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Molecular phylogeny of mangroves V. Analysis of genome relationships in mangrove species using RAPD and RFLP markers

Abstract DNA from pooled leaf samples of 11 true major mangrove, three true minor mangrove, two mangrove associate, two mangrove parasite, three terrestrial and one cultivated species were isolated for the present study. In total, 198 random amplified polymorphic DNAs (RAPDs) and 180 restriction fragment length polymorphism (RFLP) loci were scored by using ten primers and 14 enzyme-probe combinations respectively. The polymorphism observed for these markers revealed a high degree of genetic diversity in mangroves at both inter-specific or inter-generic levels. A dendrogram, constructed after pooling both RAPD and RFLP data, using a similarity index was analysed for genome relationships among these species. The dendrogram showed clustering of all the major mangroves, except for *Nypa fruticans* (Arecaceae), into one group. All species under the tribe Rhizophorae formed a subcluster, to which *Xylocarpus granatum* was found to be the most closely related species. The clustering pattern implied that *Excoecaria agallocha* and *Acanthus ilicifolius* should be considered as true minor mangroves. The present study also provided molecular data favouring the separation of *Avicennia* spp. from the Verbenaceae to create a monotypic family the Avicenniaceae. The separation of *Viscum orientale* into the Viscaceae was also favoured.

Key words Mangroves · Genome relationship · Phylogeny · RAPD· RFLP

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Introduction

Mangroves are defined as halophytes, generally woody plants that inhabit the upper inter-tidal zones of estuaries, primarily within tropical and subtropical regions. The suggestion that 'mangal' be used as a term for the ecosystem (Tomlinson 1986), leaving the term 'mangroves' for the plants of the ecosystem (Macnae 1966), though convenient, has not generally been favoured (Mepham and Mepham 1985; Duke 1993). Therefore, the term 'mangrove' is used to refer both to the plant species and the ecosystem, and under this confusing context is referred to as mangrove species or mangrove ecosystem accordingly.

Botanists differentiate mangrove vegetation as being 'exclusive' species that are limited to the mangrove ecosystem (referred as true mangroves) and 'non-exclusive' species that are mainly distributed in a terrestrial or aquatic habitat but also occur in the mangrove ecosystem (referred as mangrove associates). Authors generally agree on which taxon belongs to which group. The true mangroves are further distinguished as major mangroves, which are tree species capable of forming dense pure stands, and minor mangroves, denoted by their inability to form a conspicuous element of the mangrove vegetation (Tomlinson 1986). However, no consensus among scientists could be reached in favour of this classification. For example, *Heritiera fomes* (Sterculiaceae), treated as a minor mangrove (Tomlinson 1986), is a tree species and forms dense stands in the Sunderban mangroves of India and Bangladesh. On the other hand, the predominant mangrove tree species *Rhizophora* do not form pure stands in many places such as the Godhavari (Andhra Pradesh), Muthupet (Tamil Nadu) mangrove forest in India.

The mangrove ecosystem harbours many terrestrial and marsh-land species. The number of such species

and their frequency of occurrence in a mangrove ecosystem varies to such an extent that few species are considered as mangrove associates by some authors (e.g. *Sesuvium portulacastrum*, Tomlinson 1986). Though forest disturbance is considered to be the major reason for the occurrence of these species, the fact remains that not all the species which have access to the ecosystem find a place in the 'disturbed' forest. For example, while the coastal terrestrial species *Clerodendron inerme* was recorded in the Pichavaram mangrove (Tamil Nadu, India), and was also reported to occur in other mangroves of India, Burma, Malaysia, Indochina, Philippines and the Pacific Islands (Chapman 1975), *Eichhornia crassipes*, 'visiting' the mangrove forest in bulk from the adjacent waterways during the rainy season, disappears soon after the season is over. The reason for the occurrence of only certain selected species could not be salinity tolerance alone. Therefore, research on the physiology and genetics of their ability to coexist with mangroves may shed light on the evolution of mangroves from terrestrial species both in the past and the present. As there is no acceptable classification to include such species, they are referred to as terrestrial species in this paper. In addition, species of specialised groups, like epiphytes, parasites and climbers, which are indiscriminate in their choice of host, are also found in mangroves.

The origin and distribution of mangroves is well documented. The geological history and evidence show that mangroves appeared between the Eocene and Oligocene periods (30–40 million years ago). They occupy a latitudinal range between 32°N and 38°S. This restricted distribution is due to the sensitivity of mangroves to frost and low temperature (Walter 1977). Within the mangrove habitats salinity plays a major role in the distribution pattern as each species has a specific tolerance range for salinity. The optimum salinity tolerance, therefore, varies from species to species, and at a salinity lower than the optimum other competitive species, better adapted to the local prevailing conditions, gain the upper hand (Snedekar 1978).

One of the recent reports lists 19 genera and 54 species, including a few hybrids, as true mangroves (Riclefs and Latham 1993). Twelve out of these 19 genera were included in the present study (see Table 1). The other species studied are *Porteresia coarctata* (Poaceae) reported from mangroves of India and Bangladesh; *Acanthus ilicifolius* (Acanthaceae), one of the three mangrove species of the genus *Acanthus* considered as a mangrove associate (Tomlinson 1986); *Sesuvium portulacastrum* of the Aizoaceae reported as a mangrove associate (Tomlinson 1986): the marsh-land plants *Suaeda maritima* and *Salicornia brachiata* of the Chenopodiaceae; parasite species like *Dendrophthoe falcata* (Loranthaceae, a parasite on *Rhizophora*), *Viscum orientale* (Viscaceae, ob-

served for the first time as a parasite on *Excoecaria agallocha* in Pichavaram mangroves, Tamil Nadu, India); and the terrestrial species *Clerodendron inerme* (Verbenaceae) which was frequently observed in mangrove forests. *Pandanus fascicularis* (Pandanaceae) and L*ycopersicon esculentum* (Solanaceae) were included as outgroups for the monocots and dicots respectively.

Although mangroves have been studied extensively, their taxonomy has been subject to revision (Duke and Jacks 1987; Juncosa and Tomlinson 1988; Duke 1991). Until recently, observation on the non-morphological characteristics of mangroves was practically nil, excepting some occasional reports on chromosome analysis (Kumar and Subramanian 1988) and isozyme studies (McMillan 1986). For various reasons molecular taxonomy using DNA markers was not taken up in mangroves (Parida et al. 1995). However, physical constraints and technical difficulties in conducting such studies in mangrove species have been overcome to some extent and DNA marker-based population studies have been reported recently (Parida et al. 1995; Parani et al. 1996; Lakshmi et al. 1997; Parani et al. 1997a, b; Parida et al. 1997).

There are a number of DNA-based marker systems for studying phylogeny, each with its own pros and cons. These markers are phenotypically stable and are not prone to environmental change. Generally, the Restriction Fragment Length Polymorphism (RFLP; Botstein et al. 1980) markers were considered acceptable for systematic studies and have been used extensively for depicting genetic relationship between taxa, as well as elucidating phylogenetic trends in a number of species like potato (Debener et al. 1990), tomato (Miller and Tanksley 1990), rice (Zheng et al. 1994), and fescue (Xu and Sleper 1994). However, the recently developed Random Amplified Polymorphic DNA markers (RAPDs, Williams et al. 1990; AP-PCR, Welsh and McClelland 1990) have tremendously increased the application of molecular-marker technology in plant genome analysis and evolutionary studies, mainly because they are quick, easy to perform and economical. A comparison of RAPDs and RFLPs in determining genetic similarity among *Brassica oleracea* genotypes proved that these markers provide equivalent levels of resolution for determining genetic relationships (Santos et al. 1994; Lakshmi et al. 1997). The successful application of RAPD markers in taxonomic and evolutionary studies has been documented in a variety of species including wheat, barley, rice, peanut, mustard, radish (see the review by Demeke and Adams 1994), rose (Millan et al. 1996) and pea (Hoey et al. 1996). In the present study, both RFLP and RAPD markers were used to describe variation in 22 species, representing an equal number of genera, at the molecular level, and a dendrogram depicting the genome relationship between important mangrove species has been established.

Materials and methods

Plant material

Twenty two species, each representing a genus, were included in the present investigation. The name of the species, their place(s) of collection, reported status, life form and its level of taxonomic isolation (of mangrove and mangrove-associate species) are described in Table 1. Leaf samples from individual plants of each species were collected separately and stored at $-70°C$ for DNA isolation.

Isolation of genomic DNA

An equal quantity of leaf tissue from ten individual genotypes of each species was pooled and total genomic DNA was isolated. DNA from *P. fascicularis* was isolated following the method described by Walbot (1988), except that the sample in suspension buffer was incubated at $45°C$ for 30 min as against $70°C$ for 15 min. For DNA isolation from *Rhizophora* and *Sonneratia*, 5 g of leaf tissue were ground to powder under liquid nitrogen and the powdered tissue was suspended in 30 ml of suspension buffer (pH 8.0) containing 50 mM EDTA, 100 mM Tris-HCl, 0.8 M NaCl, 0.5 M sucrose, 2% Triton X 100 and 0.1% β-mercapto ethanol, and incubated at $60°C$ for 30 min. The suspension was centrifuged at 5000 g for 10 min at room temperature. This process was repeated twice by re-suspending the pellet in the same buffer. The pellet was suspended in 20 ml of extraction buffer (20 mM EDTA, 100 mM Tris-HCl, 1.5 M NaCl, 2% cetyl trimethyl ammonium bromide and 1% β-mercapto ethanol, pH 8.0). The suspension was incubated at $60°C$ for 45 min followed by chloroform:isoamylalcohol (24:1) extraction and isopropanol precipitation at $-20°C$ for 2 h. DNA from the other species was isolated using the CTAB method (Saghai-Maroof et al. 1984) with minor modifications (Parani et al. 1997 a).

PCR amplification

Amplification of genomic DNA was carried out in 25-μl reaction mixture containing 10–20 ng of template DNA, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.001% gelatin, 2 mM $MgCl_2$, 100 μM dNTPs, 15 ng of primer and 1.0 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 45 cycles with 1 min (3 min for the first cycle) at $94°C$ for template denaturation, 1 min at $40°C$ ($37°C$ for microsatellite primers) for primer annealing,

Table 1 Details of the species used in the present study. T = Tomlinson 1986, S = Seanger et al. 1983, A = present authors

No.	Species	Family	Place(s) of collection	Status	Life form/level of taxonomic isolation
1	*Rhizophora mucronata* Lamk.	Rhizophoraceae	Pichavaram, Sunderban, Bhitarkanika	True mangrove	Tree/tribe
2	*Kandelia candel* L. Druce	Rhizophoraceae	Bhitarkanika, Goa	True mangrove	Tree/tribe
3	*Bruguiera cylindrica* L.	Rhizophoraceae	Pichavaram, Coringa, Goa	True mangrove	Tree/tribe
4	*Ceriops decandra* (Griff.) Ding Hou	Rhizophoraceae	Pichavaram, Bhitarkanika, Sunderban	True mangrove	Tree/tribe
5	*Hertiera fomes* Buch. - Ham	Sterculiaceae	Bhitarkanika, Sunderban	True mangrove	Tree/species
6	*Xylocarpus granatum* Konig	Meliaceae	Pichavaram, Bhitarkanika, Coringa	True mangrove	Tree/species
7	*Sonneratia apetala* Buch. - Ham	Sonneratiaceae	Coringa, Bhitarkanika	True mangrove	Tree/genus
8	*Avicennia marina* (Forsk.) Vierh.	Avicenniaceae	Pichavaram, Bhitarkanika, Goa, Calicut	True mangrove	Tree/family
9	*Aegiceras corniculatum* L.	Myrsinaceae	Pichavaram, Coringa, Bhitarkanika	True mangrove	Tree or shrub/tribe
10	*Lumnitzera racemosa* Willd.	Combretaceae	Pichavaram, Goa	True mangrove	Shrub or tree/genus
11	*Excoecaria agallocha* L.	Euphorbiaceae	Pichavaram, Calicut, Coringa, Muthupet	Minor mangrove[T]	Tree or shrub/species
12	*Acanthus ilicifolius* L.	Acanthaceae	Pichavaram, Calicut, Goa, Muthupet	Minor mangrove[S]	Shrub /species
13	*Suaeda maritima* Dumort.	Chenopodiaceae	Pichavaram, Coringa	Mangrove associate[A]	Herb/species
14	*Salicornia brachiata* Roxb.	Chenopodiaceae	Pichavaram, Madras, Pondicherry	Terrestrial	Herb
15	*Sesuvium portulacastrum* L.	Aizoaceae	Pichavaram, Madras, Goa	Mangrove associate[T]	Herb/species
16	*Nypa fruticans* (Thumb.) Wurmb.	Arecaceae	Madras (not a natural population)	True mangrove	Tree/genus
17	*Porteresia coarctata* Tateoka	Poaceae	Porto Novo, Pichavaram, Coringa, Goa	Minor mangrove[A]	Grass/genus
18	*Pandanus fascicularis* Lamk.	Pandanaceae	Pichavaram	Terrestrial	Herb
19	*Viscum orientale* Willd.	Viscaceae	Pichavaram	Parasite [A]	Herb
20	*Dendrophthoe falcata* (L.f.) Etting.	Loranthaceae	Pichavaram	Parasite	Herb
21	*Clerodendron inerme* Gaertner	Verbenaceae	Pichavaram, Pondicherry	Terrestrial	Herb
22	*Lycopersicon esculentum* Mill.	Solanaceae	Madras	Cultivated	Herb

and 2 min (15 min for the final cycle) at 72°C for primer extension. The amplified products were separated by agarose-gel (1.5%) electrophoresis in $1 \times$ TAE buffer (40 mM Tris-acetate and 1 mM EDTA, pH 8.0) and stained with ethidium bromide. Twenty primers with arbitrary sequences of ten nucleotides (random primers) and three microsatellites, $(GATA)_4$, $(GACA)_4$ and $(GTG)_6$, were tested for amplification.

Southern hybridisation

Genomic DNA was restriction digested with the enzymes E*co*RI and *Hin*dIII under the conditions specified by the supplier (Amersham, UK). About 8 μg of the digested DNA was fractionated in 1% agarose gel and transferred onto nylon membrane (Hybond N^+, Amersham, UK) by Southern blotting (Sambrook et al. 1989). The membranes were pre-hybridised in pre-hybridisation solution ($6 \times$ SSC, $5 \times$ Denhardt's reagent, 0.5% SDS, and 100 μg/ml denatured salmon sperm DNA), and hybridised in the same buffer with random prime labelled (Rediprime Labelling Kit, Amersham) probes overnight at 60°C. After overnight hybridisation, the membranes were washed once in $2 \times$ SSC plus 0.1% SDS and $1 \times$ SSC plus 0.1% SDS at room temperature for 15 min, followed by once in $1 \times$ SSC plus 0.1% SDS, $0.5 \times$ SSC plus 0.1% SDS and $0.1 \times$ SSC plus 0.1% SDS at 60°C for for 15 min. The membranes were exposed to X-ray film with intensifying screens at -70°C overnight. Each membrane was re-probed the same way to seven genomic clones from mangroves (Table 2).

Data analysis

Both RAPD and RELP bands were scored for presence/absence, ignoring the intensity of the bands (fragments). Similarity index in all pair wise combinations was calculated as $2m_{xy}/(m_x + m_y)$, where m_{xy} was the number of fragments shared by two species and m_x and m_y were the number of fragments in each species. Similarity index was calculated separately for RAPD and RFLP data as well as for the pooled data from both. Relatedness based on percentage similarity was established by constructing a dendrogram using MultiVariate Statistics Package (Kovach 1986) following unweighted pair group with arithmetic mean average (UPGMA) method (Sneath and Shokal 1973).

Results

In a preliminary study using RAPD markers, a dendrogram showing the genetic relationship between 20 mangrove species belonging to nine genera was constructed (data not shown). The dendrogram revealed that two species within a genus shared a common node, whereas if there were more than two species in a genus they formed a cluster at the node with only a narrow difference in similarity (data not shown). Later, a detailed study on three of the 20 species, namely *Avicennia marina*, *A. officinalis* and *A. alba*, using RAPD and RFLP markers also revealed close inter-specific relationships with a 63–84% similarity between the species (Parani et al. 1997 a). One species from each genus, therefore, was arbitrarily selected for the present study. It represented 11 true major mangroves, three true minor mangroves (including *P. coarctata*), two mangrove associates (including *S. maritima*), two mangrove parasites, three wild terrestrial species and one cultivated species (Table 1). Samples from these species were collected from different mangrove forests of India. Ten individuals from each species were selected at random, leaf tissues were pooled, and total DNA isolated as described in Materials and methods.

Among the 23 primers tested for PCR amplification, only nine random primers and $(GATA)_4$ showed distinct amplification products in all the species. These primers had either a 60 or a 70% G + C content, except for $(GATA)_4$ which had a 25% G + C content. Amplification with these ten primers was carried out three times and only consensus amplification products were scored for further analysis. The size of the amplified products ranged from 0.2 kb to 3.5 kb. The sequence of the primers, and the number of amplification products, are listed in Table 3. For RFLP analysis, genomic clones developed from the mangrove species *A. ilicifolius*, *A. marina*, *A. officinalis*, *E. agallocha*, *Bruguiera cylindrica* and *Rhizophora apiculata* were used as probes (Table 2). The membranes were washed at high stringency and exposed overnight. Since polymorphism with different enzymes for the same RFLP probe is not considered as independent, 14 different probes for the two restriction enzymes were used so as to have 14 independent enzyme-probe combinations. The probes and the number of loci detected are listed in Table 3.

The number of RAPD and RFLP loci detected per primer or enzyme-probe combination was much higher than that estimated earlier at the intra- and inter-specific levels in some mangrove species (Parani et al. 1996, 1997 a, b; Lakshmi et al. 1997). The number of RAPDs amplified by the primers varied from 16 to 24. Out of the 198 amplified products only two fragments amplified by the primer OPD06 were monomorphic across all the species. Each primer, except OPD06, produced

Table 2 Name and source of the genomic clones used as probes. Figures in paranthesis indicate the size of the inserts

No.	E*co*RI blot	*Hin*dIII blot	Source
1	ACP 101 (2.0)	ACP 201 (2.0)	*A. ilicifolius Pst* I/pUC18 library
2	AMP 105 (1.8)	AMP 262 (1.8)	*A. marina Pst* I/pUC18 library
3	EXP 202 (1.5)	EXP 102 (1.5)	*E. agallocha Pst* I/pUC18 library
4	BCP 012 (1.8)	BCP 006 (1.8)	*B. cylindrica Pst* I/pUC19 library
5	RAP 002 (3.2)	RAP 112 (3.2)	*R. apiculata Pst* I/pUC19 library
6	AOP 252 (2.5)	AOP 152 (2.5)	*A. officinalis Pst* I/pUC18 library
7	AME 082 (2.0)	AME 082 (2.0)	*A. marina EcoR* I/pUC18 library

Table 3 The number of loci scored among the 22 species against each primer and enzyme-probe combination

| RAPD analysis			RFLP analysis			
			*Eco*R I blot		*Hin*d III blot	
Primer	Sequence	No. of loci	Probe	No. of loci	Probe	No. of loci
OPA03	5′AGTCAGCCAC	24	ACP101	14	ACP201	16
OPA07	GAAACGGGTG	20	AMP105	8	AMP262	11
OPA11	CAATCGCCGT	18	EXP202	13	EXP102	17
OPA18	AGGTGACCGT	16	BCP012	16	BCP006	15
OPD04	TCTGGTGAGG	24	RAP002	15	RAP112	13
OPDO6	ACCTGAACGG	22	AOP252	12	AOP152	14
OPD08	GTGTGCCCCA	18	AME082	6	AME082	10
OPAR13	GGGTCGGCTT	21				
OPAF08	CTCTGCCTGA	19				
(GATA)$_4$	(GATA)$_4$	16				
Totals		198		84		96
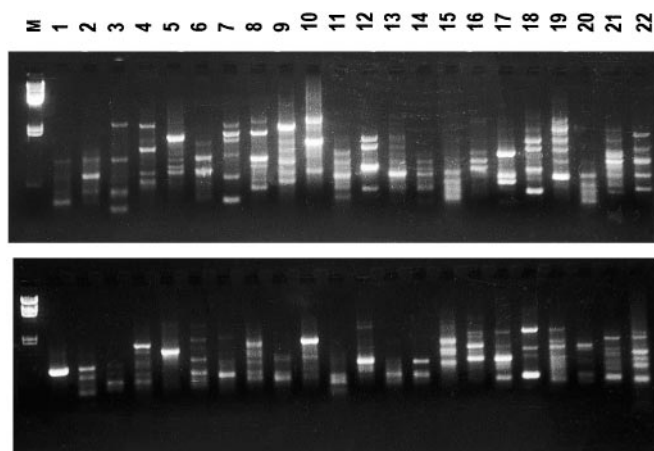
Fig. 1 RAPD profile of total genomic DNA from 22 species amplified by using random primer OPA11 (above) and microsatellite (GATA)$_4$ as a primer (below). *Lanes* 1–22 refer to the name of the species as given in Table 1. *M* is the lane loaded with *Hin*dIII-digested λ DNA as a size marker
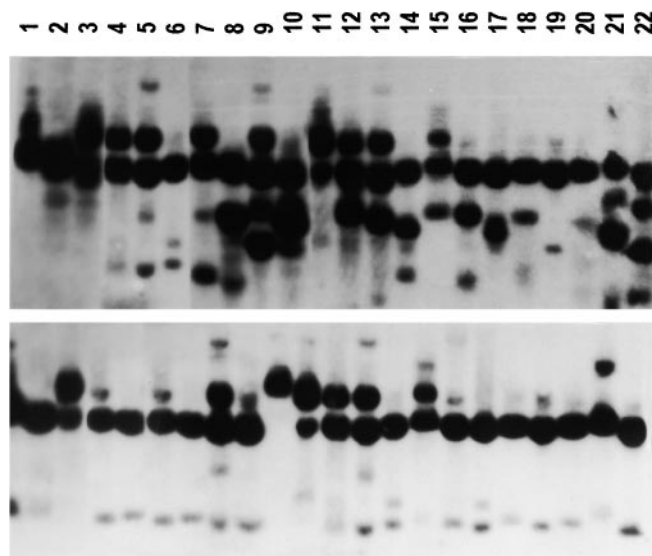

Fig. 2 RFLP profile of the total genomic DNA from 22 species digested with *Eco*RI and hybridised to the genomic clone from *A. ilicifolius*, ACP101 (above), and from *A. marina*, AMP105 (below). *Lanes* 1–22 refer to the name of the species as given in Table 1

species- specific fingerprints which were diagnostic. The RAPD profiles of the 22 species amplified by the primers OPA11 and (GATA)$_4$ are shown in Fig. 1. The number of RFLPs detected by individual enzyme-probe combinations varied from 6 to 17. In total, 180 RFLP loci were identified. All the fragments detected by the probes were polymorphic, except for six monomorphic fragments detected by AME 082, BCP 006 and EXP 202. However, RFLPs detected by single enzyme-probe combinations were not sufficient to obtain a species- specific pattern for all the species. RFLP profiles of the 22 species detected by the hybridisation of *Eco*RI-digested DNA to the clones ACP 101 and AMP 105 are shown in Fig. 2.

The presence/absence of amplification products in 198 RAPD and 180 RFLP loci was analysed independently, as well as by pooling the data, and three separate dendrograms were constructed. Although, a comparison of the dendrograms generated by the analysis of RAPD and RFLP data, both individually and in combination, revealed differences in the degree of relationship between certain groups, the pattern of clustering, by and large, remained the same (data not shown). Therefore, for the purpose of discussion on genome relationships, the dendrogram constructed after pooling the RAPD and RFLP data has been used (Fig. 3). This dendrogram showed a clustering of 12 species representing ten major mangrove genera and two mangrove associates, *Excoecaria* and *Acanthus*, into one major group. Within this group, *Bruguiera* and *Ceriops*, *Rhizophora* and *Kandelia*, *Avicennia* and *Aegiceras*, and *Excoecaria* and *Acanthus* shared common nodes.
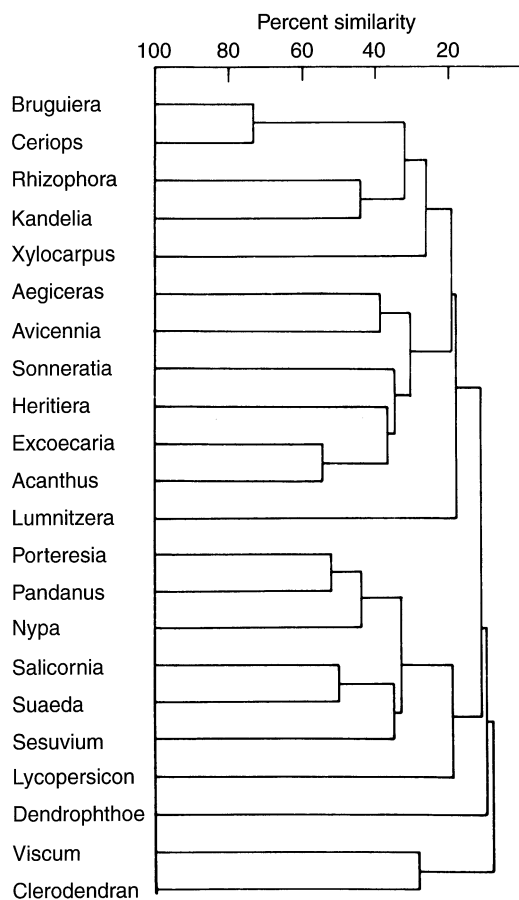
Percent similarity

Fig. 3 Dendrogram depicting the genomic relationship among 22 genera, each represented by one species, as given in Table 1. The dendrogram was constructed based on the percentage similarity calculated after pooling the data from 198 RAPD and 180 RFLP loci

Another major cluster included the mangrove species *Nypa fruticans*, the mangrove associates, the parasites, *P. fascicularis* and *L. esculentum*.

Discussion

Units of study in biology (from genes through organisms to higher taxa) do not represent statistically independent entities if we are to interpret rather than simply describe the results but rather are interrelated through their historical connections. Therefore, almost any comparative statistical analysis in biology requires information on phylogeny (Hillis 1997). Clades occupying the mangrove habitats which arose independently in at least 15 families (Ricklefs and Latham 1993) are taxonomically 'unrelated'. Diversity in mangroves is a widely accepted phenomenon which has been further supported by the molecular data from different mangrove species accrued in the recent past. RAPD analysis revealed 34, 74 and 76% intra-specific polymorphisms in *A. ilicifolius* (Lakshmi et al. 1997),

E. agallocha (Parani et al. 1996) and *A. marina* (Parani et al. 1997a) respectively. Added to this, the data from the present study showed that more than 95% of the RAPD and RFLP loci to be polymorphic at the intergeneric level. Nevertheless, mangroves have similar physiognomical, physiological and structural similarities as a result of convergent evolution (Yanney-Ewusie 1980). The mangrove community has a distinct commonality in respect of characteristics like tolerance to salinity and submergence, susceptibility to frost and low temperature etc. Therefore, despite the rich diversity in its forms there must be a line of relatedness among the flora which is as yet rather unexplored.

Exploring the phylogeny and genomic relationships in mangroves by conventional morphological and cytological means is difficult given the level of taxonomic isolation between the families from which they have evolved. In this context, RAPD and RFLP analyses, which basically testify to the degree of homology between any taxa irrespective of their taxonomic isolation, could effectively be employed to elucidate interrelationships at the molecular level. In many cases, the molecular data have supported, complemented, and helped to extrapolate the data from cytological and morphological observations. The present study, being the first of its kind in mangroves, provides molecular data from both RAPD and RFLP analyses of 22 species including 11 major mangroves. The primers used for RAPD analysis in the present study showed more discriminatory power than the enzyme-probe combinations used in RFLP analysis. This could be due to the nature of the probes and/or the limited number of enzyme-probe combinations used. Higher estimates of similarities at the inter-specific level using RAPD markers have also been reported before (Powel et al. 1996). For analysing the genomic inter-relationship among mangroves, as well as between mangroves and other terrestrial genera, a dendrogram constructed after pooling the data from 198 RAPD and 180 RFLP loci (Fig. 3) was employed.

The dendrogram showed the clustering of ten major mangroves, and two minor mangroves, *E. agallocha* and *A. ilicifolius*, into one group. A close examination of this group revealed many interesting features of association. At the 60% similarity level, a cluster between *Ceriops decandra* and *B. cylindrica* was observed and possessed the highest similarity (73%) among the mangroves. *Rhizophora mucronata* and *Kandelia candel* shared a node with 44% similarity and formed a cluster with the former at a 28% similarity level. These four species, representing the four genera of the tribe Rhizophorae (which is sometimes referred to as the Rhizophoraceae mangrove), are characterised by viviparity which is the most distinguishing feature of mangroves. They also belong to the salt-excluding type of mangrove which is reported to have an ultra-filtration mechanism in the roots for excluding salt. Among the other true mangroves analysed in the present study,

the genus *Xylocarpus* of the Meliaceae, represented by *X. granatum*, was found to be closest to the *Rhizophora* complex. The Rhizophoraceae and Meliaceae were earlier placed under same order, Myrtales; however, the Rhizophoraceae is now placed under a separate order, the Rhizophorales. This *Rhizophorae - Xylocarpus* complex was related to another cluster formed by *Aegiceras corniculatum*, *A. marina*, *Sonneratia apetala* (representing exclusive mangrove genera), *Heritiera fomes*, *E. agallocha* and *A. ilicifolius*. *Lumnitzera racemosa*, representing the exclusive mangrove genus of the Combretaceae, was found outside this cluster.

A. marina, which was earlier placed in the family Verbenaceae, shared a node with *A. corniculatum* of the Myrsinaceae which is another true mangrove, rather than with *C. inerme* which is a member of the Verbenaceae. This observation supports the separation of *Avicennia* species into a monotypic family, the Avicenniaceae, based on phenotypic characters (Moldenke 1967), though biochemical evidence does not favour such segregation (Reddy et al. 1993). While *Aegiceras* perpetuates itself through viviparous propagules, incipient viviparity in the seeds of *Avicennia* has been frequently reported. Both genera belong to the salt-excreting type of mangrove which possesses salt glands on the leaf surface that secrete excess salt out of the system. Moorthy and Kathiresan (1997) also reported a close relationship between *A. corniculatum* and *Avicennia* species in terms of photosynthetic efficiency.

Another node at 54% similarity was shared by *E. agallocha* and *A. ilicifolius*, which is a part of the cluster formed by other true mangroves. These two species were closely related to the true mangroves, in fact closer than one of the major mangrove species, *L. racemosa* (Fig.3). *E. agallocha* is widely distributed throughout the Asian tropics and mangrove locations. The physiological features of *E. agallocha* also showed its close relationship with other mangrove genera like *Avicennia*, *Bruguiera* and *Ceriops* (Moorthy and Kathiresan 1997). *Acanthus* is the only genus in the Acanthaceae which has a representation in mangrove communities. *A. ilicifolius* is widespread in almost all the mangrove formations in India. Therefore, based on the present study and on observations made earlier (Seanger et al. 1983; Tomlinson 1986), these two species could be considered as true minor mangroves rather than as mangrove associates. *E. agallocha* having a larger genome size (2n = 148), greater variability in genomic DNA and in its mitochondrial genome (Lakshmi et al., unpublished), as well as recombination through natural cross pollination facilitated by its dioecious nature, may help this species to evolve as a true major mangrove if certain genotypes are favoured by selection. However, this is probably not the case for *A. ilicifolius*, because even at the species level its phenotypic features do not differ much and there has been a tendency to treat the three species of the genus

Acanthus described from mangrove habitats (Steenis 1937) as a single variable species. Additionally, a detailed analysis of *A. ilicifolius* (2n = 48) showed only 34 and 45% polymorphism for RAPD and RFLP markers respectively (Lakshmi et al. 1997).

The only true major mangrove species which did not cluster with this group was *N. fruticans* (Arecaceae). This is not surprising because, being a monocot, *N. fruticans* (Arecaceae) would have more similarity with the other monocotyledous species included in the study, and therefore formed a cluster with *P. fascicularis* (Pandanaceae) and *P. coarctata* (Poaceae) outside this group. However, among the taxanomically diverse taxa ranging from *Rhizophora* to *Lycopersicon*, the clustering of all the true mangroves (except *N. fruticans*) into one group indicated that the homology among the mangroves is more than that between the mangroves and other genera. This observation is further supported by the fact that *A. marina* is closer to another mangrove species, *A. corniculatum*, than to a taxonomically related non-mangrove species, *C. inerme*. Therefore, it may be assumed that the clades occupying the mangrove habitats may not have evolved from certain randomly chosen families. Rather, they might have possessed a certain genetic homogeneity and physiological specialisation so as to become better adapted to the prevailing environmental conditions in the mangrove ecosystem. It is, therefore, important to account for the diversity of mangroves, and at the same time to recognise features (including those at the molecular level) common to all the mangrove species. However, whether this homology existed in the ancestral terrestrial vascular species and predisposed them to evolve as mangroves, and/or is the result of convergent evolution, is a matter for further investigation.

The other major cluster in the dendrogram included mangrove associates, parasites and other terrestrial genera. Within this group, there were two sub-groups. In one of these, *P. coarctata* showed a closer relationship with another herbaceous monocot, *P. fascicularis*, than with *N. fruticans*. *P. coarctata* can be treated as a true minor mangrove because it is reported only from mangrove habitats. In the other sub-group, the mangrove associate *S. maritima* (Chenopodiaceae) was more closely related to another genus of the same family, *S. brachiata*, than to *S. portulacastrum* of the Aizoaceae. *S. maritima* and *S. portulacastrum*, which are found abundantly both in the fringes as well as deep inside the mangrove forest, may be considered as mangrove associates. *V. orientale*, which was earlier placed under the Loranthaceae, did not share a node with *D. falcata* of the same family, and thus favoured its segregation into a monotypic family, the Viscaceae, based on morphological traits. This species was recorded for the first time as a parasite on *E. agallocha*.

By employing molecular markers the present study has helped to resolve the relationship between the

taxonomically diverse mangroves species. The information that *Xylocarpus* is closely related to the tribe Rhizophorae could not be reliably obtained though other conventional methods of phylogenetic analysis. The molecular markers showed a rather distant relationship between mangrove associates and true mangroves. However, they may share, or compete, for the same pollinators, or else share the same predators or parasites so that as alternative hosts their influence may not be entirely negligible. As diversity is perceived as the outcome of ecological interactions, particularly competition within small habitats, the increasing competition between mangroves and their associates, as evidenced by their deep and wide intrusion into mangrove habitats, may ultimately lead to the evolution of new species better adapted to ecological conditions in the coastal areas, particularly the estuaries. A close association of *E. agallocha* and *A. ilicifolius* (which are not popularly considered as true mangroves) with the other true mangroves observed in the present study seems to be a positive indication towards that end. Further elaborate study on the phylogeny of mangroves using molecular markers should help us to understand the evolution of these specialised taxa, and may ultimately give clues to the evolutionary pathways by which the highly specialised adaptive characteristics of mangroves have been achieved.

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