

# Identification of Medicinal Mangrove *Rhizophora apiculata* Blume: Morphological, Chemical and DNA Barcoding Methods

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**Abstract**— "Kandal" (*Rhizophora apiculata* Blume) is a medicinal mangrove plant used to treat pain, inflammation and reduce blood glucose level in Southeast coast of India. Identification and revision of *R. apiculata* are largely based on morphological characteristics. Previously, we reported and filed a patent on anti-diabetic Glycosin from *R. apiculata*. Subsequently, for the quality and market authorization, comprehensible identification and authentication of plant material is needed to avoid misidentification of the species by non-taxonomist. In the light of successful DNA barcoding in many medicinal plants, this study explored the potential of DNA barcoding to complement classical methods of identification of *R. apiculata*. Specimens were examined by morphological, HPTLC, and DNA fingerprinting. The molecular markers *rbc L* and *mat K* regions clearly distinguished *R. apiculata* from *Rhizophoraceae* family. Moreover, the phylogenetic analysis confirmed their taxonomic position within the tribe *Rhizophoraceae*. The study indicates that DNA barcoding provides an effective and accurate strategy for increasing the reliability of species identification. The barcode sequence also was deposited in NCBI and BOLD systems. This is the first scientific information on morphology, chemical and DNA fingerprinting of medicinal mangrove *R. apiculata* for pharmacophore application.

**Index Terms**— barcoding, HPTLC, mangrove, genomic DNA, *Rhizophora apiculata*, *rbc L*

## 1 INTRODUCTION

MEDICINAL plants are more economically valuable and profitable areas of complementary and alternative medicine, which generating billions of dollars in revenue (WHO, 2010). The basic key requirements of both registration and market authorization are quality aspects it needed the identification and authentication of plant material. Traditionally, plants are identified by morphological methods through macroscopic and microscopic examination by a highly trained individual or taxonomists. However, these methods have several disadvantages such as a detailed description not available in the monograph and chemical constituents of plants vary due to climatic factors (Vlietinck *et al.*, 2009). Chromatographic fingerprinting techniques are most significant methods which can be used for the routine herbal drug analysis and for quality assurance. HPTLC offers better resolution and estimation of active constituents can be done with reasonable accuracy in a shorter time. HPTLC based methods could be considered as a good alternative, tool in routine drug analysis. It has several advantages viz., small quantity of mobile phase, less time, cost effective, minimal exposure toxic solvents and facilitates repeated detection of chromatogram with same or different parameters (Joshi *et al.*,

2004).

DNA bar-coding is an another method of identification of plant material based on sequence data from one to numerous gene regions. It presents the possibility of identifying all species by analysis of a specific region of the genome, ecological surveys, cryptic taxon identification and confirmation of medicinal plants (Lahaye *et al.*, 2008). Several chloroplast gene regions are typically used as plant barcodes, with *mat K* and *rbc L* (Hollingsworth *et al.*, 2009).

Mangrove forests are distinctiveness intertidal zones of tropical and subtropical coastlines around the world. It has essential morphological and physiological traits such as viviparous propagules, aerial roots, and salt tolerance. These traits are believed to be an adaptation to severe coastal environments. The revealing genetic structure of mangrove species provides useful information not only for management of plant biodiversity, also to use valuable species for drug development. "Rhizophora" means 'root bearing' in Greek, referring to the stilt roots characteristic of the genus, "apiculata" means 'to end abruptly' in Latin, referring to the leaf apex. Common names of *R. apiculata*: Corky stilt mangrove, tall stilt-root mangrove, tall-stilted mangrove. It is distributed along the intertidal regions of India and Sri Lanka, throughout Asia, western Pacific and northern Australia (Duke, 2006). Recently, we reported the  $\alpha$ -glucosidase and PPAR $\gamma$  inhibitory activities and also the anti-diabetic alkaloid Glycosin of *R. apiculata* (Gurudeeban *et al.*, 2014a; Gurudeeban *et al.*, 2014b). Therefore, the goal of this work was to find an approach to authenticating *R. apiculata* through morphological, chemical and DNA fingerprinting methods.

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## 2 EXPERIMENTAL SECTION

### 2.1 Chemicals

Agarose, Bromophenol blue, Ethidium bromide, Tris Base, Tris-HCl, Sodium Chloride, Triton X- 100, isopropanol, Sodium Ethylene diamine tetra acetic acid, Eliminase, Proteinase K, DNAase free water, PCR Master Mix, Taq DNA polymerase, MgCl<sub>2</sub>, dNTPs mix and analytical grade extraction solvents were purchased from Sigma-Aldrich, (U.S.A.). The Pure Fast® DNA extraction buffer, (1 kb) DNA ladder, Primers (rbc L and mat K) were obtained from HELINI Biomolecules, Chennai, India.

### 2.2 Plant Material

A total of (50) *R. apiculata* samples were collected from selected mangrove sites in Southeast coast of India viz., mangroves of Kodyampalayam coastal village (Nagapattinam district, Tamil Nadu), Pichavaram mangrove forest (Cuddalore district, Tamil Nadu), Kattumavadi mangroves (Tanjur district, Tamil Nadu), Ariyaankuppam brackish water (Pondicherry) and Bhitarkanika, (Odisha) during the monsoon season month in 2009-2010 (Figure 1). Systematic classification of *R. apiculata*, as follows,

Kingdom: Plantae

Class: Dicotyledons

Order: Rhizophorales

Family: Rhizophoraceae

Genus: Rhizophora

Species: *R. apiculata* Blume.

### 2.3 Morphological Identification

The morphological identification of *R. apiculata* was conducted at the Herbarium, maintained in Medicinal Plant Laboratory, Centre of Advanced Study in Marine Biology, Annamalai University, India (Voucher No.: AUCASMB10/2010). Three sets of morpho-groups were assigned and treated independently: leaves, seedlings and flowers. These groups were identified to species using available literature (Table 1). When the existing identification key did not match or was not available, leaves were classified as morphospecies. Seedlings and flowers were all classified as morphospecies. The specimens were separated based on variations in all observable morphological traits, such as the fruit colour, shape, seed number, and length, germination and hypocotyls shapes. For flowers, bracteoles, Calyx, Petals, Andrecium, and Gynecium (Figure 2). Once sorting was completed, specimens representing the identified species (for leaves) were selected for DNA barcoding. Depending on specimen availability for each site, at least one specimen of each species was selected for the DNA sequence analysis. For Seedlings and flowers, the treatment was carried out the opposite way, where randomly selected specimens of both groups were sequenced for DNA first, and later confirmed by their diagnostic morphological characters. The identification of all specimens relied on available keys, taxonomic revisions, and original descriptions (Kathiresan and Ramanathan, 1997). All studied specimens were deposited at the Herbarium, maintained in Medicinal Plant Laboratory, Centre of Advanced Study in Marine

Biology, Annamalai University, India.

### 2.4 HPTLC Finger-Printing

One-gram powdered material of *R. apiculata* (leaf, flower and seedling) in 10 ml of 40% (v/v) methanol containing 0.1% (v/v) 1N HCl with a Ten Broeck homogenizer. The homogenate was centrifuged at 5000 × g for 3 min and filtered through Whatmann No.2 filter paper in a Buchner funnel. Each sample was diluted four-fold and filtered through a 0.45-µm Millipore filter prior to automatic injection (Gurudeeban, 2014 is thesis ?). (3 ml) of total alkaloid extracts of *R. apiculata* was loaded at (6 mm) band length in the (5 × 10) Silica gel 60 F254 TLC plate (Hamilton syringe and CAMAG L-5). The sample loaded plate was kept in TLC twin through developing chamber with respective mobile phase (Ethyl acetate: butanol: water 5:3:1) and the band was developed in the respective mobile phase up to (80 mm). The developed band was dried. The plate was kept in Photo documentation chamber (CAMAG R- 3) and captured the images. The developed band was sprayed with (1 %) aluminum chloride reagent and dried at (120 °C). Finally, the plate was fixed in the scanner and scanning was done at a (254 nm). The chromatogram represents the concentration of the alkaloid in the samples, that was compared with standard Glycosin. The R<sub>f</sub> values and fingerprint data were recorded by WIN CATS software (Harborne, 1998; Wagner *et al.*, 1996).

### 2.5 DNA Finger-Printing

The genomic DNA isolation method was followed by Gurudeeban *et al.*, (2011) with slight modification. Fast Pure DNA extraction buffer was used instead of lysis buffer. Freshly collected leaf, seedling and flower samples (1 g) was ground using a mortar and pestle along with (0.1 g) of PVP. The grinded leaves were quickly transferred to (3 ml) of freshly prepared prewarmed (65°C) extraction buffer and shaken vigorously by inversion to form the slurry. The tubes were incubated at 65°C in hot air oven with intermittent shaking and swirling for every (30 min). An equal volume of Chloroform: Isoamyl alcohol (24:1) was added and mixed properly by inversion for (30 min) and centrifuged at (12000 × g) for (15 min) at room temperature to separate the phases. The supernatant was carefully decanted and transferred to a new tube and was precipitated with equal volumes of cold isopropanol and gently mixed to produce fibrous DNA and incubated at (-20°C). The samples were centrifuged at (12000 × g) for (15 min). The pellet was washed with (70 %) ethanol and 1/10 volume of sodium acetate were added and incubated at (-20°C) for (30 min) followed by centrifugation at (12000 × g) for (15 min). The pellet was air dried and resuspended in TE buffer. The yield of DNA per gram of leaf tissue extracted was measured using a UV Spectrophotometer (PerkinElmer, USA) at (260 nm). The purity of DNA was determined by calculating the ratio of absorbance at (260/280 nm). DNA concentration and purity was also determined by running the samples on (0.8 %) agarose gel-based on the intensities of band when compared with the Lambda DNA marker. Then, purified DNA samples was used for polymerase chain reaction for DNA amplifications and sequencing with the following

primers:

rbc La-F: ATGTCACCACAAACAGAGACTAAAGC (Levin *et al.*, 2003)

rbc La-R: GTAAAATCAAGTCCACCRCG (Kress & Erickson, 2007)

mat K-1RKIM-f: ACCCAGTCCATCTGGAAATCTTGGTTC

mat K-3FKIM-r: CGTACAGTACTTTTGTGTTTACGAG

PCR cycling parameters for each region were as follows, (25 µl) of PCR-Master mix contains 10X Taq buffer, (2 mM) MgCl<sub>2</sub>, (0.4 mM) dNTPs mix, and 2U Proofreading Taq DNA polymerase, (1 µl) Chloroplast specific primer - forward (10pmoles/µl) and reverse each and (22 µl) of nuclease-free water. The amplifications were carried out using a PCR thermal cycler (Lark, India). rbc L programme: (5 min) at (95°C) initial denaturation step, (35 cycles) consisting of (30 s) at (95 °C), (20 s) at (52 °C) and (50 s) at (72 °C), with a final extension period of (5 min) at (72 °C). mat K programme: (5 min) at (94 °C) initial denaturation step, (35 cycles) consisting of (30 s) at (95 °C), (20 s) at (46 °C) and (40 s) at (72 °C), with a final extension period of (3 min) at (72 °C). The success each PCR reaction was verified by electrophoresis. The amplified products were loaded on a (2 %) agarose gel containing (5 µl) of ethidium bromide. PCR Samples are loaded after mixed with gel loading dye along with (10 µl) Quick Ref (1 kb) DNA Ladder. Custom decanucleotide primers were synthesized from M/s Helini Biomolecules, India. Gel viewed in UV transilluminator at (50 V) till the dye reaches three fourth distances. This PCR product of (550 bp) sliced from the gel and extracted as described using a DNA Gel Extraction kit (Millipore Corporation, Bedford) and sequenced.

## 2.6 DNA Sequence Analysis and Barcode Reference

DNA sequences were trimmed and manually aligned using the software Bio Edit (Hall, 1999). A search was made using BLAST (Altschul *et al.*, 1997) through the website of the NCBI and barcode records of the BOLD (Ratnasingham & Heber, 2007) for determining the similarity and/or contamination of the sequences to any other published sequences. When the sequence identification hit a match of preferably (> 98 %) similarity, the morphology of the specimen was re-examined to establish the result of the earlier search. DNA sequences were analyzed in MEGA 5.0 (Tamura *et al.*, 2013) using the standard NJ method with pairwise distances modified using the Kimura 2-Parameter model. All ambiguous positions were removed for each sequence pair. Estimates of average evolutionary distances between sequences (species) were also calculated using the maximum composite likelihood model in MEGA 5.0 (Tamura *et al.*, 2013). Bootstrap analysis was performed with (1500 replicates). Maximum Likelihood analysis was subsequently carried out to inspect if the output of the analysis would find the same barcode clusters as NJ. The difference of seedling to leaves and flower to leaves sequences must be no greater than 4% (Hebert *et al.*, 2003), and in the taxon-ID tree each sequence had to demonstrate a monophyletic group with the corresponding leave sequence. The barcode sequences were uploaded into BOLD database under the name "AUCAS Barcoding of Indian Mangroves" to be used by researchers who are interested in medicinal

mangrove *R. apiculata* species for drug inventory.

## 3 RESULTS

*R. apiculata* is a mangrove plant which has a bio-active substances and secondary metabolites valuable for human being and habitat of marine organisms. HPTLC fingerprinting profile is very important parameter of herbal drug standardization for proper identification of medicinal plants. The respective yellow, green, and purple fluorescence zone was observed on the chromatogram, which confirmed the presence of alkaloids in leaves, seedling and flower compared with standard (Figure 3 & 4). So, it is also desirable method for identification of *R. apiculata*.

Analyses of the DNA sequences of various species are providing valuable information about their taxonomy, gene makeup and utilizations. In the present study, DNA sequence polymorphism of the chloroplast gene rbc L and mat K of *R. apiculata* was assessed to know the inter-specific and intra-specific differences between the Rhizophoraceae family. While working with a leaf and seedlings sample enriched in polysaccharides, polyphenols and other secondary metabolites, that causes difficulty in DNA extraction. During the experimental studies, we encountered difficulties from the stage of cell lysis to DNA separation in the supernatant and subsequent reactions. Major problems encountered were low DNA yield and restriction endonuclease digestion. The protocol involves several modifications with one parameter tested at a time to address the problem of phenolic compounds. Modifications included the use of different concentrations of β-mercaptoethanol (2-4%), PVP (2-4%), and sodium acetate. The usage of (3 %) β-mercaptoethanol, (0.1 g) PVP and (0.15 %) sodium acetate (1/10 volume) was found to be most appropriate. Compared to leaf and seedlings, flowers samples did not cause any difficulties in the experiment. The yield ranged from (20 to 50 µg/g) of *R. apiculata* leaf and seedlings.

The Universal barcode markers rbc L and mat K amplified product having a molecular weight of (616 bp) and (1146 bp) respectively. PCR analysis revealed rbc L and mat K primers are specific for genus and species identification of *R. apiculata* (Figure 5). The successful double-stranded amplification and partial sequences deposited in NCBI. (rbc L: JN390943; mat K: JQ042818). Of the two plastid loci examined in the study, mat K is the most variable across *R. apiculata* followed by rbc L (Figure 6). The phylogenetic tree was constructed for two different loci of Rhizophoraceae to confirm differentiation in species level. The minimum evolution criterion analyzed by the NJ method expressed the topology of least total branch length of Rhizophoraceae preferred at each step of the algorithm (Figure 7). The intraspecies genetic distance within the members of Rhizophoraceae found low due to its recent divergence from other members of the family. The intraspecific variation of the genus *Rhizophora* was found higher. Finally, the discriminating power of mat K has the highest success rate in species discrimination. Most importantly, when considering each locus, the highest success rate is provided by mat K + rbc L. In this study, we provide



the first thorough evaluation of the official CBOL land plant barcode (mat K and rbc L) loci for *R. apiculata*. The BOLD illustrative barcode of *R. apiculata* shown in Figure 8. Even within the species complexes represented by mat K and rbc L together provide the highest discriminating power, supporting their use as the official core DNA barcode. It should be noted that our mat K and rbc L sequences are longer than the barcode regions specified by CBOL. Although universal matK primers remain elusive in *R. apiculata*, we believe the primer development will be considerably improved as more sequences become available.

#### 4 DISCUSSION

The sequencing failure for mangrove species, because incomplete degradation of DNA, slow drying under moist conditions and high level of salt content. In such cases, the possible solution is to search for molecular techniques that support the extraction and sequencing of mangrove plant species. Considering that the present study provides a successive rate of DNA extraction and sequencing method compared to the method of Parani *et al* (1998). There are two proposals regarding a global DNA barcode for all land plants were recently formulated and presented to CBOL (Hollingsworth *et al.*, 2009). One consisted of rbcL and matK while the other included rbcL, matK and trnH-psbA. CBOL officially approved the rbcL+ mat combination to assess its potential as a backup Barcoding locus. Because matK had been previously thought to be unattainable in certain land plants, hence non-coding loci trn L-F was independently proposed as alternative barcoding loci (De *et al.*, 2011). *R. apiculata* is a medicinal mangrove plant species belonging to the family Rhizophoraceae was not amplified using the universal matK and rbcL primers. The family Rhizophoraceae is the largest family of the mangrove plants. The central concept in species identification is to match the sequence of the evidence item to a reference sequence, either through DNA sequence similarity searches (Altschul *et al.*, 1997) or by phylogenetic reconstruction. Except Restriction Fragment Length Polymorphism, all the molecular markers including the Barcoding genes require PCR-based protocols (Kumar *et al.*, 2009). The use of universal primers is important to retrospective species identification as they allow amplification across a wide taxonomic range governed by the PCR success rates, particularly for new or rarely studied medicinal plant species. PCR amplification of matK and rbcL gene segments is a prerequisite for subsequent sequencing of plant barcodes towards species identification. Primer bias and non-universality reported for dramatically skewing results (Soininen *et al.*, 2009). Several studies have investigated the effects of the primer-template mismatches and it has been demonstrated that PCR can be prevented by a single mismatched base (Bru *et al.*, 2008). Variation in primer specificity also affects sequence recovery at low DNA concentrations (Dawnay *et al.*, 2007). Although we observed appreciable success in amplification of rbc L and mat K genes, failure in PCR amplification in for certain species may be attributed to primer mismatch at the annealing sites. The

possibility of poor quality of extracting DNA in failed PCR is remote because we confirmed the quality of DNA on the gel as well as spectrophotometrically. Moreover, in many cases, the same DNA specimen showed negative amplification for matK but positive PCR for rbcL, negating the role of poor DNA quality or quantity in the former case. The generation of matK sequences for ferns is currently problematic because this part of the chloroplast genome underwent a strong restructuring during the evolution of the mangroves (Duffy *et al.*, 2009). Recently, rbcL and trnL-F were used (instead of mat K) for the two-locus barcoding of mangroves (De *et al.*, 2011). The first practical problem for barcoding plants is the acquirement of sufficiently clean DNA for multi-locus sequencing as isolated plant DNA contains PCR inhibitors (Vanijajiva *et al.*, 2005). The second technical issues of primer universality, sequence quality and complexity remain not clear for bar coding land plants of different region (Schneider *et al.*, 2006).

The identification and characterization of medicinal plant species based on morphological and physiological traits which are sometimes difficult (Kadkhodaei *et al.*, 2010). Molecular tools that have been developed for characterization of biodiversity may allow classification of synonyms and detection of the origin of species (Rahman *et al.*, 2009). Ongoing research concentrated on the development of universal primers, PCR conditions, and high-throughput sequencing techniques would certainly enhance the application of DNA Barcoding of plants and development of genetic databases for more efficient utilization of this technique.

#### 5 CONCLUSION

This study provides first scientific information on morphology, chemical and DNA fingerprinting of medicinal mangrove *R. apiculata* for pharmacophore application.

#### 6 ABBREVIATIONS

BLAST - Basic Local Alignment Tool; BOLD - Barcode of Life Data; CBOL - Consortium for the Barcode of Life; DNA - deoxyribonucleic acid; dNTPs - deoxynucleotide triphosphates; HPTLC - High performance thin layer chromatography; TLC - Thin layer chromatography; MgCl<sub>2</sub> - Magnesium chloride; MEGA - Molecular Evolutionary Genetics Analysis; mat K - maturase K; NCBI - National Centre for Biotechnological Information; NJ- Neighbour Joining; PCR - Polymerase chain reaction; PPAR - Peroxisome proliferator-activated receptor; PVP - Poly Vinyl Pyrrolidone; rbc L - ribulose 1,5-bisphosphate carboxylase/oxygenase large subunit; RFLP - Restriction Fragment Length Polymorphism; UPGMA - Unweighted Pair Group Method with Arithmetic Mean

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## COMPETING INTERESTS

The authors have declared there is no competing interest

### Study Area



Figure 1. Different Sampling Areas of Southeast coast of India



Figure 2. Leaf, Flower and Seedlings of *R. apiculata*

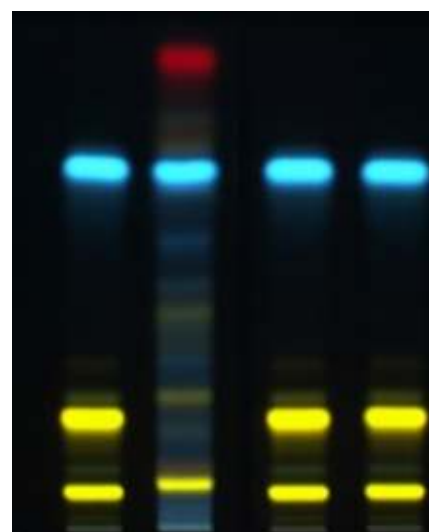


Figure 3. Documentation of Glycosin under UV 366nm derivations.

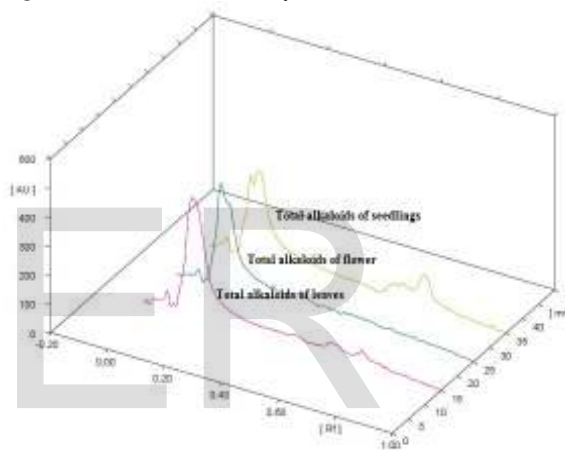


Figure 4. Chemical fingerprints of *R. apiculata* total alkaloids by HPTLC

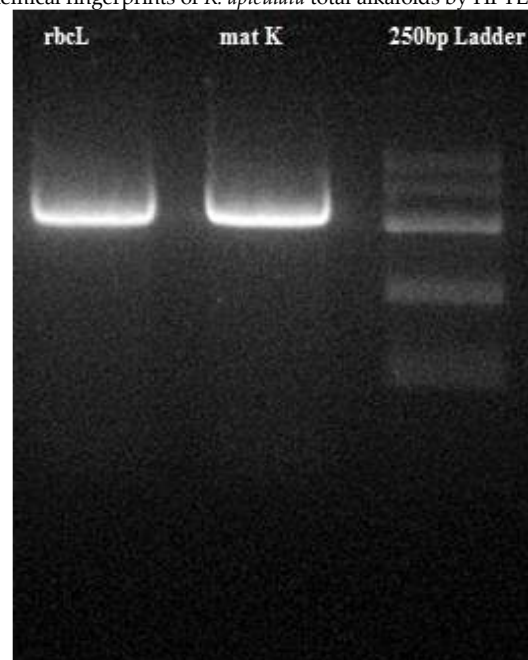


Figure 5. Genomic DNA of *R. apiculata* using *rbcL* and *mat K* markers

JM39 0943  
JQ04 2818  
ATTTAGAACTAGATAGATCTCGAAAAATGATTTCTATTCCACCTTATCTTGGGAAT 60  
-----TCCTTGGTAAATCAAG 17  
ATATTATACATTTCTCATGATCTGTTTAAATAATCTATTTTGTGGAAATGAG 120  
\* \* \* \* \*  
TCCACCAACGAGACATTC-----ATAAACCG-----43  
GT CATAACAAATAATTTAGTTAGTAAATTAATAACGTTTAACTCGAATATATCAAC 180  
\* \* \* \* \*  
-----CTCTACGTAATTCCTAGCGGATAACCCCAATTTAGGTTTAATA 87  
AGAATCATTTAAATTTCTGCAAAAGATTTCTAACCAAAATCGATTTCTGAGTACAATA 240  
\* \* \* \* \*  
G-----TACAGCCCAATAG-----101  
AGAATTTATAATTGCAATGATATCAGAGGGGTTTGCAGTCAATTGGAATTTCTATTTT 300  
\* \* \* \* \*  
-----GGGGGACACATCTTGTCAATTT-----AT 127  
GCCTACAATTAGTATCTTCTTGAAGAGTCCGAAATAGTCAATCTCATAAATTTACGAT 360  
\* \* \* \* \*  
CTCTCTCAACTTGGATGCGATGAGGTGG-----CCCTGGAAAGTTTAAATATAG 178  
CAATTGATTCATTTCTTCTTTAGAGGACAAAATTCACATTTAAATATGATCAG 420  
\* \* \* \* \*  
AAGTAGGAATTCG-----CAATCCT-----CCAGAGGTAGAGCGGTAGG 219  
ATGTAATTAGTACCTTATCTATCCAATTAGAAAAATGATTCACAACTCGCTACTCGG 480  
\* \* \* \* \*  
GCTTTGAGCCCAATACAT-----TACTACAA-----247  
TAAAGATCCCTCTTCTTGGCAATTTGTCGCTCTGTTGTTCTAGAGGAGTGGAAATGGA 540  
\* \* \* \* \*  
-----TGAAGTAAACATAT-----TAGTAAACAGAACT 276  
ACAGTCTTATTTTCAAGAAATCTATTTCTATTTTCCAAAAATCAAGATTTCT 720  
\* \* \* \* \*  
TCTT-----CAAAAGGTCTAAGGGGTAAG-----CTACATA 308  
TCTTTTCTATATAATTTATGATATGAAATGAAATCTATCTTTTCTCTCCGTA 660  
\* \* \* \* \*  
AGCAATATATGATTTCT-----TCTCCAG-----CAACTGCTCGATGTTGGTAGCA 356  
ACCAATCTTTTATTTAGATCAACATTTCTTGGGTACTCTTGGAGCGAATAAATTTCT 720  
\* \* \* \* \*  
TCCGTCCTTTTAAAGC-----ATCAAGACTGGTAAAGC-----CATCGGTCCACAGG 403  
ACGGAATTTAGAGAGTTTACAGAGCTCTTCTGCTAAATGATTTCTCGCTCTCTCTATGTT 780  
\* \* \* \* \*  
TTGTCCTATGATACAGTAAAGATTTCTAG-----C 431  
TGTTCAGGATCTCTTCTATGATTTGTTAGATCAAGGAAATTAATTTGGCTTCAA 840  
\* \* \* \* \*  
AGTACCGGACCTCTCTCT-----TCTCAGGGG-----AAGTCCAGC-----470  
AGTGTACGCCCTCTTCTGATGAAATATGAAATATTAATTTGCTCAATTTAGGTCATATGTC 900  
\* \* \* \* \*  
-----TTGAGGATTTACTCGGAATG-----CTG-----CCATGATATC-----503  
ATTTTATGTTGTTCTCAAGGAAAGATCTATATAAAGCTCTTATCCAAAGCATCTCTC 960  
\* \* \* \* \*  
-AGTATCTTTGG-----TTCATAG-TCAGAGGATATAA 534  
TAGACTTTTGGGTTATCTTCAAGTATACGACTAAATCTTCAAGTGTACGAGTCAAA  
1020  
\* \* \* \* \*  
T-----AAGTCAATTTAATCTTTAAGCAG-----CCTTAAATCCAA 574  
TGTTGAAAAATTCATTTAATATAGTATGCTATGAAAAATCGATACATAGTTCCAA  
1080  
\* \* \* \* \*  
CATTGCTTTAG-----TCTCTT-----TTTGGTGACATCTCTTCAAAAAA-----616  
TTATTCTTTAAATGATCTATTGGCAGAAACGAACTTTGTAAGCAATAGGATATCCCG  
1140  
\* \* \* \* \*  
JM39 0943  
JQ04 2818  
TTAGTA 1146

Figure 6. Multiple sequence variation between loci *rbcl* and *matK* of *R. apiculata*

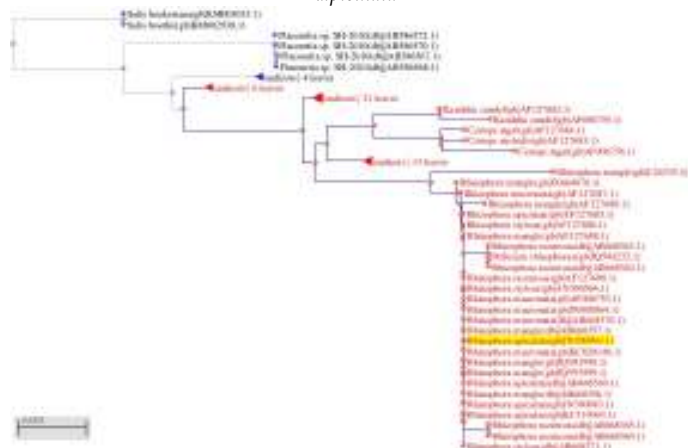


Figure 7. Minimum-evolution criterion of Rhizophoraceae was analyzed using NJ method

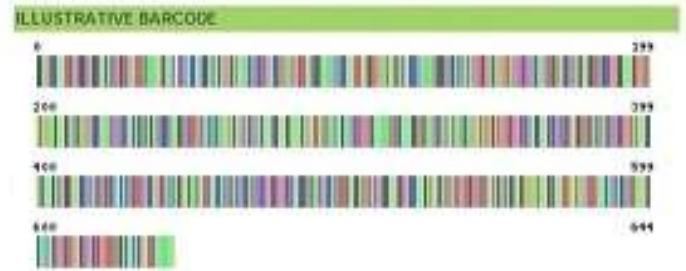


Figure 8. BOLD defined Barcode of *R. apiculata*

TABLE 1. Morphological Characters of *R. apiculata* (Kathiresan and Ramanathan, 1997)

General		
Habit - Tree or shrub, with aerial roots; Habitat -An evergreen shrub, common along with higher estuarine banks, canals, tidal forest and mangrove swamps;		
Stem -Stipules reddish, sessile, leaf like, lanceolate;		
Leaves	Flower	Fruit
Opposite or distichous; leaf blade glabrous, midvein extended into a caduceus point; margin entire or serrulate near apex; Inflorescences axillary, dense cymes	Bracteoles forming a cup just below flower; Calyx - Calyx tube adnate to ovary, persistent; lobes 5-8 Corolla, Petals 4, lanceolate; Andrecium - Stamens 8-12; filaments much shorter than anthers or absent, anthers introrse, locules many, dehiscent by an adaxial valve; Gynecium-Ovary inferior, 2-loculed, apically partly surrounded by a disk, free part elongating after anthesis; style 1, sometimes very short; stigmas 4	Fruit brown, ovoid; Seed- One per fruit; Germination- viviparous, hypocotyls protruding to (78 cm) before propagule falls



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