTGTTGTCGATATTGACATGTACAATGGGACTC<br>TATCTTTATTCTCGTCCGATTAATCAATTATCTATCAGACTATGGAGTG<br>ACTGGTTTGATTAATTATAATATTCGATCCTTTCTTCAACTTGGAATCG<br>ATTCAAAACAATTATTTTTATTTTATTTCCCTTTTTAGATAAATATCCAA<br>AAAGCAAAATTCGGGTTATCAGTAATCATTTGATATATTATTTCAGTAC<br>GTATACGTATGTATATAGGGTTATCCTTTATTTTATCTTTTCTGGCATTT<br>TATTTTGAATTTTGACAGAAGGATTCCTTTACTTTACTAATGAAAGGCA<br>GCCAACTCCATTTGTTAGAACAACTTCCATTGAGTCTCTGCACCTATCC<br>CTTTTTTTTTTCTTTTATTCTGTCTTTATAAACTTTTGTTTGTTTTCGTAA<br>AACAGGATTTGGCTCAGGATTACCCATTTTTTATTCCAGGGTTTCTCTG<br>AATTTGAAAGTTATCACTTAGTA | 904 |

**Table-5.** Consensus sequences of *trn L-F* gene in *Rhizophora mucronata* (898bp)

| Consensus sequences | bp |
|---|---|
| AAGTGATAACTTTCAAATTCAGAGAAACCCTGGAATAAAAA<br>ATGGGTAATCCTGAGCCAAATCCTGTTTTACGAAAACAAAC<br>AAAAGTTTATAAAGACAGAATAAAAGAAAAAAAAAAGGGA<br>TAGGTGCAGAGACTCAATGGAAGTTGTTCTAACAAATGGAG<br>TTGGCTGCCTTTCATTAGTAAAGTAAAGGAATCCTTCTGTCA<br>AAATTCAAAATAAAATGCCAGAAAAGATAAAATAAAGGAT<br>AACCCTATATACATACGTATACGTACTGAAATAATATATCA<br>AATGATTACTGATAACCCGAATTTTGCTTTTTGGATATTTAT<br>CTAAAAAGGGAAATAAAATAAAAATAATTGTTTTGAATCGA<br>TTCCAAGTTGAAGAAAGGATCGAATATTATAATTAATCAAA<br>CCAGTCACTCCATAGTCTGATAGATAATTGATTAATCGGAC<br>GAGAATAAAGATAGAGTCCCATTGTACATGTCAATATTGAC<br>AACAAGGAAATTGATAGTAAGAGGAAAATCCGTCGACTTTT<br>GAAATCGTGAGGGTTCAAGTCCCTCTATCCCCAAAACAAAA<br>AAGGGGCTCGTTCGACTCCCTAAATTTCTTATTATATTTTCT<br>CTTTTCGTTAATGGTTCACAATTCGTTATCTTTCTTTTCTCAT<br>CCATTCGATTCTTTCACAAACATATCCGGGCTTCATTTTTTTT<br>TTTTTTTTTATTTTCACAAGTCTTGTGATAGATATGAGACAC<br>ATACAAATAAACATTTTTGAGCAAAACAAAAGATAGAAAAC<br>ATTCCAAGTTCTGAATATAGGATTTTAGAAAACTTTGTCGTC<br>TTTTTTTTTTTAATTGACATAGACTCAAGTCCTTGAGTAAAA<br>TAAAAAAGATGACACGTTGGGAATGGTC | 898 |