ELSEVIER

Aquatic Botany 74 (2002) 201–217

Aquatic botany

www.elsevier.com/locate/aquabot

MOLECULAR PHYLOGENY OF MANGROVES IX
Molecular marker assisted intra-specific variation and species relationships in the Indian mangrove tribe Rhizophoreae

Mukkamala Lakshmi, Madasamy Parani, Ajay Parida*

M.S. Swaminathan Research Foundation, III Cross Street, Institutional Area, Taramani, Chennai 600113, India

Received 30 May 2001; received in revised form 1 May 2002; accepted 27 May 2002

Abstract

Molecular phylogeny and genetic diversity in all the nine species (*Rhizophora mucronata*, *R. apiculata*, *R. stylosa*, *Bruguira cylindrical*, *B. parviflora*, *B. gymnorriza*, *Ceriops tagal* and *C. decandra*) and a natural *Rhizophora* hybrid, of the mangrove Rhizophoraceae, occurring in the Indian sub-continent were analysed using random amplified polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP) and RFLP of polymerase chain reaction amplified chloroplast genes (PCR-RFLP) as markers. Intra-specific variability as revealed by RAPDs were low in all the analysed species. Inter-specific RFLP analysis revealed species-specific profiles in some probe-enzyme combinations. The rDNA repeat units, as flanked by the *Hind* III restriction sites was found to be very conserved within each genus and three different rDNA repeat units were observed among the four genera. Generic differences in PCR-RFLP were observed only in *rbc*L and *trn*S-*psb*C gene regions. Species differences were observed in Rhizophora in the PCR-RFLP of *trn*S-*psb*C and *trn*L-UAA with *Hae* III and *Taq* I, respectively. *Rhizophora mucronata* was found to be the chloroplast donor for the natural inter-specific hybrid. A dendrogram based on the data sets from all the three marker systems revealed that the four genera segregated into three species groups.

Keywords: Rhizophoraceae; Mangrove; Molecular markers; Phylogeny; Conservation

* Corresponding author. +91-44-254-1229/1698; fax: +91-44-254-1319.
E-mail addresses: pmb@mssrf.res.in (M. Parani), ajay@mssrf.res.in (A. Parida).

0304-3770/02/$ – see front matter © 2002 Elsevier Science B.V. All rights reserved.
PII: S0304-3770(02)00105-5

1. Introduction

The coastal wetlands of the tropics and the subtropics of the world are characterised by the presence of a unique group of plant species, the mangroves. There are about 70 species of true mangroves of which about 65 contribute significantly to the structure of mangrove formation (Spalding et al., 1997). Of these, the members of the tribe Rhizophoreae of the family Rhizophoraceae are the dominant species inhabiting all types of mangrove formation. This tribe is generally referred to as the mangrove Rhizophoraceae and includes four genera e.g. *Rhizophora*, *Ceriops*, *Bruguiera*, and *Kandelia*. These species are classified as true mangroves owing to their fidelity to the mangrove ecosystem such as presence of adaptive features like aerial roots (e.g. knee roots in *Ceriops* and *Bruguiera*) and viviparous mode of reproduction (Tomlinson, 1986). There are 14 species and three hybrids in Rhizophoreae; of which 9 species and one natural inter-specific hybrid occur in India (Spalding et al., 1997). The natural hybrid found in the Pichavaram mangrove forest has variously been described as *Rhizophora* × *lamarkii* (Spalding et al., 1997), *Rhizophora anamalai* (Kathiresan, 1995) and *Rhizophora* hybrid (Parani et al., 1997a, 1997b). In the present communication, we describe the taxa included in the present study as *Rhizophora* hybrid. Parani et al. (1997a, 1997b) has described the morphological features of the hybrid and two other *Rhizophora* species occurring in Pichavaram mangrove forest. Representative genotypes of these taxa are being maintained in the in situ conservation center at Pichavaram, Tamil Nadu, India, and voucher specimens are available at the Indian Herbarium collections of Botanical Survey of India as well as at the M.S. Swaminathan Research Foundation, Chennai.

Taxonomy of the tribe Rhizophoreae, like in most other mangrove species, has been primarily based on morphological characters and in many instances these characters tend to overlap (Tomlinson, 1986) making the species distinction difficult. The earlier work on these species has been reports of isozyme study between species of *Ceriops* (Ballment et al., 1988) and different genotypes of *Kandelia* (Sun et al., 1998). In a recent report, Schwarzbach and Ricklefs (2000), have analysed the systematic affinities of Rhizophoraceae and Anisophylleaceae, and inter-generic relationship within Rhizophoraceae, using six molecular data sets, both chloroplast and rDNA sequences from GenBanks. This study provides Information on the inter-generic diversity, however, no attempt was made to understand the nature and extent of intra-specific and inter-and intra- population diversity.

The present investigation, therefore, was designed to study the genetic diversity and species relationship among Rhizophoraceae species at both intra- and inter-population as well as species levels using molecular markers based analysis. DNA based molecular markers unlike morphological markers are considered as stable and influenced very little by environmental fluctuations (Gottlieb, 1977; Hardrys et al., 1992; Williams et al., 1990). DNA based markers such as random amplified polymorphic DNA (RAPD) and restriction fragment length polymorphism (RFLP) have been widely used in a number of plant groups for variety of purposes such as cultivar identification, diversity studies, parentage determination, developing breeding programmes and conservation strategies. Similarly, markers specific to ribosomal DNA and chloroplast genes have provided reliable information for the analysis of genomic relationships above the level of species owing to their highly conserved

nature. Nuclear ribosomal RNA genes (rDNA) and PCR-RFLP of chloroplast gene region (Zimmer et al., 1988; Wagner et al., 1987; Palmer and Zamir, 1982; Tsumura et al., 1995, 1996) have also been extensively used in taxonomic studies. The present communication details out the results of the analysis of genetic diversity and species relationship in all the nine species and a natural inter-specific hybrid of the tribe Rhizophoreae found in India.

2. Material and methods

Young leaves from nine species and a hybrid of the tribe Rhizophoreae were collected from different natural populations along the Indian coast and the Andaman and Nicobar islands. Species analysed, sites of collection and their geographical locations are given in Table 1. A total of 290 genotypes belonging to 10 species of Rhizophoreae were studied for intra-specific genetic diversity using RAPD markers. Based on these results, genotypes from each species were selected and analysed for species relationships using RAPD, RFLP and PCR-RFLP markers.

2.1. Genomic DNA isolation

The high amounts of secondary metabolites and polysaccharides present in the leaf tissues of all the members of Rhizophoraceae make the extraction of DNA difficult. Therefore, a modified protocol using cetyl-trimethyl ammonium bromide (CTAB) was used. About 5 g of leaf tissue was ground in liquid nitrogen and suspended in 15 ml of 2% CTAB buffer (2% CTAB; 0.1 M Tris (pH 8.0); 20 mM EDTA; 1.4 M NaCl; 2% PVP-40; 1% β-mercaptoethanol).The suspension was incubated at 60 °C for 15 min with gentle mixing and extracted with equal volume of chloroform: isoamyl alcohol (24:1). To the aqueous phase, 0.2 volume of 5% CTAB (5% CTAB; 0.1 M Tris (pH 8.0); 20 mM EDTA; 1.4 M NaCl; 1% PVP-40) was added and extracted with equal volume of chloroform: isoamyl (24:1). The aqueous phase was transferred to a 30 ml tube and the DNA was precipitated with 0.6 volume of cold isopropanol (−20 °C) and stored at −20 °C for 1 h. The sample was centrifuged to recover the nucleic acids and was dissolved in TE buffer (10 mM Tris–HCl, 1 mM Na_2 EDTA, pH 8.0) and subsequently treated with RNase A. The sample was then purified by phenol:chloroform:isoamyl alcohol (25:24:1) and chloroform extractions (Sambrook et al., 1989) and precipitated with ethanol. The pellet was air dried and dissolved in TE buffer. The DNA concentration was estimated by agarose gel electrophoresis and ethidium bromide staining.

2.2. Random amplified polymorphic DNA (RAPD) analysis

Polymerase chain reaction (PCR) amplification with random primers and minisatellite primers was carried out in 25 μl volume containing 10–15 ng of genomic DNA, 2.5 μl of 10× *Taq* DNA polymerase assay buffer, 0.5 μl of 25 mM $MgCl_2$, 0.5 μl of 10 mM dNTPs (USB), 15 ng of primer and 1.0 unit of *Taq* DNA polymerase. The reaction mix was overlaid with equal volume of mineral oil. PCR amplification was carried out in a DNA thermal cycler (Perkin-Elmer 480). The first cycle of the amplification consisted of a initial denaturation

Table 1
Details of the species used for the analysis

Genus	Species	Number of genotypes analysed	Site of collection	Remarks
Rhizophora L.	*R. apiculata* Blume	60	Pichavaram (11°27′N; 79°47′E); Bhitarkanika (20°40′N; 86°52′E); Muttupet (10°46′N; 79°52′E); Coringa (16°30′N; 82°20′E); Ratnagiri (17°08′N; 73°19′E); Goa (17°38′N; 73°52′E)	This genus comprised of eight species (including three putative hybrids), of which only four are found in India
	Rhizophora hybrid	10	Pichavaram	
	R. mucronata Lamk.	60	Pichavaram, Bhitarkanika, Muttupet, Coringa, Ratnagiri, Goa	
	R. stylosa Griff.	10	Andaman islands[a]	
Ceriops Arnold	*C. decandra*(Griff.) Ding Hou	40	Pichavaram, Bhitarkanika, Muttupet, Coringa	This genus comprised of two species; both of which are recorded in India
	C. tagal (Perr) C.B.Rob	20	Bhitarkanika, Coringa[b]	
*Bruguiera*Lamk.	*B. cylindrica* (L.) Bl.	40	Pichavaram, Bhitarkanika, Muttupet, Coringa,	This genus comprised of six species of which three species are found in India
	B. gymnorhhiza(L.) Lamk.	20	Bhitarkanika, Coringa[b]	
	B. parviflora wright and Arnold ex Griffith	20	Bhitarkanika, Coringa[b]	
Kandelia Wright and Arnold	*K. candel* L. Druce	10	Bhitarkanika[b]	This is a monotypic genus

[a] Species not recorded in the Indian mainland.
[b] Species not reported in Pichavaram & Muttupet mangrove forests.

Table 2
Details of the probes used for RFLP analysis

Serial number	Probe	Source	Size (kb)
1	*ver* 18	*Vicia faba*	3.7
2	*p*TA71	*Triticum aestivum*	8.95
3	18S *rrn*	*Avicennia marina*	1.8
4	26S *rrn*	*Zea Mays*	1.0
5	*ACP 21*	*A. ilicifolius*	2.0
6	*ACP 48*	*A. ilicifolius*	1.5
7	*BCP 01*	*B. cylindrica*	1.5
8	*RLE 01*	*R. apiculata*	2.0
9	*RLE 02*	*R. apiculata*	1.0
10	*CDP 10*	*C. decandra*	3.0

step of 3 min at 94 °C, 1 min at 37 °C (40 °C for $(GATA)_4$) and 2 min at 72 °C. This was followed by 44 cycles programmed for 1 min at 94 °C, 1 min at 37 °C and 2 min at 72 °C. An additional cycle of 15 min at 72 °C was used for primer extension. The amplification products were electrophoresed in 1.5% agarose gel in 1× TAE buffer. Out of 30 random primers (Kit A, and Kit D, Operon Tech. USA) and four minisatellite primers [$(GATA)_4$, $(GACA)_4$, $(CA)_8$ and $(AT)_8$] tested for amplification, only 11 random primers and the minisatellite primer, $(GATA)_4$ which gave informative and reproducible amplification across all the species were included in the data analysis.

2.3. Restriction fragment length polymorphism (RFLP)

For RFLP studies the genomic DNA were digested with three restriction enzymes e.g. *Eco*R I, *Hind* III and *Dra* I, and polymorphisms were scored using six random genomic sequences and four rDNA sequences as probes (Table 2). About 10 μg of DNA from each sample was digested with restriction enzymes according to the manufacturer's instructions (Amersham-Pharmacia, UK). The digested DNA was electrophoresed on a 1% agarose gel in 1× TAE buffer along with a size marker and transferred onto nylon membrane (Hybond N+, Amersham-Pharmacia, UK) by Southern transfer (Southern, 1975). The membranes were pre-hybridised in pre-hybridisation solution (6× SSC, 5× Denhardt's reagent, 0.5% SDS, and 100 μg/ml denatured salmon sperm DNA), and hybridised in the same buffer (Sambrook et al., 1989) with random prime labelled (Rediprime Labelling Kit, Amersham) probes overnight at 60 °C. After overnight hybridisation, the membranes were washed once in 2× SSC plus 0.1% SDS and 1× SSC plus 0.1% SDS at room temperature for 15 min, followed by once in 1× SSC plus 0.1% SDS, 0.5× SSC plus 0.1% SDS and 0.1× SSC plus 0.1% SDS at 60 °C for 15 min. The membranes were exposed to X-ray film with intensifying screens at −70 °C overnight.

2.4. PCR-RFLP of chloroplast genes

For PCR-RFLP studies, four chloroplast DNA regions consisting of the coding region for the large subunit of RuBisCO (*rbc*L), the coding and intergenic spacer sequences of

Table 3
PCR primer pairs used to amplify the chloroplast gene regions and the size of the amplified products

Gene	PCR primers	Expected size (bp)	Reference
*rbc*L	5′ TGTCACCAAAAACAGAGACT 5′ TCCATACTTCACAAGCAGC	1382	Hipkins et al. (1990)
*trn*S-*psb*C	5′ GGTTCGAATCCCTCTCTCTC 5′ GGTCGTGACCAAGAAACCAC	1600	Demesure et al. (1995)
16S	5′ ACGGGTGAGTAACGCGTAAG 5′ CTTCCAGTACGGCTACCTTG	1375	Shinozaki et al. (1986)
*trn*L-UAA	5′ CGAAATCGGTAGACGCTACG 5′ GGGGATAGAGGGACTTGAAC	400	Taberlet et al. (1991)

tRNA-serine gene and the adjacent PSII 44 kDa gene regions (*trn*S-*psb*C), the coding region for 16S rRNA (16S) and the coding region for tRNA-lysine (*trn*L-UAA) were amplified by polymerase chain reaction (PCR) using specific primer pairs (primer sequences refer Table 3) at appropriate annealing temperatures (63 °C for *rbc*L, 60 °C for *trn*S-*psb*C and 55 °C for 16S and *trn*L-UAA). The amplified PCR products were separated by agarose gel (1.2%) electrophoresis in 0.5× Tris–borate buffer and stained with ethidium bromide. About 15 μl of the PCR products were directly digested with 19 restriction enzymes (Table 6) following the manufacturer's instructions (Amersham-Pharmacia, UK). The restriction fragments along with 1 kb ladder marker DNA (Gibco-BRL, USA) were separated by agarose gel (1.5%) electrophoresis in 0.5× Tris–borate, and stained with ethidium bromide. PCR amplification and restriction of PCR products were repeated at least twice for all the samples. Amplification products as well as restriction patterns were found to be reproducible. Total size of the restricted products was always almost equal to the undigested PCR product. Wherever discrepancy was observed between the two, the PCR product was purified and then digested to identify the authentic restriction banding pattern.

2.5. Data analysis

The RAPD, RFLP, and PCR-RFLP bands were scored for presence (1) or absence (0) ignoring the intensity of the bands. The statistical analysis was carried out using NTSYS-pc software (Ver 1.8; Rholf, 1993). The fraction of common bands between two species (F) was computed according to method of Nei and Li (1979). Clustering was done following unweighted pair group with arithmetic mean average (UPGMA) method.

3. Results

3.1. Random amplified polymorphic DNA (RAPD) analysis

In all, 30 random primers and 4 minisatellite primers were tested for amplification of which 11 random primers and a minisatellite primer $(GATA)_4$ showed reproducible amplification in all the species. The details of the amplified loci and percentage polymorphism

Table 4
Percentage of polymorphism at intra- and inter-population level using 11 random and 1 minisatellite primer

Species	Total	Polymorphism (%)	
		Intra-population (Average S.D.±)	Inter-population
R. apiculata	77	17.29	32.46
R. hybrid	76	17.10	–
R. mucronata	78	18.85	33.33
R. stylosa	73	16.43	–
C. decandra	72	17.93	27.77
C. tagal	75	15.48	29.33
B. cylindrical	75	17.67	37.33
B. gymnorhiza	74	14.83	31.08
B. parviflora	73	16.76	32.67
K. candel	83	14.45	–

within and between the populations of each species are given in Table 4. The total number of amplification products within each species varied from 72 to 83. Intra-population variation ranged from 12–17% in *Bruguiera gymnorhiza* to 18–19% in *R. mucronata*. Of the six populations analysed in *R. apiculata* and *R. mucronata* lowest level of polymorphism (16.20 and 18.02%, respectively) was observed in the samples from Ratnagiri population. Muthupet population of *Ceriops decandra* and *B. cylindrica* showed the lowest percentage of polymorphism (6.23 and 16.38%, respectively). The Coringa population showed lower polymorphism in the three species, *C. tagal* (14.68%), *B. parviflora* (14.16%) and *B. gymnorhiza* (12.68%). The Bhitarkanika populations showed the highest variation in respective species (Table 4).

The percentage of polymorphic amplification products at inter-population level varied between 28% in *C. decandra* and 37% in *B. cylindrica* (Table 4). Analysis of polymorphism at inter-specific level showed that 72% of the RAPDs were polymorphic. Each primer showed at least one amplification product specific to each genus. Inter-specific and inter-generic RAPD profiles with four random primers are given in Fig. 1A–D.

3.2. Restriction fragment length polymorphism (RFLP) analysis

Genomic DNA from nine species and one hybrid were analysed for variations in RFLP using three restriction enzymes and 10 probes (4 ribosomal DNA and 6 genomic DNA probes). In total, out of the 167 fragments observed and 162 were polymorphic. Genus-specific RFLP profiles were observed with most of the enzyme-probe combinations with *Rhizophora* and *Kandelia* being distinct (e.g. 18S rDNA/*Eco*R I combination; Fig. 2a). Differences among the four genera were observed with the combination of *p*TA71 and *Dra* I. Species-specific profile was observed in the genus *Ceriops* with the combination of *ver* 18/*Eco*R I, wherein *C. tagal* showed a total of three fragments as compared to one fragment found in *C. decandra* (Fig 2b). The *Dra* I/*p*TA71 combination revealed species-specific RFLPs in *Rhizophora* and *Bruguiera* (Fig. 2c). The total number of fragments in three enzyme digests and the number of polymorphic ones within each genus (except for the montypic *Kandelia*) are given in Table 5. Polymorphism among the species of *Rhizophora*,

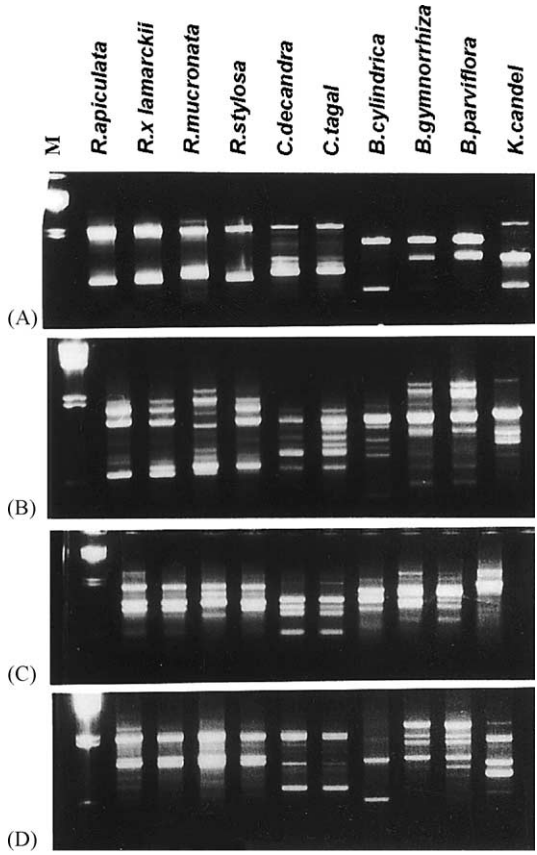

Fig. 1. Intra- and Inter-generic RAPD profiles of the 10 species of Rhizophoreae with four random primers (A) OPD 18; (B) OPD 20; (C) OPA 04; (D) OPA 01.

Ceriops and *Bruguiera* were observed to be 71.08, 12.24 and 52.38%, respectively. RFLP analysis of the genomic DNA with *p*TA71 probe revealed that the rDNA repeat units in all the species are flanked by the restriction sites for *Hind* III. The length of the rDNA repeat units as flanked by *Hind* III was estimated to be 10.2 kb in *R. mucronata*, 7.2 kb in *R. apiculata*, *Rhizophora* hybrid and *R. stylosa*, 10.2 kb in *Ceriops* and *Bruguiera* and 6.7 kb in *Kandelia candel*.

3.3. PCR-RFLP analysis

Amplification of the cpDNA regions using the respective primer pairs given in the Table 3 produced fragments without any size variation. The apparent size of the amplified fragments of *rbc*L, *trn*S-*psb*C, 16S and *trn*L-UAA were 1.4, 1.6, 1.3 and 0.4 kb, respectively. Nineteen restriction enzymes were tested for the presence of sites within the four amplified regions of the cpDNA. The details of the restriction enzymes used and the enzymes for which restriction sites were observed are given in Table 6.

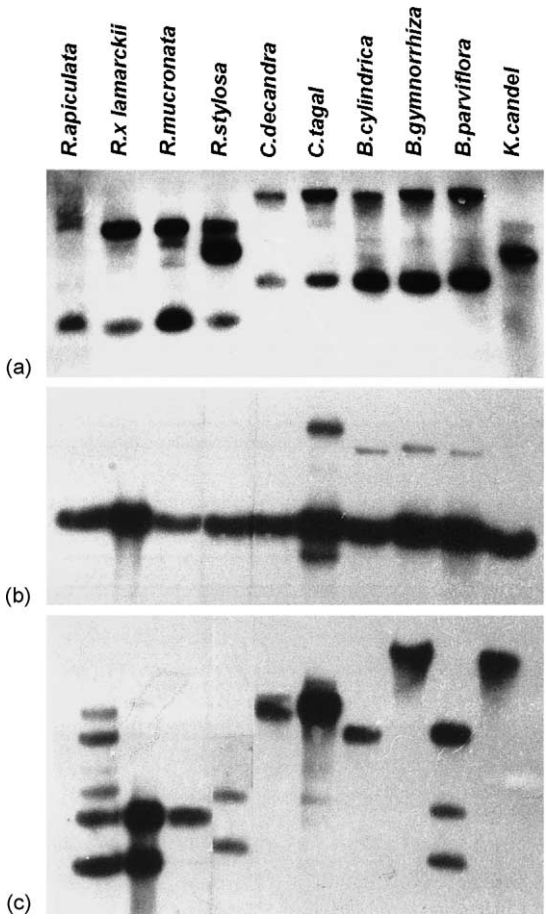

Fig. 2. Intra- and inter-generic RFLP profiles of the 10 species of Rhizophoreae with different probe/enzyme combination: (a) 18S rDNA/*Eco*R I; (b) *ver* 18/*Eco*R I; (c) *Dra* I/*p*TA71.

PCR-RFLP of *rbc*L showed the presence of restriction sites for 6 out of the 19 enzymes tested. Among these, *Hae* III, *Bgl* II and *Taq* I showed informative restriction profiles. While *Hae* III and *Bgl* II distinguished only the genus *Bruguiera* from the other genera, *Taq* I showed genus-specific profiles (Fig. 3b). PCR-RFLP of *rbc*L with *Sau*3A I, *Taq* I and *Hae* III is shown in Fig. 3. PCR-RFLP of *trn*S-*psb*C showed the presence of restriction sites for seven enzymes. While *Hind* III could distinguish only the genus *Bruguiera* from other genera, *Hae* III and *Taq* I showed genus-specific profiles (Fig 4a and b). *Hae* III also showed species-specific restriction patterns within the genus *Rhizophora* and the restriction pattern of the natural hybrid was similar to that of *R. mucronata*. *Pst* I and *Puv* II showed two restriction patterns and of which one was shared by *Rhizophora* and *Bruguiera* and the other was shared by *Ceriops* and *Kandelia*. Though *Sma* I did not have a site in *Ceriops* and *Kandelia*, it could clearly differentiate *Rhizophora* and *Bruguiera* with two and three restriction fragments, respectively (Fig. 4c)

Table 5
Total number and polymorphic RFLP at inter-specfic level in *Rhizophora*, *Ceriops* and *Bruguiera*

Probe	*Rhizophora*		*Ceriops*		*Bruguiera*	
	Total	%	Total	%	Total	%
*p*TA71	10 (8)	80	8 (2)	25	8 (6)	75
ver 18	6 (4)	66.6	5 (2)	40	10 (6)	60
18S	9 (6)	66.6	6 (0)	0	6 (2)	33.3
26S	11 (8)	72.7	6 (0)	0	9 (3)	33.3
BCP 01	15 (14)	93.3	7 (2)	28.5	10 (7)	70
CDP 10	4 (1)	25	6 (0)	0	6 (4)	66.6
ACP 21	5 (3)	60	4 (0)	0	7 (5)	71.4
ACP 48	6 (2)	33.3	2 (0)	0	2 (0)	0
RLE 01	9 (7)	77.7	3 (0)	0	3 (0)	0
RLE 02	8 (6)	75	2 (0)	0	2 (0)	0
Total	83 (59)	71.0	49 (6)	12.2	63 (33)	52.38

PCR-RFLP of 16S showed the presence of restriction sites for two enzymes and both of them were not informative as all the restriction products were monomorphic across all the species. PCR-RFLP of the *trn*L-UAA showed the presence of restriction sites for *Sau*3A I and *Taq* I. *Sau*3A I produced monomorphic pattern across all the species while *Taq* I showed species-specific profiles within the genus *Rhizophora* and the natural inter-specific hybrid was similar to *R. mucronata*, as observed from the PCR-RFLP of *trn*S-*psb*C with *Hae* III.

Table 6
Details of the presence of restriction sites for various enzymes in PCR-RFLP analysis of Rhizophoraceae

Serial number	Enzyme	Restriction site	*rbc*L	*trn*S-*psb*C	16S	*trn*L-UAA
1	*Bam*H I	G↓GATCC	–	–	–	–
2	*Bgl* I	GCCNNNN↓NGGC	–	–	–	–
3	*Bgl* II[a]	A↓GATCT	+	–	–	–
4	*Dra* I	TTT↓AAA	–	–	–	–
5	*Eco*R I	G↓AATTC	–	–	+	–
6	*Eco*R V	GAT↓ATC	–	–	–	–
7	*Hae* III	GG↓CC	+	+	+	–
8	*Hind* III	A↓AGCTT	–	+	–	–
9	*Kpn* I	GGTAC↓C	+	–	–	–
10	*Pst* I	CTGCA↓G	–	+	–	–
11	*Puv* II	CAG↓CTG	–	+	–	–
12	*Sac* I	GAGCT↓T	–	–	–	–
13	*Sal* I	G↓TCGAC	–	–	–	–
14	*Sau*3A I	↓GATC	+	+	+	+
15	*Sma* I[b]	CCC↓GGG	–	+	+	–
16	*Stu* I	AGG↓CCT	–	–	+	–
17	*Taq* I	T↓CGA	+	+	+	+
18	*Xba* I	T↓CTAGA	+	–	–	–
19	*Xho* I	C↓TCGAG	–	–	–	–

+: Presence of site for the enzyme; –: absence of site for the enzyme.
[a] *rbc*L/*Bgl* II: *Ceriops* and *Kandelia* lacked sites.
[b] *trn*S-psbC/*Sma* I: *Ceriops* and *Kandelia* lacked sites.

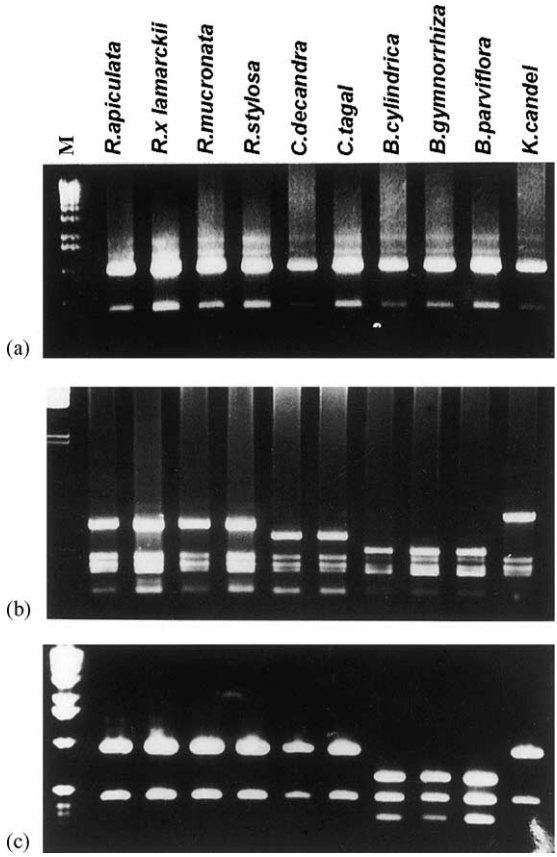

Fig. 3. PCR-RFLP of *rbc*L gene region of the chloroplast digested with enzymes: (a) *Sau*3A I; (b) *Taq* I; (c) *Hae* III.

3.4. Species relationships

The data scored for the presence and absence of bands in each marker system were used separately individual dendrograms were constructed. The clustering obtained in the three analyses remained the same, therefore, data from the three marker systems were pooled and a composite dendrogram was constructed and it is shown in Fig. 5. Clustering based on genetic distances, showed that the nine species and a natural hybrid of Rhizophoreae grouped into three distinct clusters. The four species of *Rhizophora* formed the first cluster wherein *the natural hybrid* was clustered to *R. apiculata* (distance of 0.42) and *R. stylosa* clustered with *R. mucronata* at 0.68. The two species groups formed a node at 0.77. The two species of *Ceriops* showed minimal distance of 0.09 and formed the second cluster along with *Kandelia*. The three species of *Bruguiera* formed the third cluster. *B. gymnorhiza* and *B. parviflora* formed a node at 0.25 to which *B. cylindrica*was clustered at 0.32. The *Rhizophora* complex was closely related to *Ceriops-Kandelia* cluster (1.032) with *Bruguiera* cluster forming an outgroup.

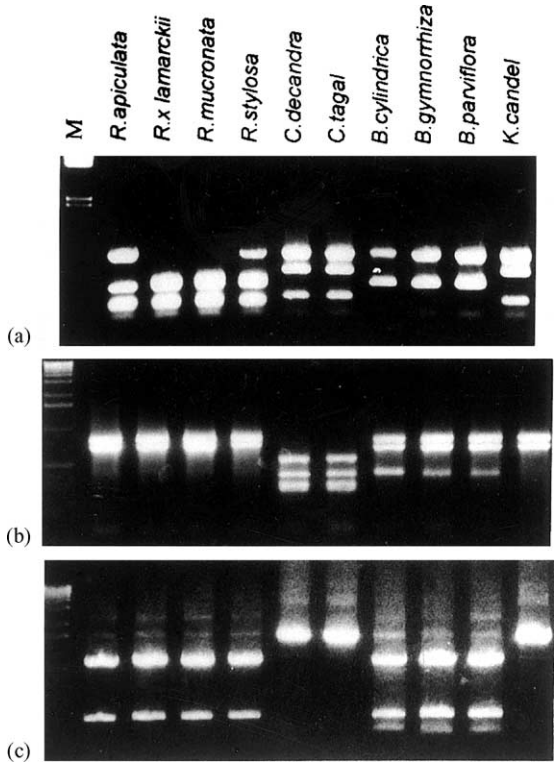

Fig. 4. PCR-RFLP of *trn*S-*psb*C gene region of the chloroplast digested with enzymes: (a) *Hae* III; (b) *Taq* I; (c) *Sma* I.

4. Discussion

For long diversity in mangrove species has been studied based on ecology, morphology, floral physiology and structure of each vegetation types. All of these parameters vary extensively depending on the physical and environmental conditions of the habitat. Therefore, assessing the genetic diversity in these species using environmentally stable DNA markers will help in explaining the wide distribution of individual species and also the genetic potential of selection and evolution in the future (Spalding et al., 1997). It is only of late that some information is available on non-morphological genetic attributes of the mangrove species (Lakshmi et al., 1997, 2000; Parani et al., 1997a,b, 1998; Schwarzbach and Ricklefs, 2000). In the present study, we have analyzed intra-specific diversity, inter-specific and inter-generic relationships between all the nine species and the natural inter-specific hybrid of the mangrove tribe Rhizophoreae reported from the Indian sub-continent using DNA based molecular markers such as RAPD, RFLP and PCR-RFLP.

For population studies, samples were collected from geographically well isolated locations from east and west coast of Indian subcontinent. These locations showed enormous variation in their micro-climatic conditions and physical parameters. A low percentage

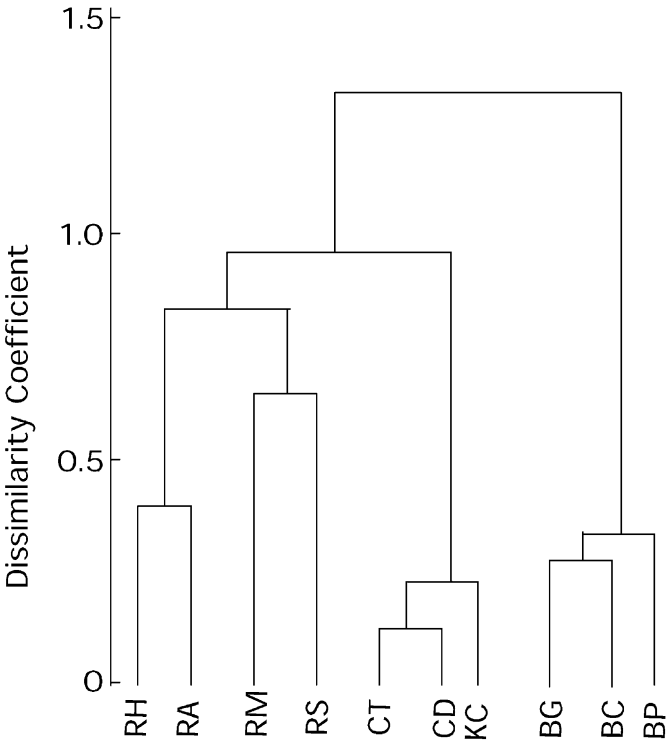

Fig. 5. Dendrogram depicting the species relationship between the 10 species of Rhizophoreae.

of intra-specific polymorphism was detected in these species and it ranged narrowly between 27.7% (*Ceriops decandra*) and 37.3% (*Bruguiera cylindrica*). It was noticed that Bhitarkanika populations wherein mangroves species are found in relatively undisturbed condition showed highest variations for all the species. Ratnagiri population of the west coast and the Muttupet and Coringa populations on the east coast of India where most of the mangrove vegetation is severely degraded due to various anthropogenic pressures. Though the species of the mangrove Rhizophoreae were collected from diverse geographical locations, the percentage of intra-specific variation did not differ drastically as was observed in case of cross-pollinated mangrove species like *Acanthus ilicifolius* (Lakshmi et al., 1997), *Excoecaria agallocha* (Lakshmi et al., 2000) and *Avicennia* species (Parani et al., 1997a). However, higher inter-specific polymorphism as detected by RAPD's is indicative of high level of genetic divergence and lack of gene flow between these species. It is evident from the fact that inter-specific hybrids were reported only in the genus *Rhizophora* despite that molecular markers showed very narrow genetic distance among the four genera of the mangrove Rhizophoraceae (e.g. *Ceriops decandra* and *C. tagal*; *Bruguiera gymnorhiza* and *B. parviflora*). It is generally believed that for long term survival and its adaptation a greater genetic diversity is a must for a species. Schaal et al. (1991) have stressed that species without adequate genetic diversity are at a greater risk of extinction. Given the low level

of intra-specific genetic variation observed in the populations of the analysed species, it is possible that self-pollinated nature of the species might have hindered the variation in this species complex.

RFLP analysis at inter-specific level revealed that 162 out of the 167 loci scored were polymorphic. Though genomic clones derived from mangrove species did not show species-specific RFLPs, the heterologous rDNA probes were useful as markers for species identification. The rDNA repeat unit in the Rhizophoraceae species was found to be flanked by the restriction sites for the enzyme *Hind* III. There were three rDNA repeat unit length classes among the nine species and one hybrid. The four species of *Rhizophora* alone showed two repeat unit length classes indicating high level of speciation in this oldest mangrove genus. The four species of *Rhizophora* and the three species of *Bruguiera* gave species-specific profiles for the rDNA probe *p*TA71 in combination with the enzyme *Dra* I. The two species of *Ceriops*, which were indistinguishable in other marker systems showed species-specific profiles with the rDNA probe *ver* 18 in combination with *Eco*R I. In most probe-enzyme combination, the natural hybrid showed an intermediate profile between *R. apiculata* and *R. mucronata* while *R. stylosa* remained distinct. This indicates that the putative parents of this hybrid are *R. mucronata* and *R. apiculata* and not *R. stylosa.* As the rDNA regions are much conserved these markers therefore, could be considered as much stable and useful in species identification.

For PCR-RFLP studies, four chloroplast gene regions e.g. *trn*L-UAA, *rbc*L, *trn*S-*psb*C, and 16S were amplified and digested with 19 restriction enzymes. Although *trn*L-UAA gene region is reported to be of variable size in other species (Taberlet et al., 1991), a 0.4 kb amplified product was observed in all the species included in the present study. Analysis of the restriction banding pattern revealed markers for the identification of species and genera. Digestion of *rbc*L gene region with *Taq* I, and digestion of *trn*S-*psb*C gene region with *Taq* I and *Hae* III distinguished each genus. Identification of different species and the hybrid of *Rhizophora* was possible upon digestion of *trn*S-*psb*C with *Hae* III and *trn*L-UAA with *Taq* I. The similar profiles observed for *R. mucronata* and the hybrid indicate the former as the chlorplast donor to the hybrid.

PCR-RFLP of *trn*S-*psb*C gene region indicates relative recent origin of *Ceriops*, *Bruguiera*, and *Kandelia* compared to *Rhizophora*. This is because of the observation that the number of bands seen in *Rhizophora* was more than that in other species analysed. The present observations are in line with that of Ziegenhagen and Fladung (1997), wherein they observed that evolution of primitive to advanced species seems to correlate with a reduction in the number of restriction fragments obtained for the amplified gene region. The *trn*S-*psb*C gene seems to be evolutionarily relevant for *Rhizophora*; as a number of different patterns reflect different degrees of systematic sub-divisions in it.

It was noticed that the species clusters in the mangrove tribe Rhizophoreae were consistent in all the three marker systems used in the present study. Genus *Ceriops* and *Kandelia* were clustered together which inturn joined the *Rhizophora* species cluster in all the analysis while genus *Bruguiera* was clustered at very high distances with other three genera. Although species-specific profiles were observed in two species of *Ceriops* the distances separating them were minimal, there were no varietal differences noticed in the genotypes collected from various locations on the Indian coast as was observed for *C. tagal var australiansis* on Australian coast where the differences observed led to the proposal that the variety

be considered as a separate species (Ballment et al., 1988). The distance of separation of each genus was seen to be very high, thereby, indicating a high degree of separation of the four genera of Rhizophoreae over evolutionary time. This dendrogram constructed based on all the molecular marker analyses showed an overall agreement with the earlier cladogram produced for the genera belonging to Rhizophoraceae based on 45 morphological characters (Juncosa and Tomlinson, 1988) and 16 seed characters (Tobe and Raven, 1983); where in all the tribes or sub-families of Rhizophoraceae (Macariseae, Gynotrocheae which are the terrestrial genera and Rhizophoreae which is the mangrove genera) were found to be well differentiated but were considered as paraphyletic. The only point of deviation from the earlier reports is the position of the genus *Ceriops*. *Ceriops* was considered closer to *Rhizophora* by the earlier authors than to *Kandelia*. This could be due to the fact that the species relationship is based only on the morphological characters, which in most mangrove Rhizophoraceae members tend to overlap. The species relationship revealed through the present study is in agreement with the recent report by Schwarzbach and Ricklefs (2000) that analysed the systematic affinities of Rhizophoraceae and Anisophyllaceae using both morphological and DNA based marker systems.

Low level of intra-specific polymorphism encountered in this tribe in combination with their very low population density especially of genera *Ceriops*, *Kandelia* and *Bruguiera*, could lead to regional extinction of these species unless any concerted conservation measures are taken up. In this context, an assessment of the level of intra-specific diversity will no doubt help in the identification of vulnerable species and also the location from where these species could be collected for further conservation programmes. Furthermore, in plant systematics, phylogenetic studies of chloroplast DNA restriction variation are becoming increasingly common, which may or may not reflect the variation of the species as a whole (Doyle, 1992). Therefore, a consensus analysis of genomic DNA and organellar DNA have, in the present study, helped to arrive at the basic understanding of the nature and extent of genetic diversity at intra- and inter-population level as well as resolving the intriguing genomic relationship between the analysed species. These present analyses are of practical utility in providing insight into the nature of genetic differences within and between populations in India, and have helped in identifying priority species and habitat requiring urgent conservation measures. The fragmented distribution of these species across the world makes their study difficult, however, there is a need for studying the range of species to reach a consensus about species identification and classification.

Acknowledgements

This work was carried out with the financial assistance from the Department of Biotechnology, Government of India.

References

Ballment, E.R., Smith, T.J., Stoddart, J.A., 1988. Sibling species in the mangrove genus *Ceriops* (Rhizophoraceae) detected using biochemical genetics. Aust. Syst. Bot. 1 (4), 391–397.

Demesure, B., Sodzi, N., Petit, R.J., 1995. A set of universal primers for amplification of polymorphic non-coding regions of mitochondrial and chloroplast DNA in plants. Mol. Ecol. 4, 129–131.

Doyle, J.J., 1992. Gene trees and species trees: molecular systematics as one-character taxonomy. Syst. Bot. 17 (1), 144–163.

Gottlieb, L.D., 1977. Electrophoretic evidence and plant systematics. Ann. Missouri Bot. Garden 64, 161–180.

Hardrys, H., Ballick, M., Schierwater, B., 1992. Applications of RAPD. Mol. Ecol. 1, 55–63.

Hipkins, V.D., Tsai, C.H., Strauss, S.H., 1990. Sequence of the gene for large subunit of ribulose 1,5-biphosphate carboxylase from gymnosperm. Douglas fir. Plant Mol. Biol. 15, 505–507.

Juncosa, A.M., Tomlinson, P.B., 1988. Systematic comparison and some biological characteristics of Rhizophoraceae and Anisophylleaceae. Ann. Missouri Bot. Garden 75 (4), 1296–1318.

Lakshmi, M., Rajalakshmi, S., Parani, M., Anuratha, C.S., Parida, A.K., 1997. Molecular phylogeny of mangroves I. Use of molecular markers in assessing the intra-specific genetic variability in the mangrove species *Acanthus ilicifolius* Linn. (Acanthaceae). Theor. Appl. Genet. 94, 1121–1127.

Lakshmi, M., Parani, M., Nivedita, R., Parida, A.K., 2000. Molecular phylogeny of mangroves VI. Intra-specific genetic variation in mangrove species *Excoecaria agallocha* L. (Euphorbiaceae). Genome 43 (1), 110–115.

Nei, M., Li, W.H., 1979. Mathematical model for studying genetic variation in terms of restriction endonucleases. Proc. Natl. Acad. Sci. U.S.A. 76, 5269–5273.

Palmer, J.D., Zamir, D., 1982. Chloroplast DNA evolution and phylogenetic relationships in Lycopersicon. Proc. Natl. Acad. Sci. U.S.A. 79, 5006–5010.

Parani, M., Lakshmi, M., Elango, S., Nivedita, R., Anuratha, C.S., Parida, A.K., 1997a. Molecular phylogeny of mangroves II. Intra- and inter-specific variation in *Avicennia* revealed by RAPD and RFLP markers. Genome 40, 487–495.

Parani, M., Rao, C.S., Mathan, N., Anuratha, C.S., Narayanan, K.K., Parida, A.K., 1997b. Molecular phylogeny of mangroves III. Parentage analysis of a *Rhizophora* hybrid using random amplified polymorphic DNA (RAPD) and restriction fragment length polymorphism (RFLP) markers. Aquat. Bot. 58, 165–172.

Parani, M., Lakshmi, M., Senthilkumar, P., Ram, N., Parida, A.K., 1998. Molecular phylogeny of mangroves VI: Analysis of genome relationship in mangrove species using RAPD and RFLP markers. Theor. Appl. Genet. 97 (4), 617–625.

Rholf, F.J., 1993. NTSYS-pc. Numerical taxonomy and multivariate analysis system, Ver 1.8, Applied Biostatisics. New York.

Sambrook, J., Fritsch, E.F., Maniatis T., 1989. Molecular Cloning, A Laboratory Manual. Cold Spring Harbor Lab Press, U.S.A.

Schaal, B.A., Levirich, W.J., Rogstad, S.H., 1991. A comparision of methods foe assessing genetic variation in plant conservation Biology. In: Falk, D.A., Holsinger, K.E. (Eds.), Genetics and Conservation of Rare Plants. Oxford University Press. London.

Schwarzbach, A.E., Ricklefs, R.E., 2000. Systematic affinities of Rhizophoraceae and Anisophylleaceae, and inter-generic relationships within Rhizophoraceae, based on chloroplast DNA, nuclear ribosomal DNA, and morphology. Am. J. Bot. 87, 547–564.

Shinozaki, K., Ohme, M., Tanaka, M., Wakasugi, T., Hayashida, N., Matsubayashi, T., Zaita, N., Chunwongse, J., Obokata, J., Yamaguchi-Shinozaki, K., Ohta, C., Torazawa, C., Meng, B.Y., Sugita, M., Dera, H., Kamogashiva, T., Yamada, K., Kusuda, J., Takaiwa, F., Kato, A., Tohdoh, N., Shimada, H., Sugiura, M., 1986. The complete nucleotide sequence of tobacco chloroplast genome: its organisation and expression. EMBO J. 5, 2043–2049.

Southern, E.M., 1975. Detection of specific sequences among DNA fragments seperated by gel electrophoresis. J. Mol. Biol. 98, 503–517.

Spalding, M.D., Blasco, F., Field, C.D., (Eds.), 1997. World Mangrove Atlas: The International Society for Mangrove Ecosystem. Okinawa, Japan, p. 178.

Sun, M., Wong, K.C., Lee, S.Y., 1998. Reproductive biology and population genetic structure of *Kandelia candel* (Rhizophoraceae), a viviparous mangrove species. Am. J. Bot. 85, 1631–1637.

Taberlet, P., Gielly, L., Pauton, G., Bonvet, J., 1991. Universal primers for amplification of 3 non-coding regions of chloroplast DNA. Plant Mol. Biol. 17, 1105–1109.

Tobe, H., Raven, P.H., 1983. An embryological analysis of Myrtales: its definition and characteristics. Ann. Missouri Bot. Garden 70, 71–94.

Tomlinson, P.B., 1986. Botany of Mangroves. Cambridge University Press, Cambridge.

Tsumura, Y., Kawahara, T., Wickneswari, R., Yoshimura, K., Symington, C.F., Ashton, P.S., 1996. Molecular phylogeny of Dipterocarpaceae in southeast Asia using RFLP of PCR-amplified chloroplast genes. Theor. Appl. Genet. 93 (1-2), 22–29.

Tsumura, Y., Yoshimura, K., Tomaru, N., Ohba, K., 1995. Molecular phylogeny of conifers using RFLP analysis of PCR-amplified specific chloroplast genes. Theor. Appl. Genet. 91 (8), 1222–1236.

Wagner, D.B., Furnier, G.R., Saghai-Mariif, M.A., Williams, S.M., Dancik, B.P., Allard, R.W., 1987. Chloroplast DNA polymorphisms in lodgepole and jack pines and their hybrids. Proc. Natl. Acad. Sci. U.S.A. 84, 2097–2100.

Williams, J.G.K., Kubelik, A.R., Livak, K.J., Rafalski, J.A., Tingey, S.V., 1990. DNA polymorphism amplified by arbitary primers are useful as genetic markers. Nucl. Acids Res. 18, 6531–6535.

Ziegenhagen, B., Fladung, M., 1997. Variation in the *psb*C gene region of gymnosperms and angiosperms as detected by a single restriction site polymorphism. Theor. Appl. Genet. 94 (8), 1065–1071.

Zimmer, E.A., Jupe, E.R., Walbot, V., 1988. Ribosomal gene structure, variation and inheritance in maize and its ancestors. Genetics 120, 1125–1136.