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RESEARCH ARTICLE

PHYLOGENETIC RELATIONSHIP OF MANGROVE SPECIES FOUND IN SRI LANKA BASED ON *rbcL* GENE SEQUENCE

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ABSTRACT

A fertile mangrove community can be seen along the sheltered coastline of Sri Lanka, which is an island in the Indian Ocean. Currently there are 21 species of mangroves that have not yet been genetically classified. Therefore, we intended to perform the Sri Lankan mangrove classification using the *rbcL* gene marker. Cetyl-trimethyl ammonium bromide (CTAB) protocols for DNA extraction from mangroves and Silica extraction method were optimized to extract DNA. All the sequence data obtained in this study were deposited in the NCBI GenBank. Maximum Likelihood and Bayesian phylogenetic trees revealed that, the Sri Lankan mangrove community can be classified genetically under 21 species and the *Rhizophora anamalayana* plant was identified as a hybrid of two species, *R. apiculata* and *R. mucranata*. As far as *Ceriops* is concerned, the vegetation of the Trincomalee and Puttalam areas seems to be distinctly different. Therefore, need to do more research on this clade in the future. The results of this study can be used as the baseline data for mangrove taxonomy in Sri Lanka and will be supported in future conservation and management actions.

Keywords: Mangroves, *rbcL* marker, *Rhizophora anamalayana*, Sri Lanka

INTRODUCTION

Mangals, sometimes called mangroves, are a plant community found in tropical and subtropical coastal areas and includes about sixteen families and about fifty species (Naskar and Guha Bakshi 1987). Sundarbans, the world's largest mangrove forest, covers approximately 10, 200 square kilometers and is located near the India-Bangladesh border. There are 26 species of true mangroves, 29 species of associated mangroves and 29 species of mangroves in 40 groups and 60 genera (Naskar and Guha Bakshi 1987). Sri Lanka has a very productive coastal environment with about 15,000 hectares of mangroves divided into true mangroves and mangrove associates. *Bruguiera cylindrica*, *Ceriops decandra*, *Sonneratia alba*, *Xylocarpus granatum* and *Lumnitzera littorea* are five rare mangrove species found in Sri Lanka (Kariyawasam 2015). There are 21 species of mangroves in Sri Lanka (Arulnayagam *et al*. 2021) and all these species have been identified and classified according to their vegetative morphology such as flower shape, flower color, leaf shape, leaf structure, mangrove shape. branched stems, root system, etc. However, many species are morphologically similar in the same environment, for example, *Rhizophora* spp and *Bruguiera* spp. *Rhizophora* thrives due to its ability to reproduce with wind pollination, but because of its fragrant and colorful flowers it can reproduce with the help of insects. According to Tomlinson (1986), wind

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-pollinated breeding increases the occurrence of putative hybrids, especially in *Rhizophora* pollen of different species with a very common morphology. This may be the reason for the active evolution of *Rhizophora* by natural hybridization.

Although the nomenclature of animals and plants based solely on morphological features has been used in the past, it provides ample or misleading information about genetic variation in the identification of many subspecies and species. Therefore, efforts to conserve "endangered mangrove species" in countries such as Sri Lanka may not work well towards the goal of conserving mangrove biodiversity. As a result, the lack of accurate taxonomy is a major drawback in assessing and identifying the diversity of mangrove species in Sri Lanka in terms of mangrove conservation activities. In such cases, DNA marker techniques offer a solution for more specific and accurate identifications, as well as the conservation and recovery of these species. There are three *Rhizophora* spp in Sri Lanka and *Rhizophora annamalayana* is morphologically very similar to the other two spp and is considered a hybrid. This makes it difficult to identify the genetic link between the species and the mangroves in Sri Lanka. Consequently, this research focused on identifying mangroves in Sri Lanka with DNA markers to fill this gap.

MATERIALS AND METHOD

In this study, leaf samples of 21 mangrove species were collected along the coast (4 replicates of each species) and taken to the faculty laboratory in sample bags for basic morphological identification. Cetyl-trimethyl ammonium bromide (CTAB) protocols (Parani *et al*. 1997; Porebski *et al*. 1997) and silica extraction method (Boom *et al*. 1990) for DNA extraction from mangroves have been optimized for DNA extraction. The quality and quantity of DNA were confirmed by agarose gel electrophoresis and nanodrop.

PCR and sequence analysis of *rbcL* gene were carried out according to the Saddhe *et al*. (2016). Sequence chromatograms were analyzed with Chromas 2.6.6 (Technelysium Pty Ltd) and low-quality leading and trailing ends were trimmed before the sequence alignment. Consensus sequences for the *rbcL* gene region were generated using DNAStar v.5.1. Reference sequences were downloaded from GenBank referring to Daru *et al.* (2013) and are mentioned in (Figure 1, Figure 2, Figure 3). *rbcL* data set were aligned using the default settings of MUSCLE (Edgar 2004) in AliView (v. 1.28). The alignments were further edited manually using AliView (v. 1.28) where necessary and leading or trailing gaps were trimmed. The resultant *rbcL* alignment was analyzed using Maximum Likelihood (ML) and Bayesian Inference (BI).

ML analysis was carried out using RAxML-HPC2 on XSEDE (v. 8.2.8) (Stamatakis *et al*. 2008; Stamatakis 2014) in the CIPRES Science Gateway platform (Miller *et al*. 2010) using GTR+G+I model of evolution with 1000 bootstrap iterations.

BI analysis was executed in MrBayes 3.2.7a (Ronquist *et al.* 2012) in the CIPRES Science Gateway platform (Miller *et al.* 2010). The model TIM1+I+G for the comprehensive dataset and TIM3+I+G for our sequence only dataset were selected for analysis using jModelTest (Posada 2008). Two independent analyses with six Markov chains were run in parallel, with sampling at every 1000^{th} generation. The Markov Chain Monte Carlo (MCMC) analyses were run until the average standard deviations of split frequencies reach below 0.01. Twenty-five per cent of the trees representing the burn-in phase was discarded. The remaining trees were used to calculate the posterior probabilities (PP) in the majority rule consensus tree. The resulting trees were viewed in the FigTree v. 1.4.0 (Alexei *et al*. 2012) and edited in Adobe Illustrator CC 2017 version 21.0.0 (Adobe Systems Incorporated, USA) for clear representation. All the sequence data generated in this study were deposited in the NCBI GenBank (Figure 1, Figure 2 and Figure 3).

RESULTS AND DISCUSSION

In all, eighty-four mangrove samples were collected, identified morphologically and packaged in polythene bags. Sampling was

completed from the island-wide coastal zone (Table 1). DNA extraction from the Gene *rbcL* (1350 bp length) ribulose-bisphosphate carboxylase gene or Plastid gene large subunit was completed in 55 samples.

Table 1: Sample collection locations where DNA extraction was completed

Sample ID	Species name /Common name	Location
R 1	*Bruguiera gymnorhiza*/ Mal kadol	Rekawa lagoon
R 2	*Excoecaria agallocha*/ Thela	Rekawa lagoon
R 3	*Sonneratia caseolaris*/ Kirala	Rekawa lagoon
R 4	*Lumnitzera racemosa*/ Beriya/Veraniya	Rekawa lagoon
R 5	*Aegieceras corniculatum*/ Evari kadol/Heen kesel	Rekawa lagoon
R 6	*Avicennia officinalis*/ Manda	Rekawa lagoon
TR 1	*Aegieceras corniculatum*/ Evari kadol/Heen kesel	Trincomalee
TR 2	*Sonneratia caseolaris*/ Kirala	Trincomalee
TR 3	*Avicennia marina*/ Manda	Trincomalee
TR 4	*Rhizophora apiculata*/ Maha kadol	Trincomalee
TR 5	*Lumnitzera racemosa*/ Beriya/Veraniya	Trincomalee
TR 6	*Avicennia officinalis*/ Manda	Trincomalee
TR 7	*Bruguiera gymnorhiza*/ Mal kadol	Trincomalee
TR 9	*Xylocarpus granatum*/ Mutti kadol	Trincomalee
TR 11	*Ceriops decandra* / Path kadol	Trincomalee
TR 12	*Ceriops tagal* /Pun kanda	Trincomalee
TR13	*Rhizophora apiculata*/ Maha kadol	Trincomalee
Ga 1	*Pemphis acidula*/ Muhudu wara	Rumassala, Galle
Madu 1	*Lumnitzera littorea*/ Rathamilla	Madu ganga,
Madu 2	*Lumnitzera littorea*/ Rathamilla	Madu ganga,
DUG 01	*Rhizophora mucronata*/ Maha kadol	Halawatha lagoon
DUG 02	*Rhizophora apiculata*/ Maha kadol	Halawatha lagoon
DUG 03	*Rhizophora apiculata*/ Maha kadol	Halawatha lagoon
M 1	*Nypa fruticans*/Gin pol	Gin oya
M 2	*Nypa fruticans*/Gin pol	Gin oya
M 2	*Nypa fruticans*/Gin pol	Gin oya
M 03	*Rhizophora anamalayana*	Pambala
M 04	*Sonneratia alba* / Gal kirala	Kalpitiya
M 5	*Sonneratia alba* / Gal kirala	Kalpitiya
M 10	*Rhizophora apiculata*/ Maha kadol	Kalpitiya
M 11	*Rhizophora apiculata*/ Maha kadol	Kalpitiya
M12	*Rhizophora mucronata*/ Maha kadol	Jaffna
M13	*Rhizophora mucronata*/ Maha kadol	Jaffna
M 07	*Scyphiphora hydrophylacea*/Kalu kadol	Kalpitiya
M 08	*Rhizophora mucronata*/ Maha kadol	Kalpitiya
M 09	*Rhizophora mucronata*/ Maha kadol	Kalpitiya
M 06	*Exoecaria indica* / Muhudu kaju	Halawatha
D1A	*Rhizophora apiculata*/ Maha kadol	Dikovita
D2B	*Bruguiera sexangula*/ Mal kadol	Dikovita
D3C	*Lumnitzera racemosa*/ Beriya/veraniya	Dikovita
P1A	*Pemphis acidula*/ Muhudu wara	Puttlam
P2B	*Bruguiera sexangula*/ Mal kadol	Puttlam
P3C	*Ceriops tagal* /Pun kanda	Puttlam
P4D	*Sonneratia alba* / Gal kirala	Puttlam
MAT-01	*Bruguiera gymnorhiza*/ Mal kadol	Kaluwamodara
MAT-02	*Xylocarpus granatum*/ Mutti kadol	Kaluwamodara
MAT-03	*Rhizophora apiculata*/ Maha kadol	Kaluwamodara
MAT-04	*Sonneratia caseolaris*/ Kirala	Malgasowita, Iththapana
MAT-05	*Heritiera littoralis*/ Etuna	Kaluwamodara
MAT-06	*Bruguiera sexangula*/ Mal kadol	Kaluwamodara
MAT-07	*Rhizophora apiculata*/ Maha kadol	Malgasowita, Iththapana
MAT-08	*Bruguiera sexangula*/ Mal kadol	Malgasowita, Iththapana

Table 1: *continued...*

Sample ID	Species name /Common name	Location
NA-RA,KAL01 (NK)	*Bruguiera cylindrica*	Kalpitiya
ER	*Bruguiera cylindrica*	Kalpitiya
R7	*Avicennia marina*/ Manda	Rekawa
R8	*Rhizophora apiculata*/ Maha kadol	Rekawa

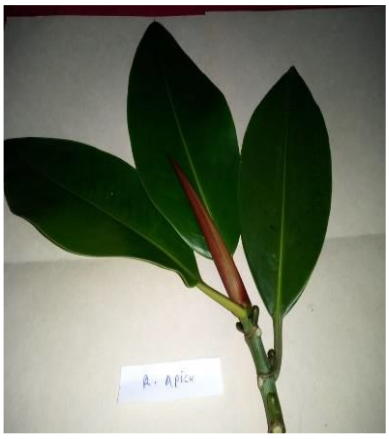
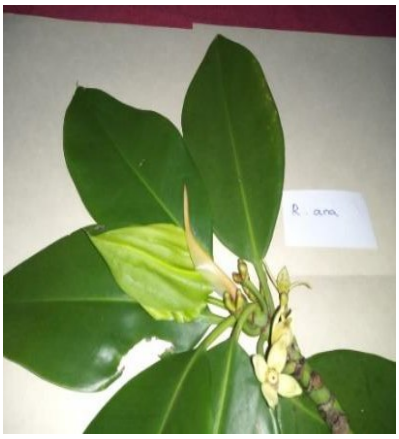
Phylogenic analysis

Out of the 84 samples collected, DNA extractions & PCR and sequencing for 55 sequences were completed. These sequences are deposited in the NCBI database and the accession numbers of these sequences are included in the phylogeny analysis. This study is the first detailed phylogenetic analysis of mangrove vegetation in Sri Lanka and has so far identified 21 species of mangroves in Sri Lanka, including 13 genera. The phylogenetic tree presented here (Figure 1) shows the phylogenetic relationship of 55 mangrove samples collected from different locations in Sri Lanka. All our collections formed 13 clades in the phylogenetic tree representing the clear separation of 13 genera into which mangrove species are classified.

The *rbcL* data set of comprehensive analysis (Figure 1) comprised 102 sequences including isolates from the current study. The *rbcL* data set of sequences generated from the current study (Figure 2) only comprised 58 sequences. The tree is rooted with *Acrostichum aureum* (AB574794), *Acrostichum danaeifolium* (EF452129), and *Acrostichum speciosum* (AB246707) (Figure 1 and Figure 2).

The best scoring ML tree for the comprehensive *rbcL* data set is shown in Figure 1 with a final ML optimization likelihood value of -3899.933826. The matrix had 276 distinct alignment patterns, with 2.08% of undetermined characters or gaps.

Table 2: Morphology of three Rhizophora species available in Sri Lanka

R. apiculata	*R. mucronata*	*R. anamalayana*
		
Leaf- thick fleshy leathery. , elliptic to sub lanceolate	Leaf- simple leathery ovate, broader at the base	Leaf- Broadly ovate to elliptic
Dark green	Yellowish green	Green
Inflorescence – 2 flowers	Inflorescence -2-8 flowers	Inflorescence – 2-4 flowers
Plant height- 30 m	Plant height – 20-25m	Plant height – 30-35 m

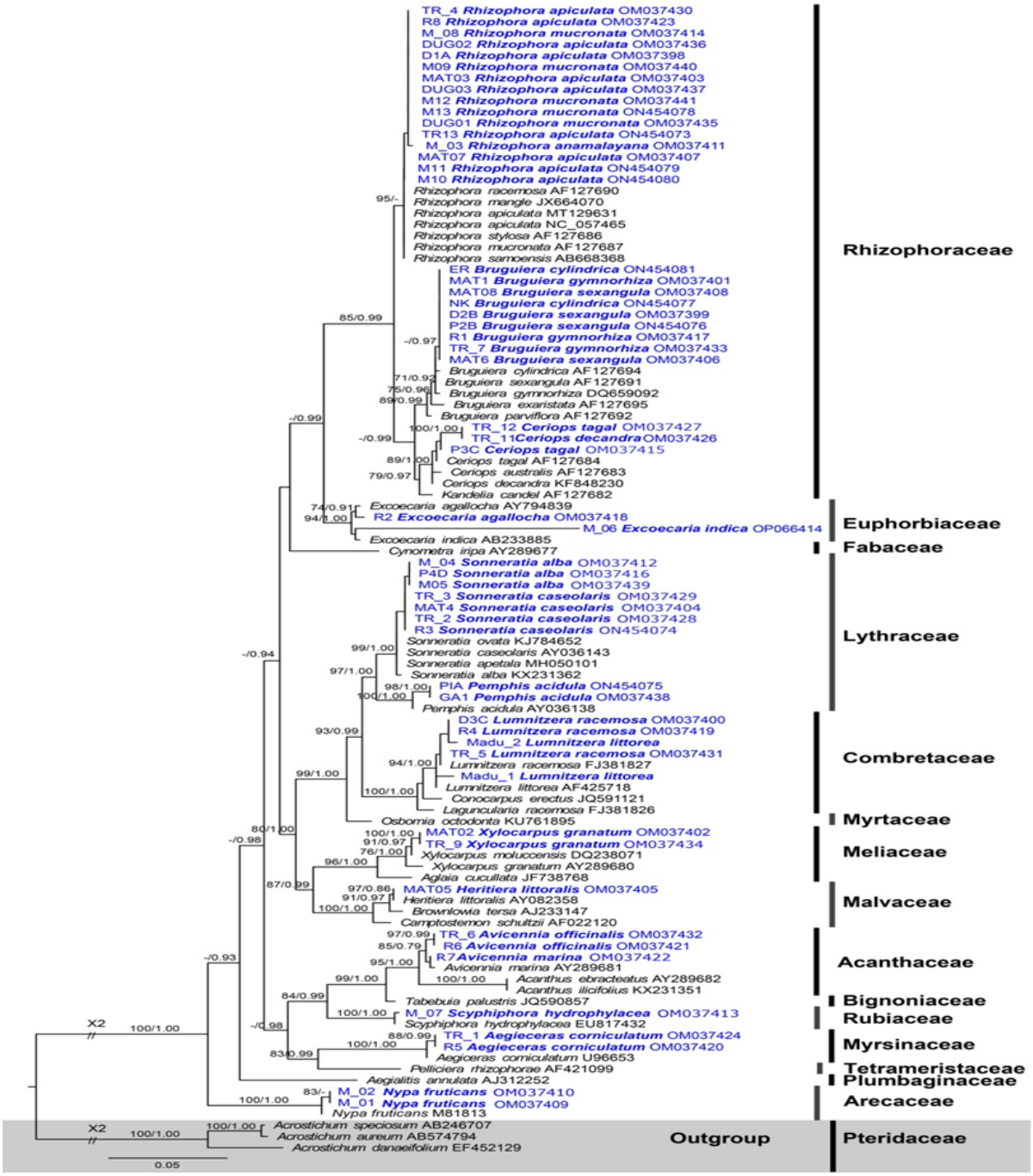


Figure 1: Maximum likelihood phylogenetic tree (RAxML) inferred from the *rbcL* comprehensive dataset. Bootstrap support (BS) for ML (> 70 %) and PP for BI (> 0.9) are given at the nodes (BS/PP). The tree is rooted with *Acrostichum aureum* (AB574794), *Acrostichum danaeifolium* (EF452129), *Acrostichum speciosum* (AB246707). Sequences generated in this study are in blue bold

Estimated base frequencies were as follows; A = 0.275553, C = 0.201141, G = 0.232065, T = 0.291240; substitution rates AC = 1.472333, AG = 3.201022, AT = 0.763235, CG = 0.954710, CT = 4.858880, GT = 1.000000; proportion of invariable sites I = 0.404097; gamma distribution shape parameter α = 0.749555. The BI analysis resulted in 1351 trees after 1350000 generations (ngen). ML and BI trees were similar in topology and significant differences between them were not observed. At the

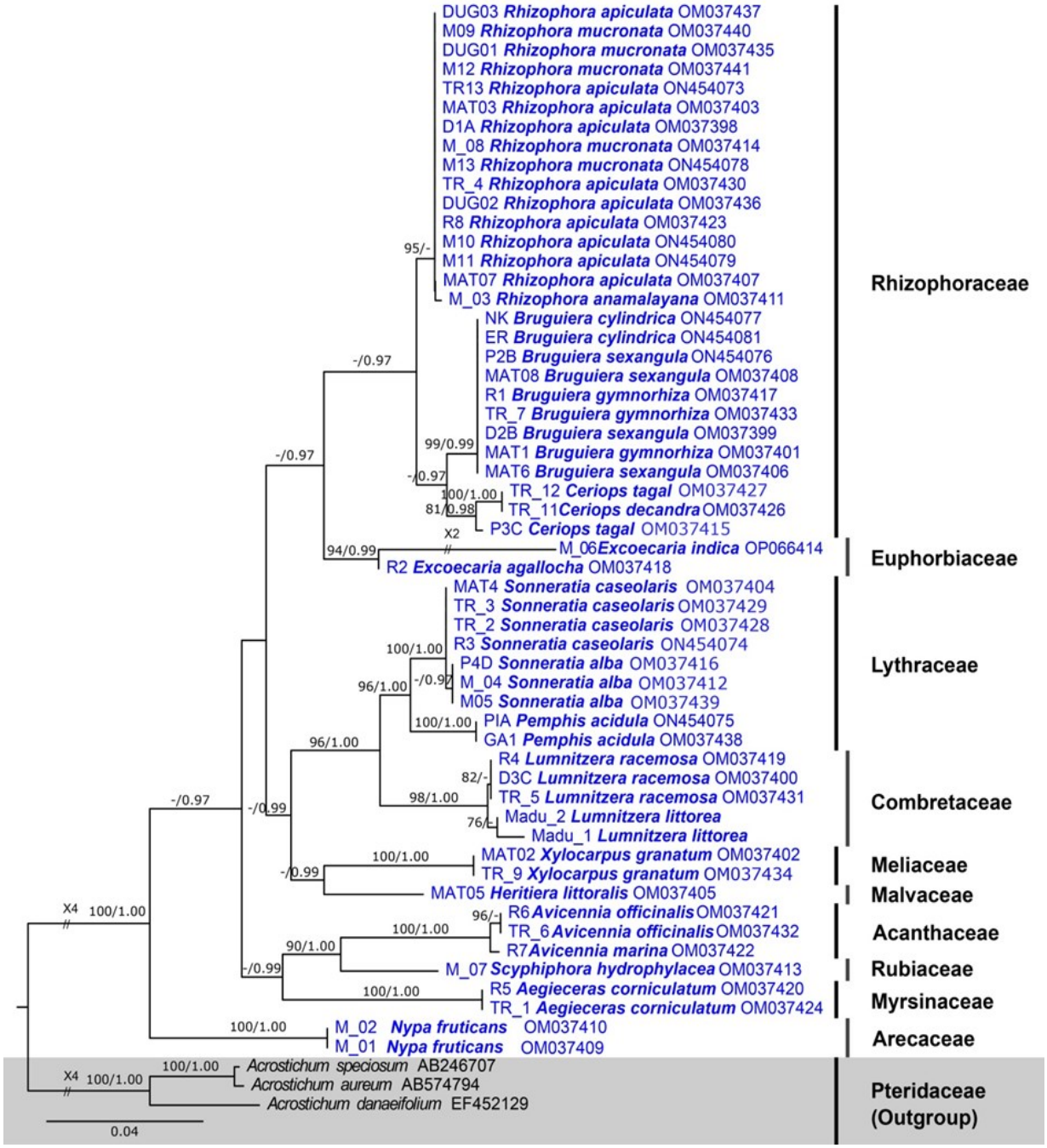


Figure 2: Maximum likelihood phylogenetic tree (RAxML) inferred from the *rbcL* our sequence only dataset. Bootstrap support (BS) for ML (> 70 %) and PP for BI (> 0.9) are given at the nodes (BS/PP). The tree is rooted with *Acrostichum aureum* (AB574794), *Acrostichum danaeifolium* (EF452129), *Acrostichum speciosum* (AB246707). Sequences generated in this study are in blue bold

generic level, relationships are in agreement with the previous study based on *rbcL* phylogeny (Daru *et al.* 2013). Our comprehensive phylogenetic analyses resulted in 17 families corresponding to mangrove species in the world. Sri Lankan mangrove isolates from this study confirmed their identities clustering within 10 families corresponding to known species.

The best scoring ML tree for the Sri Lankan mangrove species of this study is shown in

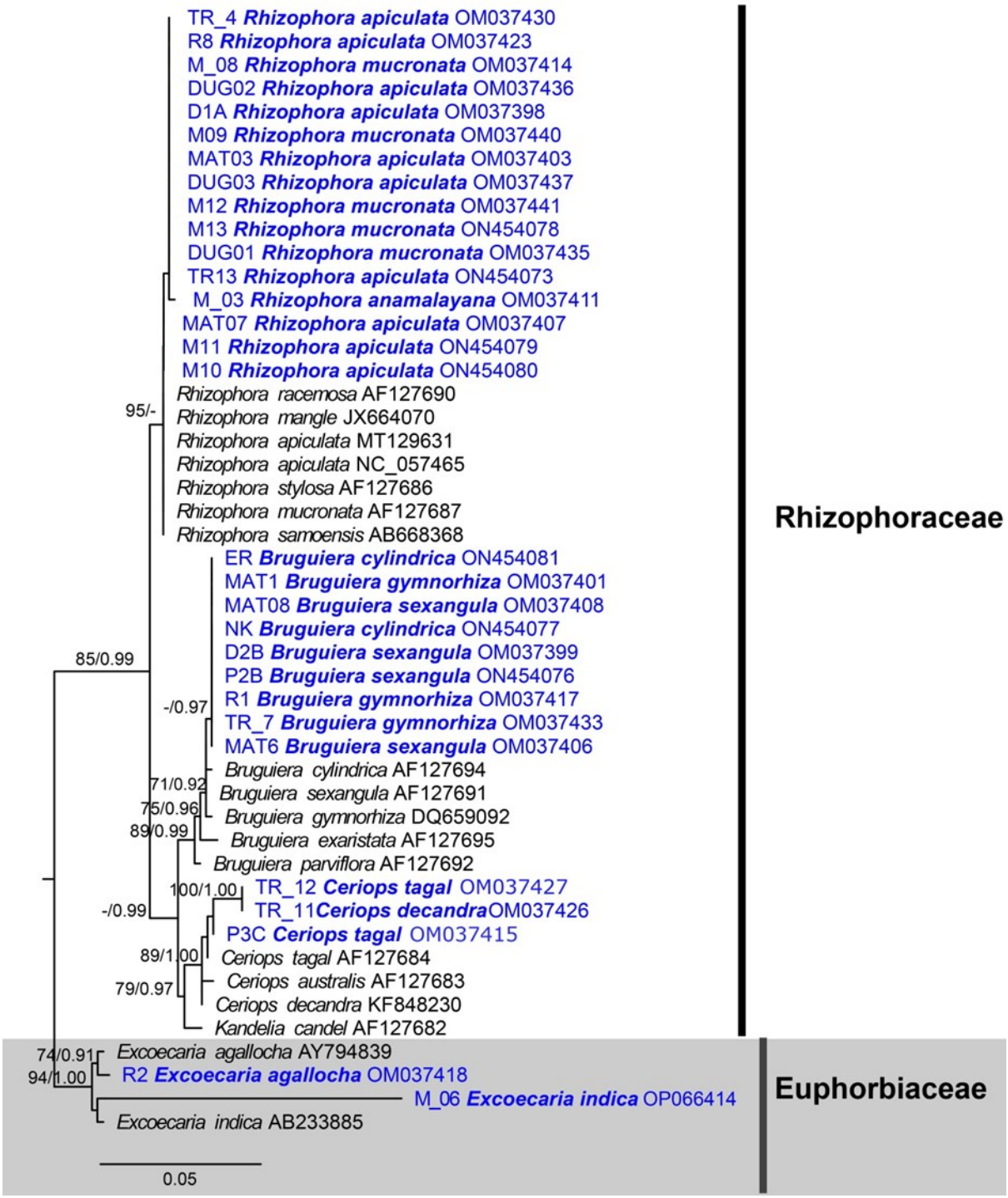


Figure 3: Rhizophoraceae clade extracted from the ML phylogenetic analysis (RAxML) of the comprehensive dataset *of rbcL* alignment. Bootstrap support (BS) for ML (> 70 %) and PP for BI (> 0.9) are given at the nodes (BS/PP). Sequences generated in this study are in blue bold

Figure 2 with a final ML optimization likelihood value of -2967.936836. The matrix had 241 distinct alignment patterns, with 2.08% of undetermined characters or gaps. Estimated base frequencies were as follows; A = 0.276407, C = 0.199580, G = 0.232321, T = 0.291693; substitution rates AC = 1.786766, AG = 3.577473, AT = 0.626542, CG = 1.271500, CT = 5.585496, GT = 1.000000; proportion of invariable sites I = 0.396670;

gamma distribution shape parameter α = 0.953272. The BI analysis resulted in 821 trees after 820000 generations (ngen). ML and BI trees were similar in topology and significant differences between them were not observed.

Collection M-3 from Sri Lanka was identified as *R. anamalayana* both morphologically and phylogenetically. In other countries, *R. anamalayana* is currently considered a hybrid of *R. apiculata* and *R. mucronata*. Our results also support this statement, with M-3, *R. anamalayana* covering a small branch between *R. apiculata* and *R. mucronata* species. The figure below shows how the gene sequence of the *R. anamalayana* plant has changed.

The number of mutations in M_03 compared to M13, MAT03 and TR_4 is one (1) and the difference is 0.16% bp (1/634 bp= 0.0016025 = 0.16% bp difference). Meantime the number of mutations in M_03 compared to M13, *R. apiculata* MT129631, *R. apiculata* NC_057465, *R. mucronata* AF127687 is two (2) and the difference is 0.32% bp (2/634 bp= 0.0032051= 0.32% bp difference).

Although the *Rhizophora* samples used here were collected from different parts of Sri Lanka, they all belong to the same clade. Several *R. annamalayana* plants were observed only in Pambala Lagoon, and are somewhat morphologically similar to both *R. apiculata* and *R. mucronata*. However, the number of flowers in the inflorescence varies and many scientists believe it is a new species. According to Setyawan *et al*. (2014), there are three species of *Rhizophora*, such as *R. apiculata, R. mucronata* and *R. stylosa*, and two other hybrids known as *R. x lamarckii* (*R. apiculata* and *R. stylosa*) and *R. x annamalayana* (*R. apiculata* and *R. mucronata*) in the Indo-Malay region.

Also, Setyawan *et al.* (2014) revealed that *Rhizophora* thrives successfully in natural habitats due to its sweet- scented nectar and colorful flowers and its ability to reproduce by wind and insects. Tomlinson (1986) revealed that breeding depends on wind pollination and that the general morphology of the pollen of different *Rhizophora* species enhances the survival of many hybrids. It is interesting to note that both the existing hybrids in the world (*R. x lamarckii and R. x*

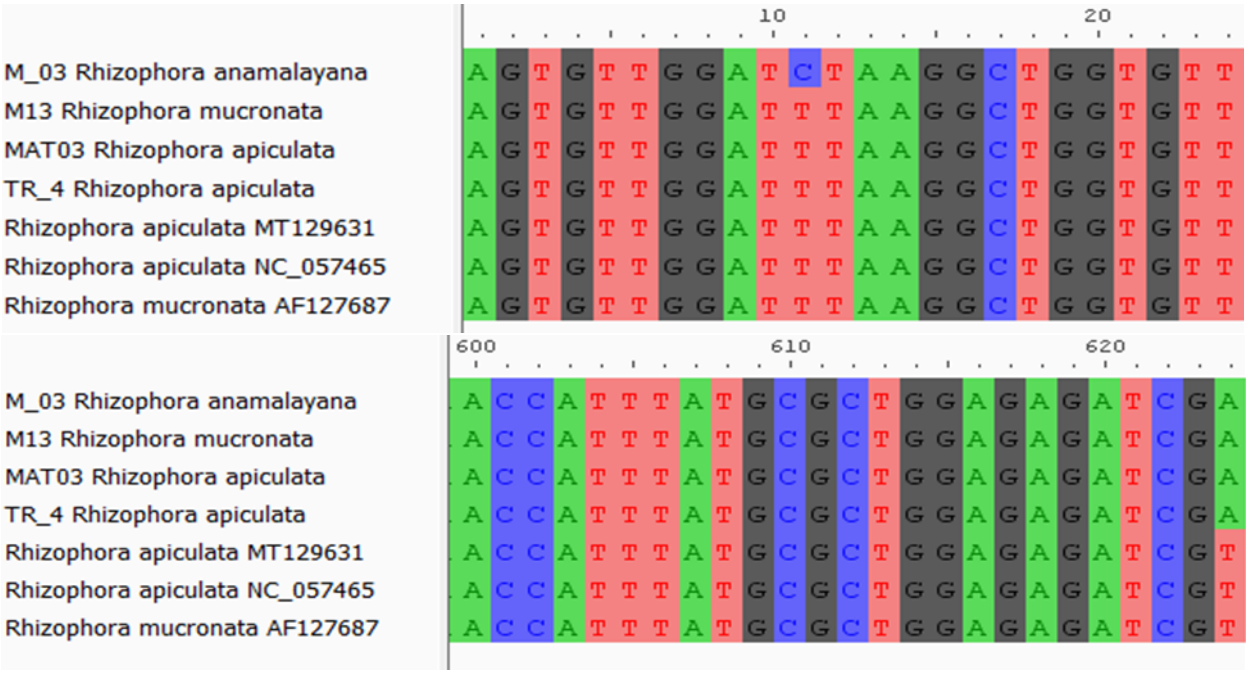


Figure 4: **Base pair difference comparison of *R annamalayana, R. apiculata* and *R. mucronata*. Total length of the alignment is 624bp**

annamalayana) were hitherto believed to be sterile due to poor pollination. Therefore, they require the presence of both parents in habitats at all times (Duke and Bunt 1979), and it is noted that *R. x annamalayana* can be observed in habitats where only *R. apiculata* and *R. mucronata* are present. The *R. x annamalayana* identification key developed by Setyawan *et al*. (2014) is currently used by many scientists in the world for the morphological classification of Rhizophora species. Also, among *R. apiculata* and *R. mucronata* plants in Sri Lanka, very few *R. annamalayana* plants can be observed only in Pambala Lagoon. This *R. annamalayana* is cladded in the *Rhizophora* clade and can therefore be considered a hybrid of *R. apiculata* and *R. mucronata.* As this plant has not been found in other parts of Sri Lanka till now, it can be described as a natural hybrid that has originated in Sri Lanka as well as other natural hybrids in other parts of the world. The small number of plants here also indicates that the infertility of the plant's pollen hinders its reproduction.

As regards *Ceriops tagal*, the flora of the Trincomalee and Puttalam areas seems to be quite different. Therefore, further investigations should be done into this.
The species *Pemphis acidula* was observed only in Rumassala, Galle and Puttalam and even in Galle it was observed in a small area of about 20 m^2. Meanwhile, *Lumnitzera littorea* was found only in the Madhu river area. A few plants of *Heritiera littoralis* were found in Trincomalee, Kadolkale and Kaluwamodara. More restoration and conservation activities should be done for these rare plants.

In addition, our results revealed that, the mangrove plants *Bruguiera cylindrica*, *Sonneratia alba*, *Xylocarpus granatum* are very rare and should be conserved.

CONCLUSIONS

Plant gene *rbcL* or ribulose-bisphosphate carboxylase gene can be successfully used to develop a phylogenic trees to confirm taxonomic levels of mangroves in Sri Lanka. The mangrove community in Sri Lanka consists of 21 mangrove species and *R. annamalayana* can be considered a hybrid of *R. apiculata* and *R. mucronata*. Our results can be used as baseline data for the classification of mangroves in Sri Lanka and will be helpful for future mangrove conservation and management activities in Sri Lanka.

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AUTHOR CONTRIBUTION

HNY and PPWA- designing the research, interpreting, summarizing, writing and revising the manuscript. VAJ and ARI- analyzing and interpreting data. PMMK and LPK – sample collection, preparation and identification.

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