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Phylogeny of Indian Rhizophoraceae Based on the Molecular Data from Chloroplast $tRNA^{LEU}_{UAA}$ Intergenic Sequences

S. Gurudeeban, K. Satyavani and T. Ramanathan
Marine Floral Biotechnology Laboratory, Centre of Advanced Study in Marine Biology,
Faculty of Marine Sciences, Annamalai University, Parangipettai 608502, Tamil Nadu, India

Abstract: Molecular identification data of unexplored Indian Rhizophoraceae an eco-friendly mangroves are an imperative aspect in molecular phylogenetics. We describe the phylogenetic relationships among the Rhizophoraceae genus *Rhizophora*, *Ceriops* and *Bruguiera* using tRNA Leu (UAA) intron sequences as a molecular marker. The results of present study reveals congeneric relationship between *R. apiculata*, *R. mucronata*, *B. gymnorhiza* indicating a high degree of gene flow within them and the pairwise distribution of study plants among Rhizophoraceae family. The phylogram constructed using tRNA Leu sequence clearly clustered the species of the same genus in individual group. The stem-loop could be divided into two classes, both built up from two base pairing heptanucleotide repeats. Size variation was primarily caused by different numbers of repeats but some strains also contained additional sequences in this stem-loop. The statistical summaries of DNA sequence data can enable to identify the structural signature of the genome as well as to classify phylogenetic relationships among different species reflected in the difference of genetic diversity distributions within their DNA sequences.

Key words: cpDNA, trnL intron, woody halophyte, phylogeny

INTRODUCTION

Mangroves are woody halophytes found in the intertidal zone of the tropical and subtropical coastlines of the world and spread over an area of 4,639 km^2 in India. Rhizophora is widely distributed in all major mangrove formations along the Indian coast. Genetic studies of this species limited to reports on chromosome number (Lakshmi *et al*., 2000). Molecular methods are important tools for identifying appropriate population sources for reforestation of these unique and important habitats. In order to elaborate a conserving and establishing a strategy of these spontaneous phytogenetic resources, we interested in the analysis of the genetic variation in *Rhizophora apiculata* and *Rhizophora mucronata*. The geomorphic changes in coastal regions and reproductive systems are important factors of mangrove distribution and genetic structure of the population. Also, several mangroves have important morphological and physiological traits such as viviparous propagules, aerial roots and salt tolerance believed to adapt to severe coastal environments (Inomata *et al*., 2009). Revealing the genetic structure of mangrove species provides useful information not only for managing the mangrove forests but also for the understanding of evolutionary forces leading to the present biodiversity and adaptation (Tana *et al*., 2005). TrnL intron has universal primers and used for phylogenetic studies among closely related genera and species of plants (Gielly and Taberlet, 1996). The trnL intron was the first group I intron positioned between the U and the A of the UAA anticodon loop described in chloroplast DNA (Simon *et al*., 2003). Group I introns are self splicing ribozymes from pre-RNA, encodes conserved primary and secondary structures required for autocatalysis. In plants, the trnL intron usually shows sequence conservation in the regions flanking both trnL exons, whereas the central part is highly variable (Bonnard *et al*., 1985). Secondary structural elements have not been found within the intergenic spacer between trnL (UAA) 3'exon and trnF (GAA) exon that could serve as splicing points. Analyzing the secondary structure in the spacer region and the trnL intron P8 loop is useful in studying phylogenetic relationships (Taberlet *et al*., 2006). The number of trnL intron sequences available in databases is the maximum available non coding chloroplast DNA sequences in the database. Primers developed for this region are highly conserved from Bryophytes to Angiosperms. Analysis of non-coding sequences of chloroplast DNA, which exhibit elevated mutation rates

Corresponding Author: T. Ramanathan, Faculty of Marine Sciences, Annamalai University, Parangipettai, Tamil Nadu, India
Tel: 04144-243223/213 Fax 04144-243555

than coding regions, can increase the resolution and permits assessment of phylogenetic relationships at the intrageneric and even at the intraspecific level (Clegg, 1993). Moreover the trnL intron represents the most variable non-coding region of chloroplast DNA and shows polymorphic phylogeny in various Rhizophora species (Lakshmi *et al*., 2002; Gurudeeban *et al*., 2012). The present investigation exposed the chloroplast DNA tRNA Leu (UAA) intron sequence instructive to evaluate phylogenetic relationships of the Indian Rhizophoraceae species.

MATERIALS AND METHODS

Chemicals: The experimental chemicals, fine chemicals were obtained from Sigma (USA) and primers procured from Helini Biomolecules, India.

Plant material and genomic DNA isolation: Plant species *Rhizophora apiculata*, *Rhizophora mucronata*, *Ceriops decandra*, *Ceriops tagal* and *Bruguiera gymnorhiza* were freshly collected from Southeast coast of India during the monsoon season month in 2010 (Table 1). Fresh and tender leaves used for DNA extraction using Pure Fast® Plant Genomic DNA purification kit, followed by the method of Gurudeeban *et al*. (2011).

PCR amplification and sequencing: Twenty five microlitter of PCR-Master mix contains 10X Taq buffer, 2 mM $MgCl_2$, 0.4 mM dNTPs mix and 2 U Proofreading Taq DNA polymerase, 1 μL Chloroplast trnL specific primer-forward (10 pmoles μL^{-1}) and reverse each and 22 μL of nuclease free water. The amplifications were carried out using a PCR thermal cycler (Lark, India). Initial denaturation was for 3 min at 94°C followed by 30 cycles of 1 min at 94°C, 1 min at 58°C, 1 min at 72°C and a 5 min final extension step at 72°C. The success each PCR reaction was verified by electrophoresis. The amplified products were loaded in a 2% agarose gel (2 g agarose in 100 mL of 1X TAE buffer) containing 5 μL of ethidium bromide. PCR Samples are loaded after mixed with gel loading dye along with 10 μL Quick Ref 250 bp DNA Ladder. Custom decanucleotide primers were synthesized from m sec^{-1} Helini Biomolecules, India.

The primer had the following sequences:

Forward primer: 5'-CGAAATCGGTAGACGCTACG-3'
Reverse primer: 5'-GGGGATAGAGGGACTTGAAC-3'

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.

Analysis of sequence data: An electropherogram generated by the automated DNA sequencer and the sequences were carefully checked for mismatch and bases spacing. 56 barcode sequences of Rhizophoraceae extracted through FASTA format from NCBI. CLUSTAL X 2.0.6 was used to align the nucleotide sequences (Thompson *et al*., 1994). The default parameters for gap opening and gap penalty and subsequently estimated the GC content of all 56 decades estimated by using the Bio Edit (Hall, 1999). Gap position and regions that could not be aligned unambiguously excluded from the analysis. Sequence data were submitted to Gene Bank. MEGA 5.0 was used to construct phylogenetic trees through Neighbor-Joining using Kimura 2-parameter and to calculate genetic distances of the given set of sequences (Tamura *et al*. 2007). The trn L sequence of *Avicennia marina* (AY008821) from NCBI used as an out group in phylogenetic tree construction.

Intron folding: Secondary structures of RNA are less complex than protein structures. But the single stranded RNA structures are often stabilized hydrogen bonds (Zamaratski *et al*., 2001). MFold Version 2.3 was used is a distinguished tool used for predicting the secondary structure. Default settings were used, except for the temperature parameter, which was set to 208°C (Zucker, 1989). RNA secondary structure plays an important role in evolution of microorganisms, plants and animals (Simmonds and Smith, 1999). Prediction of RNA folding of the conserved domains of mangrove Rhizophoraceae was completed using the online MFold package. MFold is widely used algorithms for RNA secondary structure prediction, which are based on a search for the minimal free energy state. The algorithm

Table 1: Collection of Indian mangrove Rhizophoraceae from different parts of India

Name of the plant species and No.	Herbarium voucher No.	Region	Year	Collector
Rhizophora apiculata (N = 9)	AUCASMB10	Vellar and Coleroon river, Pichavaram Thondai Nadu	2011	S.G and K.S
Rhizphora mucronata (N = 9)	AUCASMB11	Vellar and Coleroon river, Pichavaram Thondai Nadu	2011	S.G and K.S
Bruguiera gymnorhiza (N = 9)	AUCASMB14	Brahmani and Baitarani rivers, Bhitarkanika, Odisa	2011	T.R
Ceriops decandra (N = 9)	AUCASMB06	Vellar and Coleroon river, Pichavaram Thondai Nadu	2011	S.G and K.S
Ceriops tagal (N = 9)	AUCASMB16	Brahmani and Baitarani rivers, Bhitarkanika, Odisa	2011	T.R

S.G: S.Gurudeeban, K.S: K. Satyavani, T.R: Ramanathan

allows structures to be removed in later stages of the simulation if other pairings are found to be more favorable. In addition to that chance of growing new stems and permits the prediction of definite tertiary interactions.

RESULTS AND DISCUSSION

Mangrove plants are the sources of bio-active substances also produce large amounts of secondary metabolites valuable for human being and habitat of marine organisms. Thus, while working with a leaf sample enriched with polysaccharides, polyphenols and other secondary metabolites in the lysate and the DNA preparations. The secondary compounds may impede DNA isolation as well as any further reaction to be carried out on DNA preparations. The amplified product is having a molecular weight of around 550 to 1000 bp. The resulting PCR-RFLP analysis reveals trnL primers are specific to species identification and differentiation (Fig. 1). The successful double stranded amplification and partial sequences obtained from the study plants, the size of the tRNA Leu (UAA) intron ranging from 587 bp for *Rhizophora mucronata* (GenBank, ACC. No. JF318960), 538bp for *Rhizophora apiculata* (GenBank, ACC. No. JF318959), 550bp for *Ceriops decandra* (GenBank, ACC. No. JN871232), 552 bp for *Ceriops tagal* (GenBank, ACC. No. JN882013) and 554bp for *Bruguiera gymnorhiza* (GenBank, ACC. No. JN882014). The percentage of GC content in *Rhizophora apiculata* and *Rhizophora mucronata* and sequences retrieved from NCBI showed in the Table 2. The base composition of translational stop codons is biased toward a low GC content, a different density of these termination signals is expected in random DNA sequences of different base compositions. The expected length of reading frames in random sequences is thus a function of GC content (Oliver and Marin, 1996). GC content is also vastly sealed in the plastomes of land plants and is typically in the range of 30-40%, with a GC content being lower in noncoding intergenic regions than in coding regions (Bock, 2007). The total GC was higher in *Rhizophora mucronata* compared to *Rhizophora apiculata*, *Ceriops decandra*, *Ceriops tagal* and *Bruguiera gymnorhiza* it reveals a property of noncoding tRNA Leu spacer region.

Multiple sequences of *R. apiculata*, *R. mucronata, Ceriops decandra*, *Ceriops tagal* and *Bruguiera gymnorhiza* nucleotide sequences compared with similar sequences of Rhizophora species retrieved from NCBI using Clustal W (Fig. 2). The results are revealing 90% of the base pairs present in both *R. apiculata* and *R. mucronata*, the remaining 10% has been deleted/varied

Table 2: tRNA-Leu (UAA) sequences of various species of Rhizophoraceae from different countries used to construct the phylogram in the present study

Species	No. of sequences used	Country	G+C content (%)
Ceriops tagal	17	India and China	29.96
Ceriops australis	13	Taiwan, China	29.58
Ceriops zippeliana	13	Taiwan, China	27.44
Ceriops decandra	8	India and China	28.30
Rhizophora mucronata	2	India and China	31.98
Rhizophora apiculata	2	Indiaand China	29.54
Bruguiera gymnorhiza	2	Indiaand China	30.40
Rhizophora mangle	1	Germany	32.09
Ceriops sp. KD Rh-74	1	Taiwan, China	27.34
Ceriops sp. KD Rh-75	1	Taiwan, China	27.34
Ceriops sp. KD Rh-76	1	Taiwan, China	29.25
Ceriops sp. KD Rh-77	1	Taiwan, China	28.19
Ceriops sp. KD Rh-84	1	Taiwan, China	27.34
Carallia brachiata	1	Taiwan, China	29.87
Kandelia obovata	1	Taiwan, China	29.74

60 sequences belonging to 12 species representing 5 genera of Rhizophoraceae

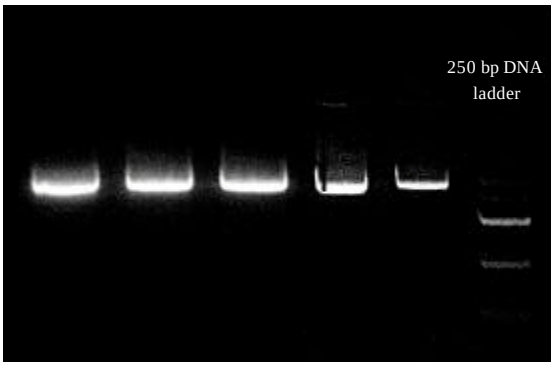

Fig. 1: RFLP analysis of genomic DNA of Rhizophoraceae species using trnL sequences compared with DNA ladder

due to the geographical distribution of the plants. Two phylogenetic trees constructed to confirm differentiation in species level. The first tree constructed to test the efficacy of trnL in delineating the members of *Rhizophora* sp. to its species level in Fig. 3. The trnL sequence of *Avicennia marina* (Avicenniaceae) from the NCBI database used as an out group and this has been clearly distinguished as an out group in the phylogenetic tree. Unlike the UPGMA algorithm for tree reconstruction, neighbor-joining does not assume that all lineages evolve at the same rate and produces an unrooted tree. Rooted trees would be created by using the out group and the root can then effectively be placed on the point in the tree where the edge of the out group connects. The minimum-evolution criterion analyzed by the Neighbor-Joining method and the result reveals (Fig. 4), the topology that gives the least total branch length of Rhizophoraceae

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Multiple sequence alignment of Rhizophoraceae
AUCASMB06   --------GGATTGAGCCTTGGTATGGAAACTTACTAAGTGATAACTTTCAAATTCAGA                51
AUCASMB14   GACTTAATTGGATTGAGCCTTGGTATGGAAACTTACTAAGTGATAACTTTCAAATTCAGA              60
AUCASMB16   ----------TTGAGCCTTGGTATGGAAACTTACTAAGTGATAACTTTCAAATTCAGA                48
AUCASMB10   ---------AACATCCCCTT----------TTTTTTAAGAAAAAA                            26
AUCASMB11   -----GGTTGAATTAACCTTGGGGAGAGAAAAAATTAAATGAAACTTTTTCAATGCAGA              54
:.******** *.:.*.*
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Fig. 2: Clastal W Multiple sequence alignment of Rhizophoraceae tRNA Leu introns showed moderate difference in the nucleotide level indicated by .: symbols

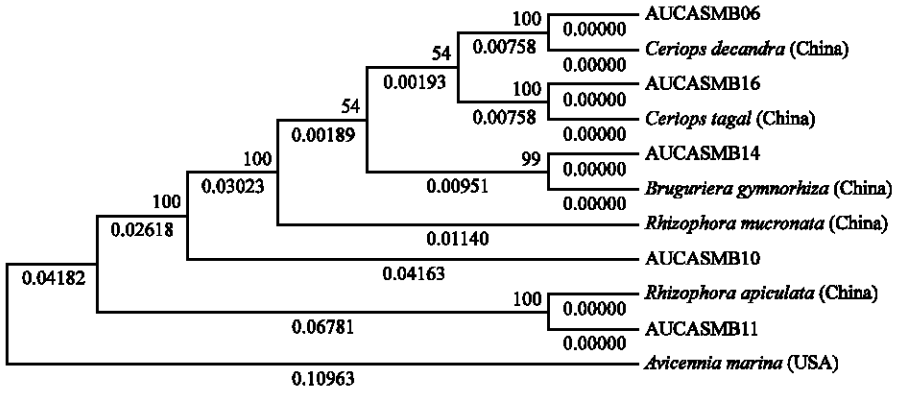

Fig. 3: Phylogram of UPGMA algorithms to test the efficacy of tRNA Leu gene in delineating the members of Rhizophoraceae to its species level. *Avicennia marina* (Avicenniaceae) from NCBI data base was used as an out group

Table 3: Average inter-generic variations determined in trnL of Rhizophoraceae family

	Bruguiera	*Carallia*	*Rhizophora*	*Kandelia*	*Ceriops*
Bruguiera					
Carallia	0.9205				
Rhizophora	0.9303	0.8472			
Kandelia	0.5251	0.8650	0.9003		
Ceriops	0.5297	0.8359	1.0056	0.5445	

Genomic DNA of Rhizophoraceae species

preferred at each step of the algorithm. The pairwise comparisons among dicots and monocots for rbcL and noncoding sequences of cpDNA the trnL (UAA) intron regions evolve faster than rbcL, as previously reported (Gielly and Taberlet, 1994). In Table 3, the genetic distance within and between the species and genera of family Rhizophoracea calculated using pairwise distance analysis via Maximum likelihood method. The intraspecies genetic distance within the members of Rhizophoracea found low due to its recent divergence from other members of the family. The intra specific variation of the genus Rhizophora was found higher. The overall genetic distance of Rhizophoraceae family found 1.221.

The alignment of Rhizophoraceae tRNALeu (UAA) introns exposes greater sequence similarity. Sequence disparity is mostly restricted to certain regions that, when the alignment is compared with the secondary structure predictions, are localized in some of the loops or hairpin structures. All size variations within the genus of the Rhizophoraceae family can be found in the hairpin by structural element P6b. The nature of the size variation in Rhizophoraceae will be discussed subsequently. Size variations in hairpin extensions are crucial when considering aligning introns of different lengths in order to obtain a correct alignment of homologous positions. All the five species share a highly conserved intron sequence having only few variable positions. With one exception, a base pair in element P9, all these variable positions also show variability when different species from different subsections are compared. Also, we determined tRNA Leu folded structure of *R. apiculata*, *R. mucronata*, *C.dencandra*, *C. tagal* and *B. gymnorhiza* with loop free energy decomposition -113.70, -127.80, -113.20, -106.80 and -118.90, respectively.

Intron evolution: MFold Version 2.3 was used is a distinguished tool used for predicting the hairpin structure formed by the first variable region of the intron in the Rhizophoraceae species (Fig. 5). The trnL (UAA) intron has been important in this debate. In contrast to

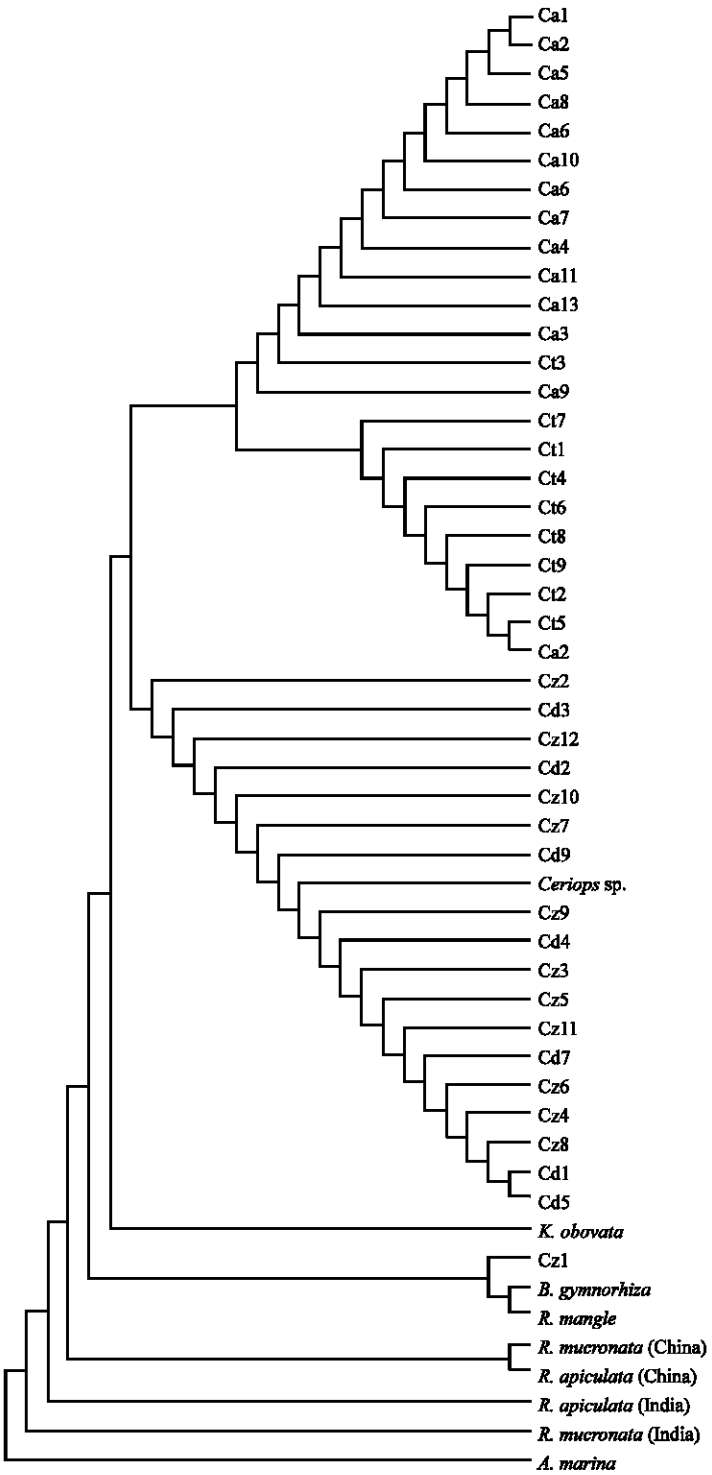

Fig. 4: The minimum-evolution criterion of Rhizophoraceae was analyzed using Neighbor-Joining method. *Avicennia marina* (Avicenniaceae) from NCBI data base was used as an out group

other bacterial introns interrupting tRNA genes, the tRNALeu (UAA) intron has features expected for an evolutionarily old genetic element. The tRNALeu (UAA) introns from plant chloroplasts are more closely related to

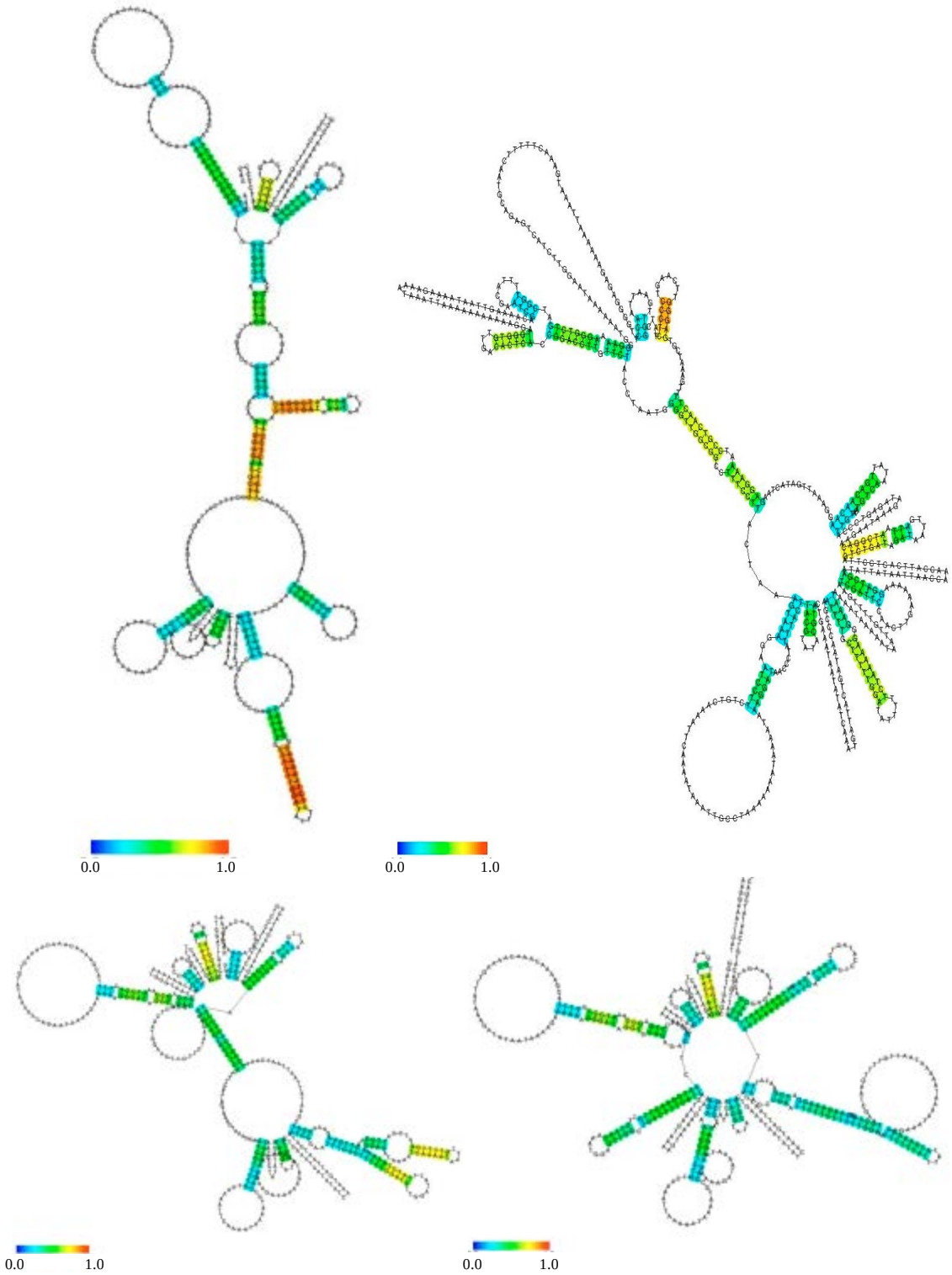

Fig. 5: Continue

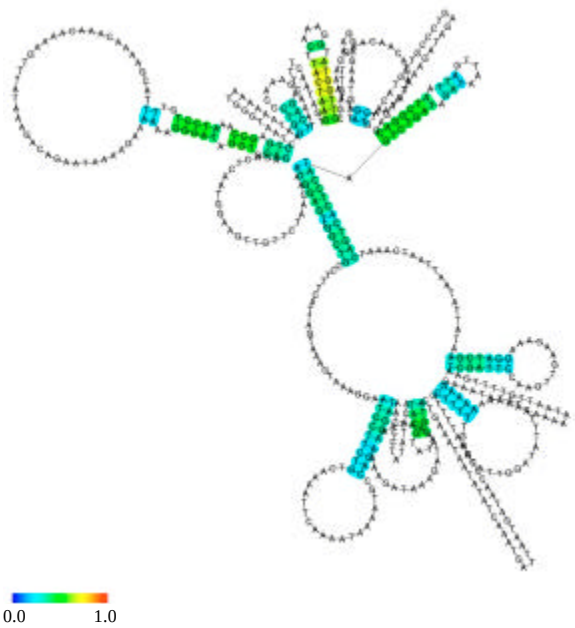

Fig. 5: tRNA Leu folded structure of *R. apiculata*, *R. mucronata*, *C.dencandra, C. tagal* and *B. gymnorhiza* with loop free energy decomposition -113.70, -127.80, -113.20, -106.80 and -118.90, respectively

each other than with any other introns. Their position between the second and third base of the anticodon is conserved, which also implies evolutionary age. The absence of the intron in some plant species would be explained by a loss of the intron (Besendahl *et al*., 2000).

CONCLUSION

The present analysis showed the close congeneric relationship among Rhizophoraceae indicating a high degree of gene flow within them and they do not support differentiation at species level. Until new phylogenetic groups are fully identified and implemented, the present species status should preserve to minimize risks of loss of the important component of wetland floral biodiversity. Our results confirm the idea that tandemly repeated tRNA gene can be used as taxonomic and phylogenetic markers. According to the comparative analysis of tRNALeu (UAA) intron sequences in the databases we found interesting evolutionary patterns. Single nucleotide differences are, as expected, restricted by the secondary structure of the intron. This is seen in different ways: (1) Different stem loops contain different degrees of variation and (2) Much of the variation retains the secondary structure in such a way that nucleotides in base pairing positions only change to a new base pairing nucleotides. When analyzing size differences between closely related Rhizophoraceae sequences, these were found to be caused primarily by different numbers of copies of heptanucleotide repeats and, in some cases, because of additional sequences. The tRNA Leu sequence in the phylogram constructed clearly clustered the species of the same genus in individual group.

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