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Identification of medicinal mangrove *Acanthus ebracteatus* Vahl., morphological, phytochemical and DNA barcoding methods

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Abstract

Sea Holly *Acanthus ebracteatus* Vahl. belongs to Acanthaceae family is a medicinal mangrove plant used as astringent, expectorant, and stimulant. Little is documented on this plant. In this study, aim was to investigate morphological, anatomical, phytochemical and molecular characterstics. Anatomy showed xerophytic characters to withstand extreme environmental conditions. Toluene: Ethyl Acetate: Formic acid: Methanol: 7:5:1:0.5 as solvent system is the best solvent system for measuring the establishment of phytochemicals of *A. ebracteatus.* HPTLC analysis of methanol extract showed twelve peaks and the R_f values ranged in between 0.07 to 0.85 at 254 nm, eight peaks and R_f values ranged in between 0.07 to 0.85 at 366 nm. The molecular markers *rbc*L and *mat*K regions clearly distinguished. Moreover, the phylogenetic analysis confirmed their taxonomic position in Acanthaceae. The study denotes DNA barcoding provides successful and precise strategy for increasing the authenticity of species identification. The barcode sequence also was deposited in NCBI. This is the first scientific information about morphology, chemical and DNA fingerprinting of medicinal mangrove *A. ebracteatus* for medicinal application.

Keywords: acanthaceae, anatomy, phytochemicals, fingerprinting

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Introduction

The genus *Acanthus* L. belonging to the family Acanthaceae is an Old World genus native of tropics and subtropics with about 30 species. It is often distinguished from the related genera by spiny leaves, spicate terminal inflorescences, two bracteoles and uniform anthers. Generally *Acanthus* species occur either as an under storey in inner mangrove area or frontal thickets on edges of the tidal creek in middle to upper estuarine areas, although they do occur in lower estuarine position; Duke (2006) [4].

Acanthus has been used as folk medicine for a wide range of tropical diseases in Ayurveda. In India, whole plants are used as astringent, expectorant, and stimulant, the roots are used as a cough remedy, and the tender shoots and leaves are used as a snakebite cure; (Subudhi & Choudhury, 1992) [3]. In China, the stem and roots are useful against coughs, chronic fever, paralysis, asthma hepatomegaly, hepatitis, and lymphoma; Perry (1980) [14]. This plant consists of various components including alkaloids, triterpenoids, steroids, glycosides, polysaccharides Pharmacological studies focusing on antioxidant, hepatoprotective, anti-inflammatory, antitumor, antimutagenic and anticarcinogenic activities of Acanthus plants have been reported; Yahaufai & Siripong (2010) [19].

DNA barcoding is a relatively new concept that has been developed for providing rapid, accurate and automatable species identification using standardized DNA sequences as tags; Tsou &Vijayan (2010) [16]

Materials and Methods

Leaf samples were collected from the intertidal zones of Thalassery (11^0 7' N: 75^0 41' E)of Kannur district in Kerala. The plants were identified by Botanical survey of India, Coimbatore. One of the healthy plant were selected and the mature leaves from fifth and sixth node and stem (T.S, T.L.S, R. L.S.) were taken for anatomical studies. Sections were made at a position approximately half way between the base and apex of a sector from one side of the lamina, stained with Toluidine blue 0 and mounted in 50% glycerin. The slides analyzed by trilocular compound microscope model number 10093409 and imaged by using the camera Olympus E-PL3. The Scanning Electron Microscopic images of leaf sample were taken Zeiss ultra 55.

HPTLC analysis carried out using methanol leaf extract. CAMAG HPTLC used for analysis. These were carried out Arya Vaidya sala Kottakkal.

The DNA extraction carried out using Nucleospin Plant II Kit (Macherey-Nagel). PCR amplification was performed using forward and reverse primers (Table 1&2). Sequencing of PCR product was carried out in Gene Amp PCR system 9700. All these were carried out from RGCB, Thiruvanathapuram. Obtained DNA sequence was subjected to NCBI.

Phylogenetic analysis done by gene sequence of *rbc*L and *mat*K regions of DNA were obtained from GenBank (NCBI BLAST). Parameters of Clustal X 2.012 software in Bio edit used for alignment ; Hall (1999) [5] Phylogenetic trees of both *rbc*L and *mat*K constructed separately. The Maximum Likelihood (ML) method using MEGA5; Tamura *et al.* (2013) using Kimura 2-Parameter model.

Table 1: Primers used

Target	Primer Name	Direction	Sequence (5' → 3')	Reference
matK	390	Forward	CGATCTATTCATTCAATATTTC	CBOL Plant working group (http://www.barcoding.si.edu.
	1326	Reverse	TCTAGCACACGAAAGTCGAAGT	
*rbc*L	rbcLa_	Forward	ATGTCACCACAAACAGAGACTAAAGC	
	rbcL724	Reverse	GTAAAATCAAGTCCACCRCG	

Table 2: PCR amplification profile

step	Tem.(°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 Ext.	72	72	60	60	1	1
Hold	4	4	∞	∞	-	-

Result

Morphology of leaves showed leathery, coriaceous and glabrous surface. Oblong to lanceolate in shape. Dentate margins with spines. Apex spinous and narrowed at the base. Margins serrated and ended with spines. Numerous glandular trichomes present at the adaxial and abaxial region. Petiole cylindrical in shape, pinkish green in colour and 3 cm in length. (Plate C&D)

Anatomy of leaf showed dorsiventral with thick cuticle. Epidermis single layered in both sides. Adaxial cells larger in size compare to those of abaxial cells. The epidermal cells are polygonal in outline with more or less straight walls. Salt glands present in both surfaces of the lamina. Hypodermis or water storage tissue two layered thick below the epidermis.. Mesophyll with upper two layered palisade and lower spongy tissue consisting of loosely arranged cells. Stomata are enclosed by a pair of subsidiary cells is at right angles to long axis of guard cells; it is caryophyllaceous type. (Plate G, H &I)

Micro anatomy showed surface with ridges and furrow like appearance in the outer surface. Epidermal cells were straight, glandular trichomes and stomata intermittently seen in the abaxial region. Stomata deeply sunken. (Plate J)

Petiole anatomy showed, outline boat shaped, slightly concave at the adaxial side and convex at the abaxial side, epidermis uniseriate, cortex composed of 7-8 layers of polygonal angular collenchymas and chlorophyllated thin walled parenchyma cells. Vascular bundles 3, amphicribal, arranged in the form of an arc. Glandular trichomes and simple, uniseriate multicellular hairs present. Sclereids present in cortex. Pith consists of thin walled parenchyma cells. (Plate K&L)

Morphology of stem was greenish red in colour. Outer surface smooth. Nodes with a pair of spines present at the base of the petiole. (Plate B)

Anatomy of young stem showed primary stage of growth beginning secondary thickening. The epidermis thin, small cells and covered by thick cuticle. The cortex was broad consists of polygonal, thin walled, parenchyma cells. The vascular cylinder consists of several segments of xylem and phloem in collateral position; the segments are separated from each other by minute parenchymatous medullary rays. The xylem elements were narrow, radially stretched and consist of a few radial multiples or chains. The vessels are somewhat circular, thick walled and wide lumened. Xylem fibres are thick walled and lignified. Phloem was narrow conical cap on the outer end of each xylem segment. Few sclereids present in phloem. Pith was wide and composed of thin walled parenchyma with varying size and shape. Average diameter of stem is.09 cm. (Plate M)

Anatomy of old stem with one layered thick epidermis. Cortex multilayered with compactly arranged parenchymatous cells. Outer cortex with collenchyma cells and inner cortex consist of parenchyma with air cavities. Stem have secondary growth. Growth rings are indistinct therefore classified under diffuse porous wood. Vessels are small to medium sized, indistinct, even very numerous solitary and round to angular in outline. Vessel lines are indistinct. Vessel cylindrical in nature with alternate inters- vessel pitting and simple perforation plate. Rays biseriate, homogenous and moderately broad to fine. Xylem showed spiral and annular thickening. Solitary sclereids scattered in the cortex that was seen around phloem cells. Central parenchymatous pith with sclereids and tannin cells. Pith was very large with compactly arranged parenchymatous cells. Average diameter of stem is.1.2 cm. (Plate N, O&P)

Nodal anatomy showed the leaves are developed in opposite manner at the nodal region. The vascular cylinder consists of broad arc-shaped median trace leaving behind a gap and small accessory trace. The two traces derived from the vascular strand. The median trace extends into the petiole. The node unilacunar one-traced. (Plate Q)

Fruit morphology showed it was square shaped capsule, dehisces along dorsi-ventral line. Greenish in colour, outer surface shiny and coriaceous. Seeds white colour and flat., length 1.2 cm and width 1.07 cm. (Plate F)

Fruit anatomy showed outer cuticle. Epidermis unilayered. Cortex consists of sclerieds followed by chlorophyllated polygonal shaped parenchyma cells. Inner portion consists of sclerieds with groove present in centre. Tracheids present in inner cortex. (Plate R)

HPTLC profile the best results were shown using Toluene: Ethyl Acetate: Formic acid: Methanol: 7:5:1:0.5 as solvent system. TLC plate of *Acanthus ebracteatus* methanol (leaf) extract scanned at 254 nm wavelength signified the existence of twelve phytoconstituents whose Rf values ranged from 0. 07 to 0.85. Peak one showing with an R_f value of 0.07 with area 3.97%. Peak two with an R_f value of 0.17, area of 1.82 %. Peak three with an R_f value of 0.20 and area 4.69%. Peak four showing R_f value of 0.35 with area 2.57%. Peak five showing an R_f value of 0.39with an area of 0.97%. Peak six showing R_f value of 0.41 with 3.40 % area. Peak seven showing R_f value of 0.47 with area 19.42%. Peak eight showing R_f value of 0.53 with area 12.56%. Peak nine showed R_f value of 0.62 with area of 8.51%. Peak ten showing R_f value of 0.66 with area 6.73%. Peak eleven showing an R_f value of 0.81 with an area of 9.20%. Peak twelve showing R_f value of 0.85 with 26.16 % area. The total peaks present in HPTLC profile of of *Acanthus ebracteatus* is twelve with an area of 4750.8(AU).

The methanol (leaf) extract scanned at 366 nm wavelength signified the existence of eight phytoconstituents whose Rf values ranged from 0.07 to 0.85. Peak one showing with an R_f

value of 0.07 with area of 1.42%. Peak two with an R_f value of 0.17with area of 0.40 %. Peak three with an R_f value of0.21 and area 0.47%. Peak four showing R_f value of 0.40 with area 1.45%. Peak five showing an R_f value of 0.46 with an area of 8.48%. Peak six showing R_f value of 0.53 with area of 4.44% Peak seven showing R_f value of 0.62 with area 5.47%. Peak eight showing R_f value of 0.85 with area 77.87%. The total peaks present in HPTLC profile of *Acanthus ebracteatus* is eight with an area of 18473.2 (AU). (Figure 1&2, Table 5&6)

The barcode markers *rbc* L and *mat* K having the molecular weight of (697bp) and (866bp). The sequence deposited in NCBI with accession numbers. (*rbc*L : KX231352; *mat*K : KX231340). Consensus sequences showed in table 3&4.

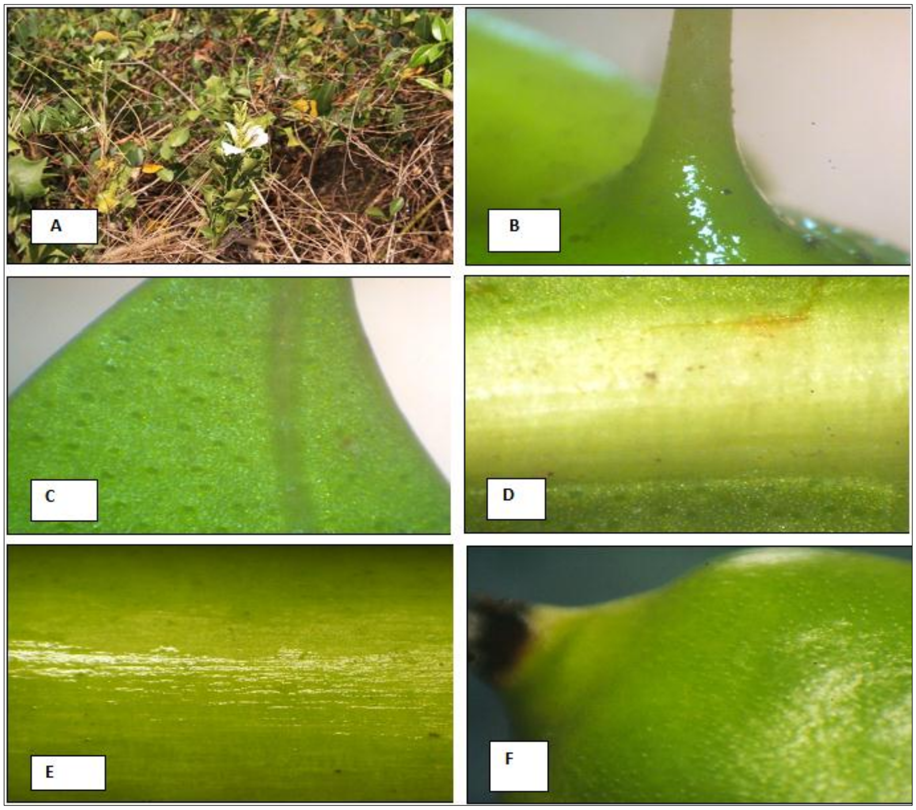

Plate: A- Habit of *Acanthus ebracteatus* Vahl, Plate B- Stereo microscopic image of stem, Plate C&D - Stereo microscopic image of leaf apex and base, Plate E &F - Stereo microscopic image of petiole and fruit.

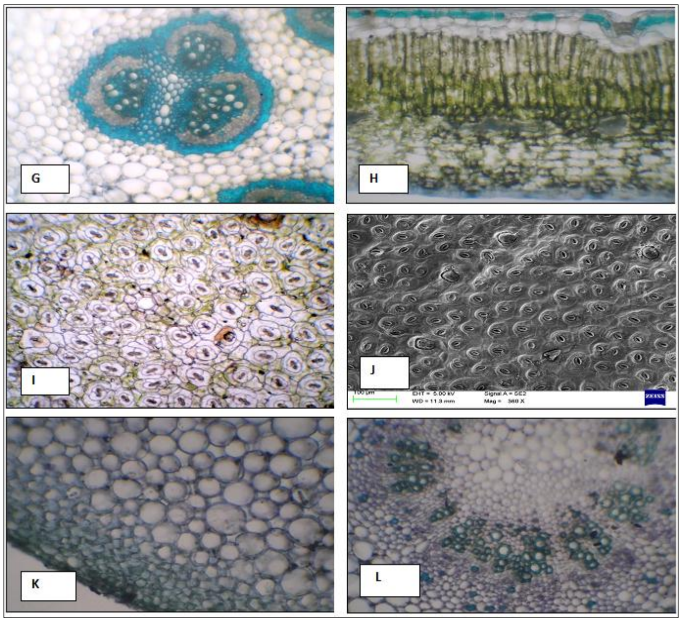

Plate: G&H- Light microscopic image of midrib and lamina, PlateI - Leaf abaxial side showing stomata, Plate J- SEM showing abaxial side of leaf, Plate K& L- Petiole T.S.

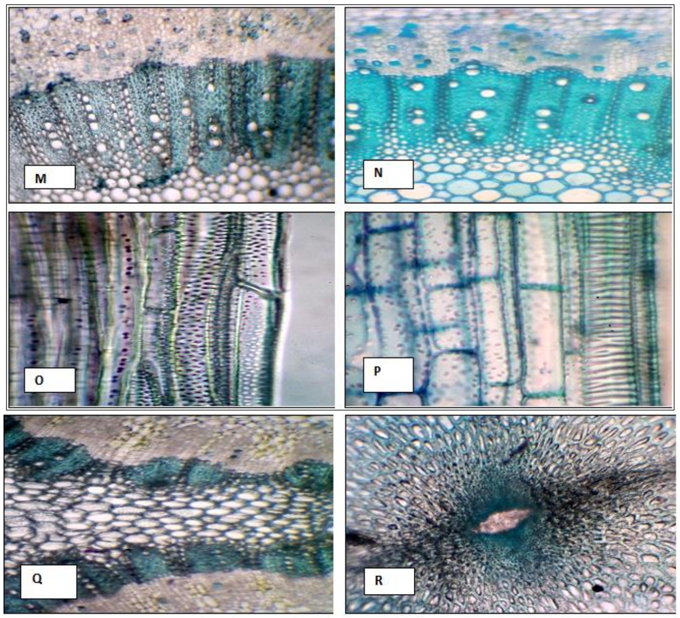

Plate: M- T.S. of primary stem, Plate N- T.S. Of old stem, Plate O- T.L.S. of old stem, Plate P- R.L.S. of old stem, Plate Q- Nodal anatomy, Plate R-T.S. of fruit.

Table 3: Consensus sequences data of *mat*K gene in Acanthus *ebracteatus* (866bp)

Consensus sequence	bp
CATGTGGAAATCTTGGTTCAAACCCTTCGCTATTGGTTAAAAGATGCCCCCTCTTTGCATTTATTACGATTCTTTTTCAACGAGTATTGTAATTGGAATAGTCTTATTACGCCAAAGAGAGCAAGTTCCTTCTTTTCAAAAAGAAATCAAAGATTATCTTTATTCTTATATAATTCTCATGTGTGGGAATATGAATCCATTTTCGTCTTTCTGTGTAACCAATCTTCTCATTTACGATCAACATCTTCTGGAGTTCTTCTTGAACGAATCCATTTCTATCGAAAAATAGAACATCTTGGGAACGTCTTTGTTAAGATTAAGTATTTTCAGGTGAACCTATGGTTTGTTAAGGAACCTTGCATGCATTGTATTAGATATCAAAGAAAGTGCATTCTGGCTTCAAAAGGGACATCACTTTTCATGAATAAATGGAAATCTTACCTTATGATTTTTTGGCAATCGTATTTTTCGTTGTGGTTTCTTCAAAGAAGGATTTATAGAAACCAATTAGCCAAACATTTCCTCGAATCTTTGGGCTATCTTTCAAGTGTACGCATGACCCCTTCAGTGATACGTAGTCAAATTCTCGAAAATGCATTTCTAATCAATAATGCTATTAAGAAGTTCGATACCTTTGTTCCAATTATTCCCTTGATTGCATCATTGGCTAAATCGAAATTTTGTAACGTATTAGGGCATCCTATGAGTAAGCCGGTTTGGGCTGATTTATCAGATTCTAATATTATTGATCGATTTGTGCGTATATGCAGAAATCTCTCTCATTATCATAGCGGATCCTCAACAAAAAAGAGTTTGTAT	866

Table 4: Consensus sequences data of *rbcL* gene in Acanthus *ebracteatus* (697bp)

Consensus sequences	bp
TTTTGTTGGATTCAAAGCGGGTGTTAAAGAGTACAAATTGACTTATTATACTCCTGAATACGAAACTAAAGATACTGATATCTTGGCAGCATTCCGAGTAACTCCTCAACCCGGAGTTCCAGCCGAAGAAGCGGGGGCAGCGGTAGCTGCCGAATCTTCTACTGGTACATGGACAACCGTGTGGACCGACGGGCTTACCAGCCTTGATCGTTATAAAGGGCGATGCTACAACATCGAGCCCGTTCCTGGCGAAACAGATCAATATATCTGTTATGTAGCTTACCCTTTAGACCTTTTTGAAGAAGGTTCTGTTACTAACATGTTCACTTCCATTGTAGGAAATGTATTTGGATTCAAAGCCCTGCGTGCTCTACGTCTGGAAGATCTGCGAATCCCTACTGCTTATATTAAAACTTTCCAAGGTCCGCCTCATGGGATCCAAGTTGAGAGAGATAAATTGAACAAGTATGGTCGTCCCCTGCTGGGATGTACTATTAAACCTAAATTGGGGTTATCTGCTAAAAACTACGGTAGAGCGTGTTATGAATGTCTTCGCGGTGGACTTGATTTACCAAAGATGATGAGAACGTGAACTCCCAACCATTTATGCGTTGGCGAGATCGTTTCTTATTTTGTGCCGAAGCCATTTATAAAGCACAGGCTGAAACAGGCGAAATCAAAGGGCATTACTTGAAT	697

Table 5: Area and peak of *A. ebracteatus* AT 254nm

Peak No	R_f Value	Area (AU)	% Area (AU)
1	0.07	188.6	3.97
2	0.17	86.2	1.82
3	0.20	223.0	4.69
4	0.35	122.1	2.57
5	0.39	46.2	0.97
6	0.41	161.5	3.40
7	0.47	922.6	19.42
8	0.53	596.8	12.56
9	0.62	404.1	8.51
10	0.66	319.7	6.73
11	0.81	437.2	9.20
12	0.85	1242.8	26.16

Table 6: Area and peak of *A. ebracteatus* AT 366nm

Peak No	Rf value	Area (AU)	% Area (AU)
1	0.07	262.2	1.42
2	0.17	73.5	0.40
3	0.21	89.0	0.47
4	0.40	267.4	1.45
5	0.46	1566.6	8.48
6	0.53	819.5	4.44
7	0.62	1009.7	5.47
8	0.85	14385.3	77.87

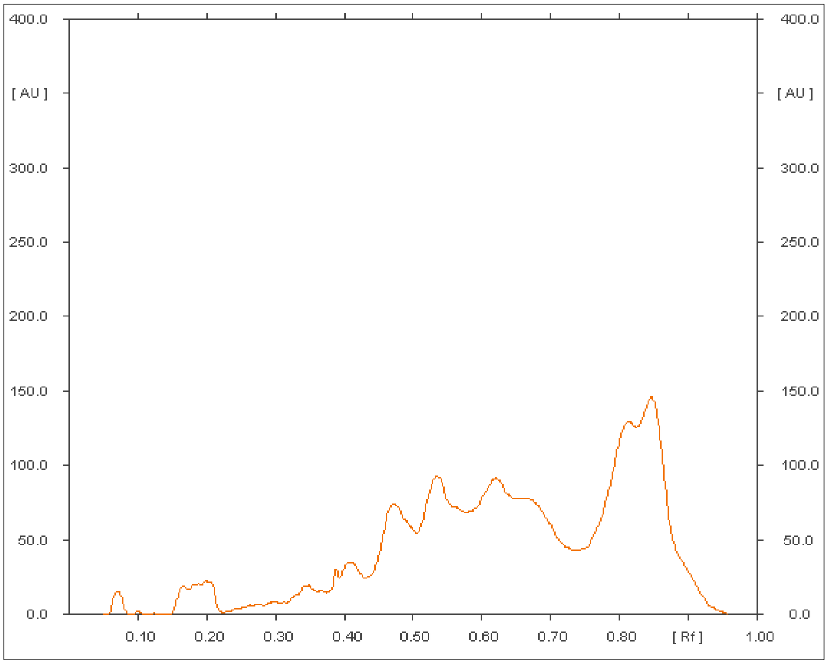

Fig 1: An overview of *Acanthus ebracteatus* Vahl., sample at 254 nm before derivatization

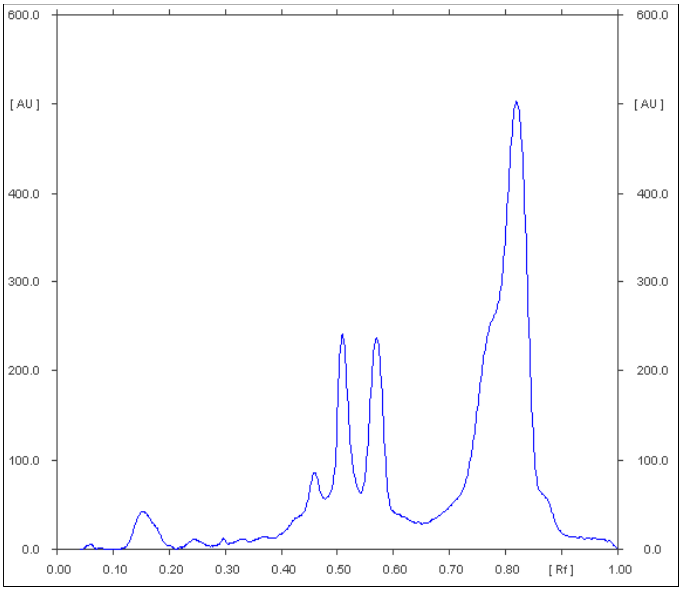

Fig 2: Overview of *Acanthus ebracteatus* Vahl., sample at 366 nm after derivatization

From the phylogram two major clusters were identified. The major cluster includes largest mangrove family Rhizophoracean members, *Sonneratia*, *Lumnitzera* and *Excoecaria.* But *Aegiceras, Avicennia and Acanthus* found in the miner cluster. All the species and members of Rhizophoraceae share the same minor cluster showing their similarity. The two species of *Sonneratia* placed slightly away but near to *Rhizophora* showed their minor difference and major similarity. *Excoecaria agallocha* was placed in the same major cluster showing more genetic relatedness to *Rhizophora* and *Lumnitzera.* The two species of *Sonneratia* placed near to *Lumnitzera* showing more similarity.

The phylogram constructed with the help of MEGA 5 helps to identify its genetic similarity and differences. *Aegiceras Acanthus*, and *Avicennia* found in second cluster. *Aegiceras* showing more genetic relatedness in *Acanthus* than *Avicennia.*

Although the Rhizophoracean members having a common ancestor, all the three genus formed distinct clades. From the result, it is clear that more genetic relatedness is shown between different genus of largest mangrove family with *Sonneratia*, *Lumnitzera* and *Excoecaria* and least genetic relatedness is shown with *Aegiceras Acanthus,* and *Avicennia.* (Figure 3)

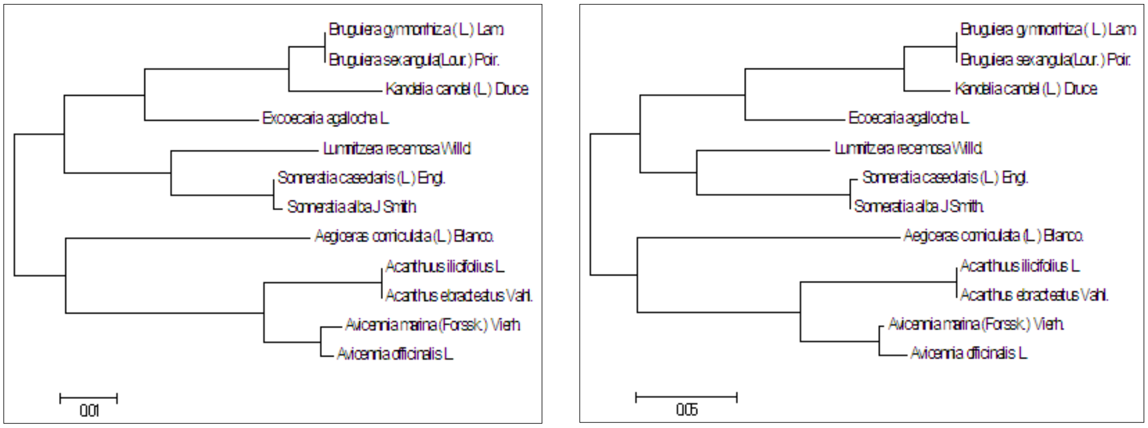

Fig 3: ML tree obtained from phylogenetic analysis based on *rbc*L and *mat*K sequences of *Acanthus ebracteatus* Vahl., with true mangrove species in Kerala

Discussion

The most important characteristic features of a DNA barcode are its universality, specificity on variation and easiness on employment. This means that the gene segment used as a barcode should be suitable for a wide range of taxa, should have high variation between species but should be conserved within the species, so that the intra-specific variation will be insignificant (Kress *et al.*, 2005; CBOL–Plant Working Group; Pennisi, 2007) [12].

Among the plastid genes, *rbc*L is the best characterized gene sequence. Therefore, most of the investigating groups tested its suitability in barcoding. As RUBISCO is a critical photosynthetic enzyme, *rbcL* was the first gene that was sequenced from the plant; Zurawski (1981) [20]. *rbcL* has been used so extensively in plant phylogenetic studies that more than 10,000 *rbcL* sequences are already available in Gen Bank (Chase, 2007; Newmaster, 2006) [2, 13]. Most of the phylogenetic studies suggest that *rbcL* is best suited to reconstruct the relationships down to the generic levels, but is not useful for specific levels; Soltis (1998) [15].

However, because of the low species discrimination, most of the investigating groups are of the opinion that *rbcL* should be used in conjunction with other markers (CBOL–Plant Working Group; Chase, 2007; Hollingsworth, 2009; Soltis, 1998) [2, 7, 15]. Therefore, the CBOL–Plant Working Group recommended a combination of *rbcL* and *matK* as the standard two-locus barcode for plants, because this combination of genes appears to be a pragmatic solution to a complex trade-off among universality, sequence quality discrimination and cost.

Among the chloroplast genes, *matK* is one of the most rapidly evolving genes. It has a length of about 1550 bp and encodes the enzyme maturase which is involved in the splicing of type-II introns from RNA transcripts (Wolfe, 1991; Neuhaus and Link, 1987) [18, 11]. *matK* has been used as a marker to construct plant phylogenies because of its rapid evolution and the ubiquitous presence in plants (Hilu and Liang, 1997; Kelchner, 2000) [6, 9]. Phylogenetically, the rate of evolution of *matK* was found suitable for resolving intergeneric as well as interspecies relationships in many angiosperms (Johnson and Soltis, 1995; Soltis and Soltis 1998) [8, 15]

Considering the high evolutionary rate of *matK*, it has been tested by several workers for suitability as a plant barcode and has been proposed either alone or in combination with other loci.

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References

1. CBOL–Plant Working Group. A DNA barcode for land plants. Proc. Natl. Acad. Sci. USA. 2009; 106:12794-12797.
2. Chase MW. A proposal for a standardized protocol to barcode all land plants. Taxon. 2007; 56:295-299.
3. Choudhury BP, Acharya BC. Some potential medicinal plants of Mahanadi delta in the state of Orissa, Journal of Economic and Taxonomic Botany. 1992; 16(2):479-487.
4. Duke NC. Australia's mangroves: the Authoritative Guide to Australia's Mangrove Plants. University of Queensland and Norman C. Duke, Brisbane. 2006.
5. Hall TA. Bio Edit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT, Nucleic Acids Symposium series. 1999; 41:95-98.
6. Hilu KW, Liang H. The *matK* gene: sequence variation and application in plant systematics. Am. J. Bot. 1997; 84:830-839.
7. Hollingsworth ML. Selecting barcoding loci for plants: evaluation of seven candidate loci with species-level sampling in three divergent groups of land plants. Mol. Ecol. Res. 2009; 9:439-457.
8. Johnson LS, Soltis DE. Phylogenetic inference in Saxifragaceae *sensu strict* and *Gilia* (Polemoniaceae) using *matK* sequences. Ann. Mo. Bot. Gard. 1995; 82:149-175.
9. Kelchner SA. The evolution of noncoding chloroplast DNA and its application in plant systematics. Ann. Mo. Bot. Gard. 2000; 87:482-498.
10. Kress JW, Wurdack KJ, Zimmer EA, Weigt LA, Janzen DH. Use of DNA barcodes to identify flowering plants. Proc. Natl. Acad. Sci. USA. 2005; 102:8369-8374.
11. Neuhaus H, Link G. The chloroplast tRNA(UUU) gene from mustard (*Sinapsis alba*) contains a class II intron potentially coding for a maturase-related polypeptide. Curr. Genet. 1987; 11:251-257.
12. Pennisi E. Taxonomy. Wanted: A barcode for plants. Science. 2007; 318:190-191.
13. Newmaster SG, Fazekas AJ, Ragupathy S. DNA barcoding in land plants: evaluation of *rbcL* in a multigene tiered approach. Can. J. Bot. 2006; 84:335-341.
14. Perry LM, Subudhi HN. Medicinal Plants of East and Southeast Asia Attributed Properties and Use, 2, The Massachusetts Institute of Technology Press, London, UK. 1980.
15. Soltis DE, Soltis PS. Choosing an approach and an appropriate gene for phylogenetic analysis. In Molecular Systematics of Plants II: DNA Sequencing (eds Soltis, D. E., Soltis, P. S. and Doyle, J. J.), Kluwer, Dordrecht. 1998, 21-24.
16. Tosu CH, Vijayan K. DNA barcoding in plants: taxonomy in a new perspective. Current Science. 2010; 99(11):1530-1541.
17. Tamura K, Dudley J, Nei M, Kumar S. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0, Molecular Biology and Evolution. 2011; 24(8):1596-1599. doi: 10.1093/molbev/msm092
18. Wolfe KH. Protein-coding genes in chloroplast DNA: compilation of nucleotide sequences, data base entries and rates of molecular evolution. In Cell Culture and Somatic Cell Genetics of Plants (ed. Vasil, K.), Academic Press, San Diego. 1991; 7BI:467-482.
19. Yahaufai Jantana, Siripong Pongpun, Limpanasithikul Wacharee. Immunomodulatory Effect of A. ebracteatus on Macrophage Function. Thai Cancer Journal. 2010; 30(2):55-68.
20. Zurawski G, Perrot B, Bottomley W, Whitefield PR. The structure of the gene for the large subunit of ribulose-1,5-bisphosphate carboxylase from spinach chloroplast DNA. Nucleic Acids Res. 1981; 9:3251-3270.