Theor Appl Genet (2000) 100:454–460

M. Parani · M. Lakshmi · B. Ziegenhagen
M. Fladung · P. Senthilkumar · A. Parida

Molecular phylogeny of mangroves VII. PCR-RFLP of *trn*S-*psb*C and *rbc*L gene regions in 24 mangrove and mangrove-associate species

Received: 20 April 1999 / Accepted: 12 May 1999

Abstract Chloroplast DNA (cpDNA) regions, *trn*S-*psb*C and *rbc*L, from 120 individuals of 24 mangrove and mangrove associate species belonging to 11 orders, 13 families and 17 genera of Angiospermae were amplified by the polymerase chain reaction (PCR) and restriction-digested with *Hae*III. Analysis of polymorphism in the restriction fragments (PCR-RFLP) revealed 18 classes of restriction banding pattern in *trn*S-*psb*C region. This has provided molecular evidence for diversity in the mangrove floral component at the above-species level. Intra-generic variations were observed in three genera, viz. *Rhizophora, Avicennia* and *Suaeda*. Species-specific restriction patterns were found in the genera *Rhizophora* and *Suaeda*. A natural hybrid belonging to the genus *Rhizophora* was also analysed, and its restriction pattern was the same as that of a putative parental species. PCR-RFLP analysis of *rbc*L gene region was less differentiating. However, it showed 13 different classes of restriction patterns and revealed the usefulness of these investigations for genome analysis at a higher taxonomic level. Intra-specific variation was not observed in any of the species in either of the cpDNA regions analysed. This is the first report which describes variations in the chloroplast genome of mangrove species.

Key words Mangroves · *trn*S-*psb*C · *rbc*L · cpDNA · PCR-RFLP · Phylogeny

Communicated by P.M.A. Tigerstedt

M. Parani · M. Lakshmi · P. Senthilkumar · A. Parida (✉)
M.S. Swaminathan Research Foundation, III Cross Street,
Taramani Institution Area, Chennai 600 113, India
e-mail: MDSAAA51@giasmd01.vsnl.net.in,
Fax: +44-91-2351319

B. Ziegenhagen · M. Fladung
Federal Research Centre for Forestry and Forest Products,
Institute for Forest Genetics, Sieker Landstraße 2,
22927 Großhansdorf, Germany

Introduction

Mangroves are defined as halophytes, generally woody plants that inhabit the upper inter-tidal zones of estuaries within tropical and subtropical regions. The mangrove ecosystem plays an important role in maintaining coastal ecological balance in these regions. It is one of the highly productive and dynamic ecosystems providing food, livelihood and ecological security to mankind. Ignorance and negligence of its significance led to a drastic reduction in its area throughout the world and local extinction of many species and populations. In mangrove ecosystems, many plant species are found exclusively (Tomlinson 1986). These species are called true mangroves; some of them play a major, and others a minor, role in establishing these ecosystems. Other species which are mainly distributed in terrestrial habitats but also occur in mangrove ecosystems are called mangrove associates. At the present time, when the possible extinction of some true mangrove species and the importance of the conservation of mangrove ecosystems are being discussed, there is virtually no information available on the genetic aspects of these species.

So far, about 54 species and hybrids belonging to 19 genera have been reported as true mangroves (Riclefs and Latham 1993). However, the classification of single populations to specific taxonomic units was not always as simple as believed due to possible intra-specific and/or inter-specific hybridisations. Therefore, there is an obvious lack of knowledge concerning the genetic characteristics within and between populations of mangrove species as well as of the genetic relationships in mangrove ecosystems during evolution. We have studied the nuclear genome of several mangrove species using molecular markers, which have provided useful information on genetic diversity, pedigree and species relationships (Parani et al. 1996; Lakshmi et al. 1997; Parani et al. 1997a, b, 1998). The present study on the chloroplast genome has been undertaken to further resolve the genetic relationships among mangrove species.

Analysis of variations in chloroplast DNA (cpDNA) is a suitable tool for studying molecular evolutionary re-

lationships among and within plant species, because chloroplast genes are assumed to have slower rates of evolution than nuclear genes (Clegg et al. 1991). The highly conserved nature of cpDNA (Palmer 1987), and its predominant maternal inheritance (Sears 1980; Harris and Ingram 1991), has facilitated its extensive usage in evolutionary and phylogenetic studies (Clegg and Zurawski 1992; Schilling 1997). In studying the hybridisation and introgression of closely related species, many investigations have been carried out by the analysis of cpDNA in various tree species (Szmidt 1991; Strauss et al. 1992; Wagner 1992; Kormutak et al. 1993; Vornam et al. 1994). A whole range of different methods have been applied in genetic studies of chloroplast DNA (reviewed by Olmstead and Palmer 1994). Recently, highly effective and simple techniques have been developed. The conserved arrangement of chloroplast genes and the availability of cpDNA sequence data from various taxa has helped in designing primers and isolating regions of interest directly by the polymerase chain reaction (PCR). Subsequent digestion of the PCR products with restriction enzymes provides a highly effective screening for variation (Tsumura et al. 1995, 1996; Ziegenahgen and Fladung 1997).

In the present study, we have investigated the cpDNA of some mangrove and mangrove-associate species by PCR amplification of two specific regions, *trn*S-*psb*C and *rbc*L, followed by restriction digestion with a four-base restriction enzyme, *Hae* III (GG↓CC). Earlier experiments on *Abies alba* by PCR-amplification of a cpDNA region consisting of both the gene and intergenic sequences of the *trn*S and *psb*C (*trn*S-*psb*C) followed by restriction digestion (PCR-RFLP) with 14 enzymes regions, revealed that this region had no sites for any of the enzymes tested except *Hae*III (Ziegenhagen et al. 1995). Subsequently, the screening of 62 woody plant species (34 species of gymnosperms and 28 species of angiosperms) for single-restriction site (GG↓CC) polymorphisms (Ziegenhagen and Fladung 1997), as well as some non-woody annuals (unpublished), demonstrated the utility of this system to differentiate groups at higher taxonomic levels than species. It has been presumed that amplifying the *trn*S-*psb*C cpDNA region from wide-ranging species, as well as the application of this technique to other chloroplast genes, would be worthwhile. Therefore, in the present study we have carried out PCR-RFLP of *trn*S-*psb*C and *rbc*L gene regions of cpDNA from 24 mangrove species representing 11 orders, 13 families and 17 genera of angiosperms. Among these 24 species two were monocots.

Materials and methods

Plant material

For the present study, 24 mangrove and mangrove associate species were included. The names and authors of these species, their place(s) of collection and the relevant taxonomic information are

Table 1 Details of the species selected for the present study. T=Tomlinson 1986, S=Saenger et al. 1983, P=Parani et al. 1998

No.	Species	Family	Order	Place (s) of collection	Status
1.	*Rhizophora apiculata* Blume	Rhizophoraceae	Rhizophorales	Pichavaram, Sunderban, Bhitarkanika	True mangrove
2.	*Rhizophora mucronata* Lamk.	Rhizophoraceae	Rhizophorales	Pichavaram, Sunderban, Bhitarkanika	True mangrove
3.	*Rhizophora stylosa* Griff.	Rhizophoraceae	Rhizophorales	Andaman and Nicobar islands	True mangrove
4.	*Rhizophora* x *lamarkii* Lamk.	Rhizophoraceae	Rhizophorales	Pichavaram, Sunderban, Bhitarkanika	True mangrove
5.	*Kandelia candel* L. Druce	Rhizophoraceae	Rhizophorales	Bhitarkanika, Goa	True mangrove
6.	*Bruguiera cylindrica* L.	Rhizophoraceae	Rhizophorales	Pichavaram, Coringa, Goa	True mangrove
7.	*Bruguiera gymnorrhiza* (L.) Lamk.	Rhizophoraceae	Rhizophorales	Coringa, Bhitarkanika	True mangrove
8.	*Ceriops decandra* (Griff.) Ding Hou	Rhizophoraceae	Rhizophorales	Pichavaram, Bhitarkanika, Sunderban	True mangrove
9.	*Hertiera fomes* Buch. - Ham	Sterculiaceae	Malvales	Bhitarkanika, Sunderban	True mangrove
10.	*Xylocarpus granatum* Konig	Meliaceae	Sapindales	Pichavaram, Bhitarkanika, Koringa	True mangrove
11.	*Amoora cucullata* Roxb.	Meliaceae	Sapindales	Bhitarkanika	Mangrove associate [T]
12.	*Sonneratia apetala* Buch. – Ham	Sonneratiaceae	Myrtales	Coringa, Bhitarkanika	True mangrove
13.	*Avicennia marina* (Forsk.) Vierh.	Avicenniaceae	Lamiales	Pichavaram, Bhitarkanika, Goa, Calicut	True mangrove
14.	*Avicennia alba* Blume	Avicenniaceae	Lamiales	Coringa	True mangrove
15.	*Avicennia officinalis* L.	Avicenniaceae	Lamiales	Pichavaram, Coringa	True mangrove
16.	*Aegiceras corniculatum* L.	Myrsinaceae	Primulales	Pichavaram, Coringa, Bhitarkanika	True mangrove
17.	*Lumnitzera racemosa* Willd.	Combretaceae	Myrtales	Pichavaram, Goa	True mangrove
18.	*Excoecaria agallocha* L.	Euphorbiaceae	Euphorbiales	Pichavaram, Calicut, Coringa, Muthupet	Minor mangrove [T]
19.	*Acanthus ilicifolius* L.	Acanthaceae	Scrophulariales	Pichavaram, Calicut, Goa, Muthupet	Minor mangrove [S]
20.	*Suaeda maritima* Dumort.	Chenopodiaceae	Caryophyllales	Pichavaram, Coringa	Mangrove associate [P]
21.	*Suaeda monoica* (Forsk.) ex J.E. Gmel	Chenopodiaceae	Caryophyllales	Pichavaram	Mangrove associate [P]
22.	*Sesuvium portulacastrum* L.	Aizoaceae	Caryophyllales	Pichavaram, Madras, Goa	Mangrove associate [T]
23.	*Nypa fruticans* (Thumb.) Wurmb.	Arecaceae	Arecales	Madras	True mangrove
24.	*Porteresia coarctata* Tateoka	Poaceae	Graminales	Porto Novo, Pichavaram, Coringa, Goa	Minor mangrove [P]

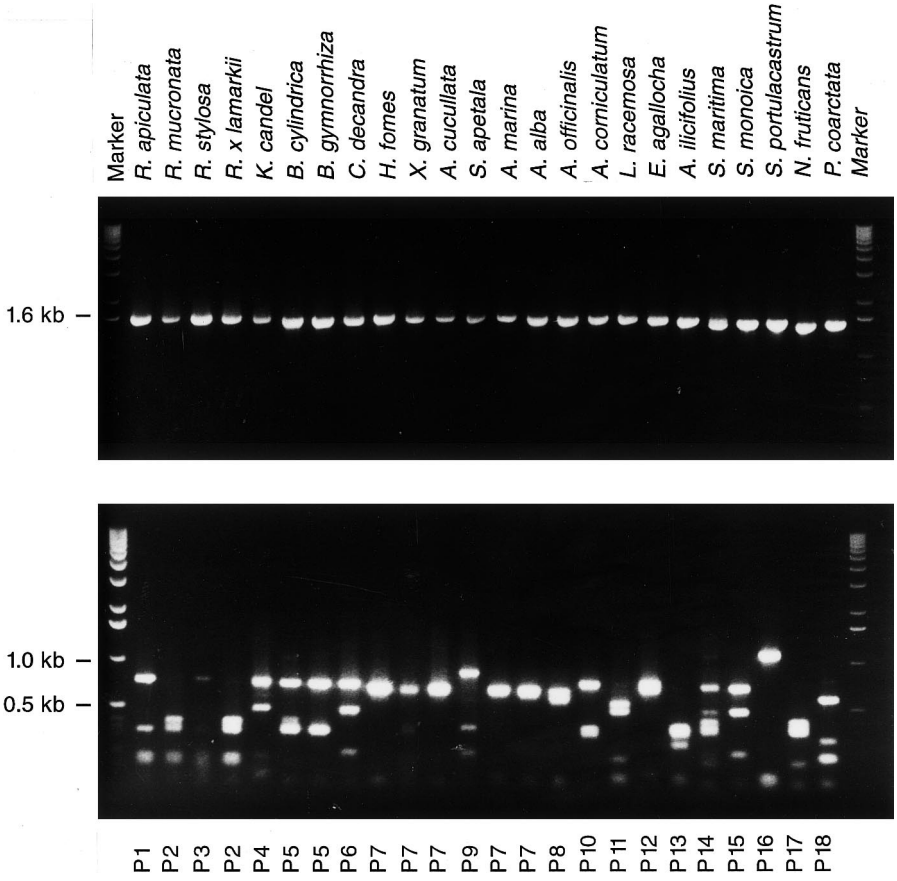

Fig. 1 DNA fragments obtained by PCR-amplification of genomic DNA from 24 mangrove and associate species with primers specific to the *trn*S-*psb*C gene region of cpDNA (above), and the restriction-banding pattern observed after restriction digestion of the amplified fragments with *Hae*III (below). P1–P18 indicate the different classes of restriction banding pattern; the marker lane was loaded with a 1-kb ladder DNA (Gibco-BRL)

all given in Table 1. Five individuals in each species were randomly selected and genomic DNA was isolated from leaf tissues. Wherever possible the individuals within a species were selected from geographically distant places.

DNA isolation

DNA isolation from *Rhizophora* sp. and *Sonneratia apetala* was carried out as described earlier (Parani et al. 1998). DNA from the other species was isolated following the CTAB method (Saghai-Maroof et al. 1984) with minor modifications (Parani et al. 1997a).

Primers

The *trn*S-*psb*C region of cpDNA was amplified by a pair of universal primers (Demesure et al. 1995); primer 1: 5′GGT TCG AAT CCC TCT CTC TC3′, primer 2: GGT CGT GAC CAA GAA ACC AC 3′. The *rbc*L region was amplified by another pair of universal primers (Hipkins et al. 1990); primer 1: TGT CAC CAA AAA CAG AGA CT 3′, primer 2: 5′ TTC CAT ACT TCA CAA GCA GC 3′. The primers were synthesised by Pharmacia Biotech Europe GmbH (Freiburg, Germany).

PCR amplification

Amplification of genomic DNA was carried out in 30-µl reaction mixture containing 20 ng of template DNA, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.001% gelatin, 2 mM $MgCl_2$, 100 µM of dNTPs, 0.5 µM of each primer and 1 unit of *Taq* DNA polymerase (USB, USA). The reaction mixture was overlaid with an equal volume of mineral oil, and amplified in a Perkin-Elmer Model 480 thermal cycler. The temperature profile consisted of a total of 35 cycles with 1 min (4 min for the first cycle) at 94°C for template denaturation, 1 min at 63°C (60°C for *rbc*L) for primer annealing, and 2 min (15 min for the final cycle) at 72°C for primer extension. The amplified products along with 1 kb ladder-marker DNA (Gibco-BRL) were separated by agarose-gel (1.2%) electrophoresis in 0.5× TRIS-borate buffer and stained with ethidium bromide (Sambrook et al. 1989).

Restriction digestion of PCR products

A part of the PCR products was directly digested with ten units of *Hae*III restriction enzyme following the manufacturer's instructions (Amersham, UK). The restriction fragments, along with 1 kb ladder-marker DNA, 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 both repeated at least twice for all the samples. Amplification products, as well as restriction patterns, were found to be reproducible. The total size of the restricted products was always almost equal to the undigested PCR product. Wherever deviation was observed between the two, the PCR product was purified and then digested to identify the authentic restriction banding pattern.

Results

PCR amplification

Amplification of genomic DNA with primers for the *trn*S-*psb*C and *rbc*L gene regions produced fragments of about 1.6 kb (Fig. 1) and 1.4 kb (Fig. 2), respectively. Al-

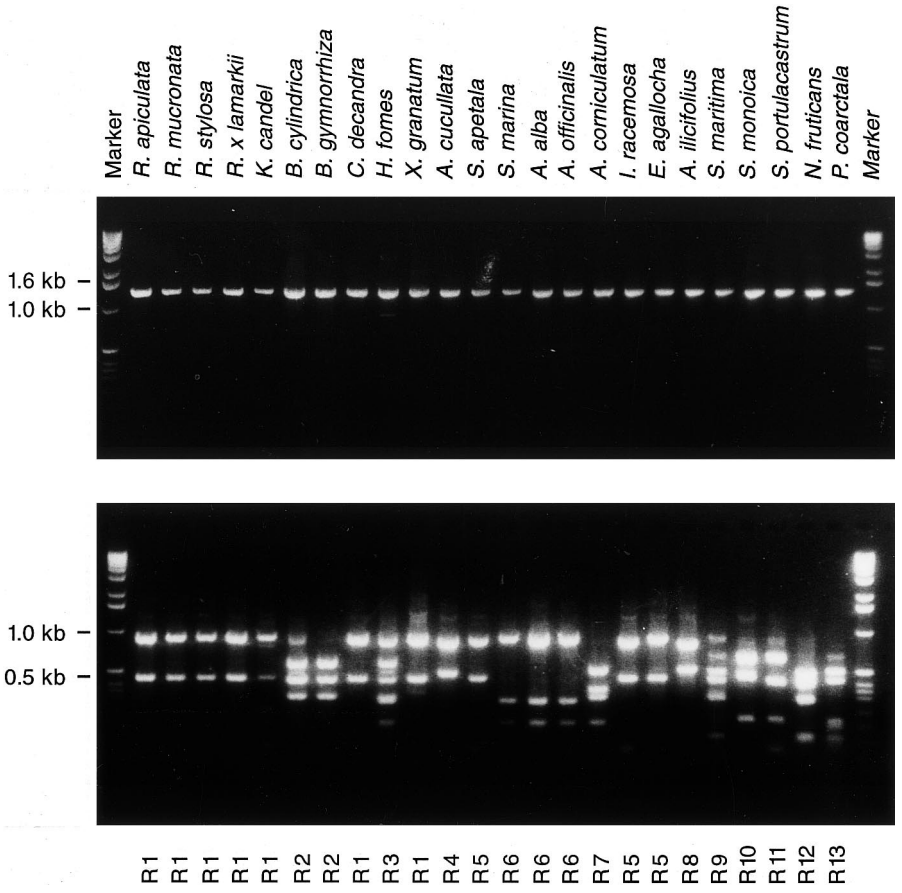

Fig. 2 DNA fragments obtained by PCR-amplification of genomic DNA from 24 mangrove and associate species with primers specific to the *rbc*L gene region of cpDNA (above), and the restriction-banding pattern observed after restriction digestion of the amplified fragments with *Hae*III (below). R1–R13 indicate the different classes of restriction banding pattern; the marker lane was loaded with a 1-kb ladder DNA (Gibco-BRL)

though we have used a stringent annealing temperature of 63°C for *trn*S-*psb*C and 60°C for *rbc*L, spurious bands bigger and smaller than the target fragment were occasionally observed. However, compared to the target fragment, the yield of spurious bands was too small to interfere with the interpretation of the results after restriction digestion. Among the 24 species, there was no variation in length for both *trn*S-*psb*C and *rbc*L (Figs. 1 and 2).

PCR-RFLP of *trn*S-*psb*C and *rbc*L

Digestion of the amplified *trn*S-*psb*C region with *Hae*III resulted in 18 different restriction patterns (Fig. 1) while that of the *rbc*L region showed 13 different restriction patterns (Fig. 2). There were no intra-specific variations for either region. The identity of the restriction patterns with subtle differences in mobility were confirmed in duplicate experiments by running the corresponding samples side by side in separate gels for longer duration. The grouping of the restriction pattern, as indicated by P1–P18 (Fig. 1) and R1–R13 (Fig. 2), was evident after repeated analyses in different gels. For example, the samples in Fig. 2, lane 6, showing a restriction pattern different from that of in lane 7, was found to be the same and, therefore, was included in the same group (R2). The species were grouped according to the restriction pattern shared among them (Table 2).

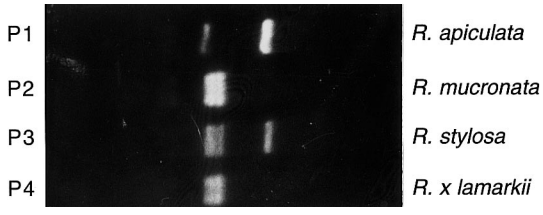

Fig. 3 *Hae*III restriction banding pattern of the *rbc*L from three species and a hybrid of the genus *Rhizoprora*. *P1*, *P2* and *P3* are the species-specific restriction banding-patterns of the three species; the restriction-banding pattern of the hybrid incates *R. mucronata* as its female parent

PCR-RFLP of *trn*S-*psb*C showed species-specific banding patterns (P1–P3) for the three species of the genus *Rhizophora*, while the banding pattern of the natural hybrid, *R* ×*lamarkii*, was the same as that of *R. mucronata* (Figs. 1 and 3). Another genus in which species-specific patterns (P14 and P15) were observed was *Suaeda*. Each of the remaining banding patterns except P7 and P8 were specific to one genus. However, it should be noted that all these genera except *Bruguiera* were represented by single species. The restriction pattern P7 was shared by more than one genus, viz. *Heritiera*, *Xylocarpus*, *Amoora* and *Avicennia*. However, one species of *Avicennia*, *A. officinalis*, showed a different banding pattern (P8). The two monocotyledonous species *Porteresia*

Table 2 Grouping of species based on the shared PCR-RFLP patterns of *trn*S-*psb*C and *rbc*L

*trn*S-*psb*C		*rbc*L	
PCR-RFLP pattern	Species	PCR-RFLP pattern	Species
P1	*R. apiculata*	R1	*R. apiculata, R. mucronata, R. stylosa, R ×lamarkii, K. candel, C. decandra, X. granatum*
P2	*R. mucronata, R ×lamarkii*		
P3	*R. stylosa*		
P4	*K. candel*		
P5	*B. cylindrica, B. gymnozhyza*	R2	*B. cylindrica, B. gymnozhyza*
P6	*C. decandra*	R3	*H. fomes*
P7	*H. fomes, X. granatum, A. cucullata, A. marina, A. alba*	R4	*A. cucullata*
P8	*A. officinalis*	R5	*S. apetala, L. racemosa, E. agallocha*
P9	*S. apetala*	R6	*A. marina, A. alba, A. officinalis*
P10	*A. corniculatum*	R7	*A. corniculatum*
P11	*L. racemosa*		
P12	*E. agallocha*		
P13	*A. ilicifolius*	R8	*A. ilicifolius*
P14	*S. maritima*	R9	*S. maritima*
P15	*S. momoica*	R10	*S. momoica*
P16	*S. portulacastrum*	R11	*S. portulacastrum*
P17	*N. fruticans*	R12	*N. fruticans*
P18	*P. coarctata*	R13	*P. coarctata*

coarctata and *Nypa fruticans* showed two different banding patterns (P17 and P18) not shared by any of the dicotyledonous species included in this study.

Unlike the PCR-RFLP of *trn*S-*psb*C, *rbc*L showed a similar restriction pattern (R1) for all three species and the natural hybrid of the genus *Rhizophora*. This pattern was shared by two other genera of the same tribe (Rhizophorae), *Kandelia* and *Ceriops*, and also by *Xylocarpus granatum* of the Meliaceae. However, another genus of the tribe Rhizophorae, *Bruguiera*, represented by *B. cylindrica* and *B. gymnorrhiza*, and another genus of the family Meliaceae, represented by *Amoora cucullata*, showed different banding patterns (R2 and R4 respectively). All the three species of *Avicennia* showed the same banding pattern. *Sonneratia apetala* gave a unique banding with *trn*S-*psb*C, whereas in the case of *rbc*L a common banding pattern was observed for *S. apetala, Lumnitzera racemosa* and *Excoecaria agallocha.* As observed with the PCR-RFLP of *trn*S-*psb*C, the two species of *Suaeda* showed different banding patterns, and banding patterns of the two monocotyledons (R12 and R13) were not shared by the other species.

Discussion

PCR-amplification of *trn*S-*psb*C and *rbc*L using universal primers, followed by restriction digestion with *Hae*III produced a discrete banding pattern in all the species studied. However, considering the taxonomic distance between the species analysed, fragments of identical length may not necessarily represent fragments of identical sequence. As only sequencing could relate to phylogenetic relationships based on single genes (Chase et al. 1993), the interpretation of our present results are based solely on the restriction banding pattern as directly observed by staining with ethidium bromide after agarose-gel electrophoresis.

The present study on PCR-RFLP of the chloroplast gene region *trn*S-*psb*C with *Hae*III in 24 species of mangrove and mangrove-associate species, belonging to 11 orders, 13 families and 17 genera of angiosperms, revealed 18 classes of restriction patterns. This was more than the 13 restriction patterns observed in 34 species of gymnosperms and 28 species of angiosperms representing 14 orders (3 gymnosperms and 11 angiosperms), 20 families (7 gymnosperms and 13 angiosperms) and 41 genera (20 gymnosperms and 21 angiosperms) with the same cpDNA region and restriction enzyme reported previously by Ziegenhagen and Fladung (1997). Moreover except for one genus, *Abies* of the gymnospermae, in which intra-generic and intra-specific variation was reported, the 14 (G5+A9) banding patterns obtained by Ziegenhagen and Fladung (1997) were different only at the genus level. These observations provided molecular evidence for the highly diverse nature of the mangrove gene pool based on morphological characters (Blasco 1984). Interspecific (intra-generic) variation was found in three genera, viz. *Rhizophora, Avicennia* and *Suaeda*. In *Rhizophora* and *Suaeda* all five species used in the present study showed species-specific restriction banding patterns. In *Avicennia, A. marina* and *A. alba* were similar but *A. officinalis* showed a distinct banding pattern. Intra-specific variation was not found in any of the species investigated. This has enabled unambiguous identification of these species, which is otherwise difficult because of overlapping phenotypes (Tomlinson 1986).

In the genus *Rhizophora*, *R. mucronata* and *R* x *lamarkii* shared a common banding pattern (Fig. 3). This points to the maternal ancestry of this hybrid from *R. mucronata*, and supports recent reports (Kathiresan 1995; Parani et al. 1997b) disputing the earlier claim that *R*×*lamarkii* was a hybrid between *R apiculata* and *R. stylosa*. As we have already reported a *Rhizophora* hybrid between *R. apiculata* and *R. mucronata*, with the former as the maternal parent based on mitochondrial DNA-RFLP (Parani et al. 1997b), the present observation indicates that reciprocal hybridisation may also be possible between the two species. Though, the random amplified polymorphic DNA (RAPD) assay has been extensively used for pedigree analysis, identification of the maternal parent requires conventional RFLP technique which is cumbersome and time consuming. Successful identification of the maternal parent of a natural hybrid in the present study, using PCR-RFLP in mangrove species, provides a PCR-based technique for parentage analysis, at least at the inter-specific level.

In the genus *Avicennia*, *A. marina* and *A. alba* shared one banding pattern (P7), while *A. officinalis* showed a different banding pattern (P8). This supports our earlier finding based on RAPD and RFLP data that, among the three species of *Avicennia* present in India, *A. marina* was closer to *A. alba* than to *A. officinalis* (Parani et al. 1997a). As the delineation of species was possible in three out of the four genera in which multiple species were used, a survey of additional genes may facilitate the application of the same approach for species identification in most of the other genera as well.

Amplification of the *rbc*L region followed by restriction digestion with *Hae*III was performed in order to increase the evidence for a highly diverse mangrove gene pool. The *rbc*L gene is one of the best-investigated chloroplast DNA genes by means of molecular techniques, and is known to exhibit extensive variation above the species level (Bousquet et al. 1992). A most comprehensive study of the phylogeny of seed plants on the basis of *rbc*L gene sequences was presented by Chase et al. (1993). Other studies deal with the inference of phylogeny from *rbc*L gene sequences among families or genera (e.g. Michaels et al. 1993; Brunsfeld et al. 1994). In the present case, restriction of the *rbc*L gene region by *Hae*III showed 13 different restriction fragment patterns compared to the 18 banding patterns observed with the *trn*S-*psb*C region. With *trn*S-*psb*C only one banding pattern (P7) was shared by multiple genera, while in case of the *rbc*L two banding patterns (R1 and R5) were shared by multiple genera. However, there was at least one case in which the monomorphic pattern observed with *trn*S-*psb*C (between *Xylocarpus* and *Amoora* of the Meliaceae, P7 in Fig. 1) could be differentiated based on PCR-RFLP of *rbc*L (see R1 and R4 in Fig. 2). Therefore, though it seems that *rbc*L is more conserved than *trn*S-*psb*C, it is too early to draw such a general conclusion based on the limited data available. However, a recent study on *rbc*L and *trn*S-*psb*C from ten different species of *Rhizophora* using 19 restriction enzymes is in favour of this observation (Lakshmi 1999)

The most-frequent banding pattern (R1) in the PCR-RFLP of *rbc*L was shared by three genera of Rhizophoraceae, v*iz.*, *Rhizophora*, *Ceriops* and *Kandelia* belonging to the order Rhizophorales, as well as *X. granatum* (Meliaceae) belonging to the order Sapindales. *Sonneratia apetala* (Sonneratiaceae) and *Lumnitzera racemosa* (Combretaceae), of the order Myrtales in which Rhizophoraceae was placed earlier, showed different banding patterns. This is in accordance with our earlier results based on RAPD and RFLP markers in which members of the Rhizophoraceae and *X. granatum* formed a cluster while members of the Myrtales were outside this cluster (Parani et al. 1998). This validates the potential application of single-restriction-site-polymorphism analysis of PCR-amplified cpDNA regions in genome relationships and phylogenetic studies.

The present study, together with our earlier investigations using RAPD and RFLP markers, indicated that single restriction site polymorphism in PCR-amplified cpDNA regions could be useful in phylogenetic studies, pedigree analysis and in the identification of mangrove species. Simplicity and reproducibility provides this approach with an edge over conventional RFLP using either isolated cpDNA or cpDNA-specific probes. Though this approach has the limitation of being related only to a small fraction of the chloroplast genome, the conserved nature of chloroplast gene arrangement (Ziegenhagen and Fladung 1997) and the commercial availability of thermostable polymerases capable of amplifying fragments of several kilobase length should obviate this limitation by simultaneous single-step amplification of multiple genes, and so make this approach more informative and useful than at present.

Acknowledgements We are grateful to Prof. M.S. Swaminathan for his valuable suggestions and guidance. The help received from Dr. N. Anil Kumar in species identification is gratefully acknowledged. This project was supported by financial assistance from the Department of Biotechnology, Government of India.

References

Blasco F (1984) Taxonomic considerations of mangrove species. In: Snedaker SC, Snedaker JG (eds) The mangrove ecosystem: research methods. UNESCO, Paris, pp 82–90

Bousquet J, Strauss ST, Doerksen AH, Price RA (1992) Extensive variation in the evolutionary rate of *rbc*L gene sequences among seed plants. Proc Natl Acad Sci USA 89:7844–7848

Brunsfeld SJ, Soltis PS, Soltis BE, Gadek PA, Quinn CJ, Strenge DD, Rankar TA (1994) Phylogenetic relationships among the genera of Taxodiaceae and Cupressaceae – evidence from *rbc*L sequences. Systematic Bot 19:253–262

Chase MW, Soltis DE, Olmstead RG, Morgan D, Les DH, Mishler BD, Duvall MR, Price RA, Hills HG, Qiu YL, Kron KA, Rettig JH, Konti E, Palmer JD, Manhart JR, Sytsma KJ, Michaels HJ, Kress WJ, Karol KG, Clark WD, Hedren M, Gaut BS, Jansen RK, Kim KJ, Wimpee CF, Smith JF, Furnear GR, Strauss SH, Xiang QY, Plunkett GM, Soltis PS, Swensen SN, Williams SE, Gadek PA, Quinn CJ, Eguiarte LE, Golenberg E, Learn GH, Graham SW, Barrett SEH (1993) Phylogenetics of

seed plants – an analysis of nucleotide sequences from the plastid gene *rbc*L. Ann Missouri Bot Gard 80:528–580

Clegg MT, Zurawski G (1992) Chloroplast DNA and the study of plant phylogeny: present status and future prospects. In: Soltis PS, Soltis DE, Doyle JJ (eds) Molecular systematics of plants. Chapman and Hall, New York, pp 1–13

Clegg MT, Learn GH, Golenberg EM (1991) Molecular evolution of chloroplast DNA. In: Selander et al. (eds) Evolution at the molecular level. Sinauer Associates, Sunderland, Massachusetts, pp 135–149

Demesure B, Sodzi N, Petit RJ (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

Harris SA, Ingram R (1991) Chloroplast DNA and biosystematics: the effects of intraspecific diversity and plastid transmission. Taxon 40:393–412

Hipkins VD, Tsai CH, Strauss SH (1990) Sequence of the gene for the large subunit of ribulose 1,5-biphosphate carboxylase from the gymnosperm, Douglas fir. Plant Mol Biol 15:505–507

Kathiresan K (1995) *Rhizophora annamalai* : a new species of mangrove. Environ Ecol 13:240–241

Kormutak A, Szmidt A, Wang X-R (1993) Restriction fragment length polymorphism of chloroplast DNAs in some species of fir (*Abies alba*). Biol Plant 35:113–119

Lakshmi M (1999) Genetic diversity in mangrove species of the family Rhizophoraceae. PhD thesis, M.S. Swaminathan Research Foundation, Chennai, India

Lakshmi M, Rajalakhsmi S, Parani M, Anuratha CS, Parida A (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

Michaels HJ, Scott KM, Olmstead RG, Szaro T, Jansen RK, Palmer JD (1993) Interfamilial relationships of the Asteraceae – insights from *rbc*L sequence variation. Ann Missouri Bot Gard 80:742–751

Olmstead RG, Palmer JD (1994) Chloroplast DNA systematics: a review of methods and data analysis. Am J Bot 81:1205–1224

Palmer JD (1987) Chloroplast DNA evolution and biosystematic uses of chloroplast DNA variation. Am Nat 130:S6- S29

Parani M, Lakshmi M, Rajalakshmi S, Anuratha CS, Parida A (1996) Genetic polymorphism in Indian mangrove species revealed through molecular markers. In: Ravishankar GA, Venkataraman LV (eds) Recent advances in biotechnogical applications of plant tissue and cell culture. Oxford and IBH Publishing Co. Pvt. Ltd New Delhi and Calcutta, India, pp 421–428

Parani M, Lakshmi M, Elango S, Nivedita R, Anuratha CS, Parida A (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 CS, Mathan N, Anuratha CS, Narayanan KK, Parida A (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. Aquatic Bot 58:165–172

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

Ricklefs RE, Latham RE (1993) Global patterns of diversity in mangrove floras. In: Ricklefs RE, Schluter D (eds) Species diversity in ecological communities: historical and geographical perspectives. University of Chicago Press, Chicago London, pp 215–230

Saenger PE, Hegrel J, Davie JDS (1983) Global status of mangrove ecosystems. Environmentalist suppl 3:1–88

Saghai-Maroof MA, Soliman KM, Jorsenson RA, Allard RW (1984) Ribosomal DNA spacer length polymorphism in barley: Mendelian inheritance, chromosomal location and population dynamics. Proc Natl Acad Sci USA 81:8014–8018

Sambrook J, Fritsch FF, Maniatis T (1989) Molecular cloning: a laboratory manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York

Schilling EE (1997) Phylogenetic analysis of *Helianthus* (Asteraceae) based on chloroplast DNA restriction-site data. Theor Appl Genet 94:925–933

Sears BB (1980) Elimination of plastids during spermatogenesis and fertilisation in the plant kingdom. Plasmids 4:233–255

Strauss SH, Bousquet J, Hipkings VS, Hong Y-P (1992) Biochemical and molecular genetic markers in biosystematic studies of forest trees. New For 6:125–158

Szmidt AE (1991) Phylogenetic and applied studies on the chloroplast genome in frost conifers. In: Fineschi et al. (eds) Biochemical markers in the population genetics of forest trees. SPB Academic Publishing,The Hague, The Netherlands, pp 185–196

Tomlinson PB (1986) The botany of mangroves. Cambridge, University Press

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 91:1222–1236

Tsumura Y, Kuwahara T, Wickneswari R, Yoshimura K (1996) Molecular phylogeny of Dipterocarpaceae in Southeast Asia using RFLP of PCR-amplified chloroplast genes. Theor Appl Genet 93:22–29

Vornam B, Herzog S, Preisig-Mafiller R, Hattemer H (1994) Restriction fragment length polymorphisms of a chloroplast Photosystem-II gene from poplar and their use for species identification. Genome 37:747–750

Wagner DB (1992) Nuclear, chloroplast, and mitochondrial DNA polymorphism as biochemical markers in population genetic analysis of forest trees. New For 6:373–390

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

Ziegenhagen B, Kormutak A, Schauerte M, Scholz F (1995) Restriction site polymorphism in chloroplast DNA of silver fir (*Abies alba* Mill). For Genet 2:99–107