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Evaluation of mangroves from Kerala, west coast India using DNA barcode

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Abstract
Mangroves are unique ecosystem exist in tropical and subtropical intertidal zones**.** They are most productive, diverse, biologically important ecosystem. Identification of mangrove species is of critical importance in conserving and utilizing biodiversity, which apparently hindered by a lack of taxonomic expertise. In the present study, we performed assessment of four mangrove species of Kerala, west coast India based on core DNA barcode markers, *rbcL* and *matK*. Present study does not support the species resolution among mangroves using plastid genes such as *rbcL* and *matK*. Our future work will be focused on evaluation of other barcode markers to characterize complete resolution of mangrove species and its identification.

Keywords: mangrove, kerala, dna barcode, *rbcl*, *matk*

Introduction
Mangroves are unique ecosystem exist along the sheltered inter-tidal coastline, in the margin between the land and sea in tropical and subtropical areas. This ecosystem endowed with productive wetland having flora and fauna adapted to local environment such as fluctuated water level, salinity and anoxic condition [1, 2]. They are most productive and biologically important ecosystems of the world which provide goods and services to human society in coastal and marine systems [3].

DNA barcoding is currently used effective tool that enables rapid and accurate identification of plant [4]. The Consortium for the Barcode of Life (CBOL) recommended *rbcL* + *matK* as the core barcode. However, these core barcode further combined with the *psb*A-*trn*H intergenic non-coding spacer region which improved discrimination power of core barcode. The noncoding intergenic region *psb*A-*trn*H exhibits high rates of insertion/deletion and sequence divergence [5]. These features make *trnH-psbA* highly suitable candidate plant barcode for species resolution.

Later on, the nuclear ribosomal internal transcribed spacer (ITS) region considered as supplementary barcode, though China Plant Barcode of Life claimed ITS region had higher discriminatory power than plastid core barcodes [6, 7, 8]. The ITS region has some limitations which prevent it from being a core barcode such as incomplete concerted evolution, fungal contamination and difficulties of amplification and sequencing [7].

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. The sequences obtained using barcode markers: *rbcL* and *matK* were submitted to the NCBI GenBank. (Accession numbers indicated in Table 1)

Table 1: Details of the mangrove species used in the present study with family, status, life form, voucher number and GenBank accession numbers obtained after sequence submission

No	Specimen	Family	Status	form	AN-*rbcL*	AN-*matK*
1	*A. ilicifolius*	Acanthaceae	TM	Shrub	KX231351	KX231339
2	*A.ilicifolius*	Acanthaceae	TM	Shrub	KX231352	,KX231340
3	*B. gymnorrhiza*	Rhizophoraceae	TM	Tree	KX231356	KX231344
4	*B. Sexangula*	Rhizophoraceae	TM	Tree	KX231357	KX231345

TR-True Mangrove, AN- Accession Number.

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 65ºC 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 hundred microlitre 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 is

added, 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 65ºC 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 4ºC.

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 V until bromophenol 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 $MgCl_2$), 0.2mM each dNTPs (dATP, dGTP, dCTP and dTTP), 1 µl DNA, 0.2 µl Phire Hotstart 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).

Table 2: Primers used

Target	Primer Name	Direction	Sequence (5' → 3')
matK	390f	Forward	CGATCTATTCATTCAATATTTC
	1326r	Reverse	TCTAGCACACGAAAGTCGAAGT
*rbc*L	rbcLa_f	Forward	ATGTCACCACAAACAGAGACTAAAGC
	rbcL724_rev	Reverse	GTAAAATCAAGTCCACCRCG
ITS	ITS-5F	Forward	GGAAGTAAAAGTCGTAACAAGG
	ITS-4R	Reverse	TCCTCCGCTTATTGATATGC
trnL	trnL-F	Forward	CGAAATCGGTAGACGCTACG
	trnL-R	Reverse	ATTTGAACTGGTGACACGAG

Table 3: PCR amplification profile

step	Tem.(^{0}C)		Time(sec)		Cycles	
	***mat* K**	***rbc* L**	***mat* K**	***rbc* L**	***mat* K**	***rbc* L**
Initial denature	98	98	30	30	1	1
Denature	98	98	5	5	40	40
Annealing	50	58	10	10	40	40
Extention	72	72	15	15	40	40
Final Extention	72	72	60	60	1	1
Hold	4	4	∞	∞	-	-

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 ethidium bromide. 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 2 µl 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 96ºC for 2 minutes followed by 30 cycles at 96ºC for 30 sec, 50ºC for 40 sec and 60ºC 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

Result and discussion

In the present study partial genome sequencing of *rbcL* and *matK* were takes place in both *Acanthus* and *Bruguiera* species. Both the genus should differentiate with *rbcL* and *matK* genome but *rbcL* + *matK* loci were not able able to adequately discriminate in species level.

(Plate – 1, 2 3 & 4). Species discrimination with plant barcodes is typically lower than with CO1 in animals. Obtaining precise figures is difficult as most studies to-date have focused on assessing the relative rather than absolute discriminatory power of different barcoding regions. Levels of discrimination vary greatly among taxa and study designs, but species discrimination figures less than 70% in plants are not uncommon. In these situations, where the barcode does not provide a unique species identification, it instead identifies to 'species group' (typically a local group of closely related congeners). Additional studies with greater sample density are required to establish the situations in which the *rbcL+matK* barcode provides 'species group' versus unique species identifiers [7].

One of the biggest challenges in reaching agreement on a plant barcode was a lack of comparative data encompassing all candidate markers and a broad taxonomic sample. The sequential timing of different proposals effectively meant that some groups were proposing new markers and primers, as others were completing their projects. Two research groups published direct comparisons of the seven candidate markers and both concluded that several different marker combinations gave equivalent performance, and that none of the proposed barcodes was perfect in every respect [5]. The same conclusion was reached the study included *rpo*C1, *mat*K and *trn*H-*psb*A [10].

Plastid gene *matK* can discriminate more than 90 % of species in the Orchidaceae but less than 49% in the nutmeg family [5, 11]. In another case, identification of 92 species from 32 genera using the *matK* barcode could achieve a success rate of 56 % [12]. However, a recent study of the flora of Canada revealed 93 % success in species identification with *rbcL* and *matK*, while the addition of the *trnH-psbA* intergenic spacer achieved discrimination up to 95 % [12]. The species discrimination was lower (<50 %) for *rbcL* + *matK* combination in the study of tropical tree species in French Guiana [13]. Lower discrimination were reported in closest and complex taxa of *Lysimachia*, *Ficus*, *Holcoglossum* and *Curcuma* using *rbcL* and *matK* [4, 14, 15, 16]. The lowest discriminatory power was observed in closely related groups of *Lysimachia* with *rbcL* (26.5–38.1 %), followed by *matK* (55.9–60.8 %) and combinations of core barcodes (*rbcL* + *matK*) had discrimination of 47.1–60.8 % [15].

Taxonomic comparisons show that the genes of closely related species usually only differ by a limited number of point mutations. These are usually found in the third (often redundant) codon positions of ORFs, resulting in a faster evolutionary rate at the third codon position compared with that at the first and second codon position. The redundancy of the genetic code ensures that nucleotide sequences usually evolve more quickly than the proteins they encode [17].

Conclusion

DNA barcoding is a system for fast and accurate species identification which will make ecological system more accessible. Present study revealed discrimination of mangroves based on DNA barcoding using *rbcL* and *matK* at species level is not an efficient tool in the case study of *Acanthus* and *Bruguiera* species.

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  1 AAGTGTTGGA TTTAAGGCTG GTGTTAAAGA TTATAAATTG ACTTATTATA CTCCTGACTA
 61 TGAAACCAAA GATACTGATA TCTTGGCAGC ATTCCGAGTA ACTCCTCAAC CTGGAGTTCC
121 GCCTGAGGAA GCAGGGGCTG CGGTAGCTGC TGAATCTTCT ACTGGTACAT GGACAACCGT
181 GTGGACCGAT GGGCTTACCA GTCTTGATCG TTATAAAGGA CGATGCTACC ACATCGAGCC
241 AGTTGCTGGA GAAGAAAATC AATATATTGC TTATGTAGCT TACCCCTTAG ATCTTTTTGA
301 AGAAGGTTCT GCTACTAATA TGTTTACTTC GATTGTGGGT AATGTATTTG GGTTCAAAGC
361 ACTACGCGCT CTACGTCTGG AGGATTTGCG AATTCCTCCT GCTTATTCTA AAACTTTCCA
421 AGGCCCGCCT CATGGCATCC AAGTTGAGAG AGATAAATTG AACAAGTATG GTCGCCCCCT
481 ATTGGGCTGT ACTATTAAAC CTAAATTGGG GTTATCCGCT AAGAATTACG GTAGAGCAGT
541 TTATGAATGT CTCCGTGGTG GACTTGATTT TACCAAAGAT GATGAGAACG TGAATTCACA
601 ACCATTTATG CGCTGGAGAG ATCGTTTCTT ATTTTGTGTC GAAGCAATTT ATAAAGCACA
661 GGCGGAAACA GGTGAAATCA AAGGGCATTA TTTGAAT 697
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Bruguiera gymnorrhiza, *rbcL* gene

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  1 AAGTGTTGGA TTTAAGGCTG GTGTTAAAGA TTATAAATTG ACTTATTATA CTCCTGACTA
 61 TGAAACCAAA GATACTGATA TCTTGGCAGC ATTCCGAGTA ACTCCTCAAC CTGGAGTTCC
121 GCCTGAGGAA GCAGGGGCTG CGGTAGCTGC TGAATCTTCT ACTGGTACAT GGACAACCGT
181 GTGGACCGAT GGGCTTACCA GTCTTGATCG TTATAAAGGA CGATGCTACC ACATCGAGCC
241 AGTTGCTGGA GAAGAAAATC AATATATTGC TTATGTAGCT TACCCCTTAG ATCTTTTTGA
301 AGAAGGTTCT GCTACTAATA TGTTTACTTC GATTGTGGGT AATGTATTTG GGTTCAAAGC
361 ACTACGCGCT CTACGTCTGG AGGATTTGCG AATTCCTCCT GCTTATTCTA AAACTTTCCA
421 AGGCCCGCCT CATGGCATCC AAGTTGAGAG AGATAAATTG AACAAGTATG GTCGCCCCCT
481 ATTGGGCTGT ACTATTAAAC CTAAATTGGG GTTATCCGCT AAGAATTACG GTAGAGCAGT
541 TTATGAATGT CTCCGTGGTG GACTTGATTT TACCAAAGAT GATGAGAACG TGAATTCACA
601 ACCATTTATG CGCTGGAGAG ATCGTTTCTT ATTTTGTGTC GAAGCAATTT ATAAAGCACA
661 GGCGGAAACA GGTGAAATCA AAGGGCATTA TTTGAAT 697
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Plate 1: *Bruguiera sexangula*, *rbcL* gene

1 AGAGGACAAA TTTCCACATT TAAATTATGT ATCAGATATA TTAGTACCTT ATCCTATCCA
61 TTTAGAAAAA TTGGTTCAAA CCCTTCGCTA CTGGGTAAAA GATCCCTCTT CTTTGCATTT
121 ATTCCGACTC GTTGTTCATG AGGAGTGGAA TTGTAACAGT CTTATTATTC CAAAAAAATC
181 TATTTCCATT TTTCCAAAAA TTAATCCAAG ATTCTTTTTC TTCCTATATA ATATTCATGT
241 ATATGAATAC GAATTCATCT TTTTTTTTCT CCGTAACCAA TCCTCTTATT TACGATCAAC
301 ATTTTCTTGG GTACTTCTTG AGCGAATAAA TTTCTACGGA AAAGTAGAAC AGTTTACAGA
361 CGTCTTTGCT AATGATTTTC AGTCCGTCCT ATGTTTGTTC AAGGATCCTT TCATGCATTA
421 TGTTAGATAT CAAGGAAAAT TAATTTTGGT TTCAAAGTAT ACGCCTCTTC TCATGAAAAA
481 ATGGAAATAT TACGTTGTCA ATTTATGTCA ATGTCATTTT TATGTGTGGT TTCAACCGGA
541 AAAGATCTAT ATAAACCTCT TATCCAAGCA CTCTCTAGAC TTTTTGGGTT ATCTTTCAAG
601 TATACAACTA AATCTTTCAG TGGTCCGGAT TCAAATGTTA GAAAATTCAT TTATACTAGA
661 TAATGCTATA AAAAAACTCG ACACAATAGT TCCAATTATT CCTTTGATTG GATCATTGGC
721 ACAGACGAAC TTTTGTAACG AAATAGGATA TCCCGTTAGT AACCCGACCC GGGCGGCTAA
781 TTCATCGGAT TCTGATATTA TCACCCGATT TGTGCATCTA TGCAGAAATC TTTTTCATTA
841 TTATAGTGGA TCCTCAAAAA AAA 863

Bruguiera gymnorrhiza, matK gene

1 AGAGGACAAA TTTCCACATT TAAATTATGT ATCAGATATA TTAGTACCTT ATCCTATCCA
61 TTTAGAAAAA TTGGTTCAAA CCCTTCGCTA CTGGGTAAAA GATCCCTCTT CTTTGCATTT
121 ATTCCGACTC GTTGTTCATG AGGAGTGGAA TTGTAACAGT CTTATTATTC CAAAAAAATC
181 TATTTCCATT TTTCCAAAAA TTAATCCAAG ATTCTTTTTC TTCCTATATA ATATTCATGT
241 ATATGAATAC GAATTCATCT TTTTTTTTCT CCGTAACCAA TCCTCTTATT TACGATCAAC
301 ATTTTCTTGG GTACTTCTTG AGCGAATAAA TTTCTACGGA AAAGTAGAAC AGTTTACAGA
361 CGTCTTTGCT AATGATTTTC AGTCCGTCCT ATGTTTGTTC AAGGATCCTT TCATGCATTA
421 TGTTAGATAT CAAGGAAAAT TAATTTTGGT TTCAAAGTAT ACGCCTCTTC TCATGAAAAA
481 ATGGAAATAT TACGTTGTCA ATTTATGTCA ATGTCATTTT TATGTGTGGT TTCAACCGGA
541 AAAGATCTAT ATAAACCTCT TATCCAAGCA CTCTCTAGAC TTTTTGGGTT ATCTTTCAAG
601 TATACAACTA AATCTTTCAG TGGTCCGGAT TCAAATGTTA GAAAATTCAT TTATACTAGA
661 TAATGCTATA AAAAAACTCG ACACAATAGT TCCAATTATT CCTTTGATTG GATCATTGGC
721 ACAGACGAAC TTTTGTAACG AAATAGGATA TCCCGTTAGT AACCCGACCC GGGCGGCTAA
781 TTCATCGGAT TCTGATATTA TCACCCGATT TGTGCATCTA TGCAGAAATC TTTTTCATTA
841 TTATAGTGGA TCCTCAAAAA AAA 863

Plate 2: *bruguiera sexangula, matK* gene

1 TTTTGTTGGA TTCAAAGCGG GTGTTAAAGA GTACAAATTG ACTTATTATA CTCCTGAATA
61 CGAAACTAAA GATACTGATA TCTTGGCAGC ATTCCGAGTA ACTCCTCAAC CCGGAGTTCC
121 AGCCGAAGAA GCGGGGGCAG CGGTAGCTGC CGAATCTTCT ACTGGTACATGGACAACCGT
181 GTGGACCGAC GGGCTTACCA GCCTTGATCG TTATAAAGGG CGATGCTACA ACATCGAGCC
241 CGTTCCTGGC GAAACAGATC AATATATCTG TTATGTAGCT TACCCTTTAG ACCTTTTTGA
301 AGAAGGTTCT GTTACTAACA TGTTCACTTC CATTGTAGGA AATGTATTTG GATTCAAAGC
361 CCTGCGTGCT CTACGTCTGG AAGATCTGCG AATCCCTACT GCTTATATTA AAACTTTCCA
421 AGGTCCGCCT CATGGGATCC AAGTTGAGAG AGATAAATTG AACAAGTATG GTCGTCCCCT
481 GCTGGGATGT ACTATTAAAC CTAAATTGGG GTTATCTGCT AAAAACTACG GTAGAGCGTG
541 TTATGAATGT CTTCGCGGTG GACTTGATTT TACCAAAGAT GATGAGAACG TGAACTCCCA
601 ACCATTTATG CGTTGGCGAG ATCGTTTCTT ATTTTGTGCC GAAGCCATTT ATAAAGCACA
661 GGCTGAAACA GGCGAAATCA AAGGGCATTA CTTGAAT 697

Acanthus ebracteatus rbcL gene

1 TTTTGTTGGA TTCAAAGCGG GTGTTAAAGA GTACAAATTG ACTTATTATA CTCCTGAATA
61 CGAAACTAAA GATACTGATA TCTTGGCAGC ATTCCGAGTA ACTCCTCAAC CCGGAGTTCC
121 AGCCGAAGAA GCGGGGGCAG CGGTAGCTGC CGAATCTTCT ACTGGTACATGGACAACCGT
181 GTGGACCGAC GGGCTTACCA GCCTTGATCG TTATAAAGGG CGATGCTACA ACATCGAGCC
241 CGTTCCTGGC GAAACAGATC AATATATCTG TTATGTAGCT TACCCTTTAG ACCTTTTTGA
301 AGAAGGTTCT GTTACTAACA TGTTCACTTC CATTGTAGGA AATGTATTTG GATTCAAAGC
361 CCTGCGTGCT CTACGTCTGG AAGATCTGCG AATCCCTACT GCTTATATTA AAACTTTCCA
421 AGGTCCGCCT CATGGGATCC AAGTTGAGAG AGATAAATTG AACAAGTATG GTCGTCCCCT
481 GCTGGGATGT ACTATTAAAC CTAAATTGGG GTTATCTGCT AAAAACTACG GTAGAGCGTG
541 TTATGAATGT CTTCGCGGTG GACTTGATTT TACCAAAGAT GATGAGAACG TGAACTCCCA
601 ACCATTTATG CGTTGGCGAG ATCGTTTCTT ATTTTGTGCC GAAGCCATTT ATAAAGCACA

661 GGCTGAAACA GGCGAAATCA AAGGGCATTA CTTGAAT 697

Plate 3: *Acanthus ilicifolius, rbcL* gene

1 AGAGGACAAT TTTTCACATT TAAGTATTGT ATTAGATATA CTAATACCCC GACCTCTCCA
61 TGTGGAAATC TTGGTTCAAA CCCTTCGCTA TTGGTTAAAA GATGCCCCCT CTTTGCATTT
121 ATTACGATTC TTTTTCAACG AGTATTGTAA TTGGAATAGT CTTATTACGC CAAAGAGAGC
181 AAGTTCCTTC TTTTCAAAAA GAAATCAAAG ATTATCTTTA TTCTTATATA ATTCTCATGT
241 GTGGGAATAT GAATCCATTT TCGTCTTTCT GTGTAACCAA TCTTCTCATT TACGATCAAC
301 ATCTTCTGGA GTTCTTCTTG AACGAATCCA TTTCTATCGA AAAATAGAAC ATCTTGGGAA
361 CGTCTTTGTT AAGATTAAGT ATTTTCAGGT GAACCTATGG TTTGTTAAGG AACCTTGCAT
421 GCATTGTATT AGATATCAAA GAAAGTGCAT TCTGGCTTCA AAAGGGACAT CACTTTTCAT
481 GAATAAATGG AAATCTTACC TTATGATTTT TTGGCAATCG TATTTTTCGT TGTGGTTTCT
541 TCAAAGAAGG ATTTATAGAA ACCAATTAGC CAAACATTTC CTCGAATCTT TGGGCTATCT
601 TTCAAGTGTA CGCATGACCC CTTCAGTGAT ACGTAGTCAA ATTCTCGAAA ATGCATTTCT
661 AATCAATAAT GCTATTAAGA AGTTCGATAC CTTTGTTCCA ATTATTCCCT TGATTGCATC
721 ATTGGCTAAA TCGAAATTTT GTAACGTATT AGGGCATCCT ATGAGTAAGC CGGTTTGGGC
781 TGATTTATCA GATTCTAATA TTATTGATCG ATTTGTGCGT ATATGCAGAA ATCTCTCTCA
841 TTATCATAGC GGATCCTCAA CAAAAA 866

Acanthus ebracteatus matK gene

1 AGAGGACAAT TTTTCACATT TAAGTATTGT ATTAGATATA CTAATACCCC GACCTCTCCA
61 TGTGGAAATC TTGGTTCAAA CCCTTCGCTA TTGGTTAAAA GATGCCCCCT CTTTGCATTT
121 ATTACGATTC TTTTTCAACG AGTATTGTAA TTGGAATAGT CTTATTACGC CAAAGAGAGC
181 AAGTTCCTTC TTTTCAAAAA GAAATCAAAG ATTATCTTTA TTCTTATATA ATTCTCATGT
241 GTGGGAATAT GAATCCATTT TCGTCTTTCT GTGTAACCAA TCTTCTCATT TACGATCAAC
301 ATCTTCTGGA GTTCTTCTTG AACGAATCCA TTTCTATCGA AAAATAGAAC ATCTTGGGAA
361 CGTCTTTGTT AAGATTAAGT ATTTTCAGGT GAACCTATGG TTTGTTAAGG AACCTTGCAT
421 GCATTGTATT AGATATCAAA GAAAGTGCAT TCTGGCTTCA AAAGGGACAT CACTTTTCAT
481 GAATAAATGG AAATCTTACC TTATGATTTT TTGGCAATCG TATTTTTCGT TGTGGTTTCT
541 TCAAAGAAGG ATTTATAGAA ACCAATTAGC CAAACATTTC CTCGAATCTT TGGGCTATCT
601 TTCAAGTGTA CGCATGACCC CTTCAGTGAT ACGTAGTCAA ATTCTCGAAA ATGCATTTCT
661 AATCAATAAT GCTATTAAGA AGTTCGATAC CTTTGTTCCA ATTATTCCCT TGATTGCATC
721 ATTGGCTAAA TCGAAATTTT GTAACGTATT AGGGCATCCT ATGAGTAAGC CGGTTTGGGC
781 TGATTTATCA GATTCTAATA TTATTGATCG ATTTGTGCGT ATATGCAGAA ATCTCTCTCA
841 TTATCATAGC GGATCCTCAA CAAAAA 866

Plate 4: *Acanthus ilicifolius matK* gene

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