Theor Appl Genet (1997) 94:1121–1127

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Molecular phylogeny of mangroves I. Use of molecular markers in assessing the intraspecific genetic variability in the mangrove species *Acanthus ilicifolius* Linn. (Acanthaceae)

Received: 5 June 1996 / Accepted: 8 November 1996

Abstract Mangroves, the intertidal ecosystems occurring primarily in the tropical regions of the world, are valuable natural resources with high productivity and unique habitat value. However, the genetic structure of plant species within the mangrove ecosystem is poorly understood. The present communication is the first report on the use of molecular markers in assessing intra-site and intra-specific polymorphism in one of the mangrove species, *Acanthus ilicifolius*, for identifying/detecting distinct genotypes for long-term conservation. Random amplified polymorphic DNAs (RAPDs) and restriction fragment length polymorphisms (RFLPs) were used to elucidate the intra- and inter-population variability in this widely distributed mangrove species. In all, 48 genotypes representing eight distinct populations were analysed. A low level of polymorphism was detected at the intra-population level through both RAPD (3.8–7.3%) and RFLP (3.2–9.1%) analyses. At the inter-population level, 25 of the 73 RAPD loci (34%) detected through the use of 13 random primers and 44 of the 96 RFLP loci (45.8%) revealed through 15 probe/enzyme combinations were polymorphic. RFLP analyses were carried out using genomic clones developed from the same species. The somatic cells of the species displayed 48 chromosomes, with no numerical changes at either intra- or inter-population levels.

Key words Mangroves · *Acanthus* · Molecular markers · RAPDs · RFLPs · Cytology · Conservation

Communicated by G. E. Hart

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Introduction

Mangroves, which are associated with estuarine and coastline ecosystems, are quite diverse in ecological structure and dynamics. The plant species that form a part of this ecosystem are constantly under environmental stress due to high saline conditions and extreme temperatures and have adapted themselves to these frequent and fluctuating changes. These plants, which are either bushy shrubs or small-to-large trees with some associated herbaceous species, are of great significance because of their potential in protecting coastlines by preventing sea water inundation and maintaining coastal ecological balance. Detailed studies of these plants are lacking in all major areas, including physiological, cytological and molecular aspects, and the substantial accumulation of mucilage, latex, phenolics and other secondary metabolites in these plants make it a difficult system for protein and nucleic acid isolations and related studies.

The mangrove ecosystem is often over-exploited for various purposes. Large areas of mangrove forests throughout the world are being converted for agriculture or exploited for wood and other forest products (Snedaker 1978). In the Indian context, due to the absence of any national plan for conservation and sustainable utilisation, mangroves along the Indian coast have almost reached an alarming stage of depletion (Untawale 1985). In an effort to protect, restore and conserve this unique ecosystem, a multi-faceted approach has been initiated at our Centre. Studies on genetic variability and relationships within the mangrove ecosystem using molecular markers were initiated as an essential pre-requisite to our ongoing conservation programme. Molecular markers have been used to quantify accurately the extent of genetic diversity within and between populations (Chalmers et al. 1992; Waugh and Powell 1992). Unlike morphological markers, molecular markers are not prone

to environmental influences and do portray the genetic relationship between plant groups (Powell 1992, Brown 1979; Gottlieb 1977; Beckmann and Sollar 1986; Tanksley et al. 1989; McCouch and Tanksley 1991). These markers could be used to select priority areas for conservation and provide vital information for the development of genetic sampling, conservation and improvement strategies (Waugh and Powell 1992; Newbury and Ford-Lloyd 1993; Chalmers et al. 1994).

The present communication describes the results of a molecular-marker-based analysis of a mangrove species, *Acanthus ilicifolius* Linn. (Acanthaceae), which is present as distinct populations along the Indian coastline. The divergence at intra- and inter-population levels of this species was assessed by both cytological and molecular [restriction fragment length polymorphism (RFLP) and random amplified polymorphic DNA (RAPD)] parameters.

Materials and methods

Eight distinct populations of *A. ilicifolius* along the east and west coasts of peninsular India were included in the present study. The geographical location and physical characteristics of the populations are given in Table 1. Young leaves were collected from individual plants for DNA isolation. The number of individuals of each population used in the RAPD and RFLP analysed is given in Table 2.

Cytological studies

Mitotic chromosome numbers were determined from the root tips. Actively growing root tips, either from the germinated seeds or from the plants growing in plastic bags were pre-treated in water at 4°C for 24 h and fixed in 1:3 acetic acid and absolute alcohol for a minimum of 24 h. Fixed root tips were washed in water, hydrolysed for 30 min in 5 *N* HCl at room temperature and stained in Fuelgen stain (pH 2.2) for 1 h. Meristematic zones were squashed in 1% acetocarmine, and observations were made from temporary mounts.

DNA extraction

Genomic DNA was isolated from leaves following the CTAB method (Saghai-Maroof et al. 1984) with minor modifications. Five grams of tissue were ground in liquid nitrogen and suspended in 3 volumes of CTAB extraction buffer [2% CTAB (Cetyl trimethyl ammonium bromide), 100 m*M* Tris-HCl (pH 8.0), 20 m*M* EDTA and 1% β-mercaptoethanol]. The suspension was incubated at 60°C for 25 min, extracted with equal volume of chloroform:isoamyl alcohol (24:1) and centrifuged at 5000 *g* for 10 min. The aqueous phase was precipitated with a two-third volume of isopropanol at −20°C for 1 h. The pellet recovered by centrifugation at 10 000 *g* for 10 min was dissolved in TE (10 m*M* Tris-HCl, 1 m*M* EDTA, pH 8.0) and treated with RNase at 37°C for 1 h. The DNA was purified by phenol:chloroform extraction and ethanol precipitated in the presence of 0.3 *M* sodium acetate (pH 5.2). The pellet was dissolved in TE buffer, and the DNA concentration was estimated in 1% agarose gel.

RAPD analysis

RAPD analysis of genomic DNA was done using 10-mer random oligonucleotide primers (Operon Tech, Calif., USA). Each polymerase chain reaction (PCR) amplification reaction mixture of 25 µl contained 50 ng of genomic DNA, 2.5 µl of 10× assay buffer, 2 µl of 5 m*M* $MgCl_2$, 0.5 µl of 5 m*M* dNTPs (Pharmacia), 15 ng of primer and 1 unit of *Taq* DNA polymerase (USB). The reaction mixture was overlaid with 25 µl of mineral oil, and amplification was carried out in a DNA thermal cycler (Perkin-Elmer 480) programmed for 45 cycles. The first cycle was programmed for 3 min at 94°C, 1 min at 37°C and 2 min at 72°C, followed by 44 cycles of 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 amplified samples were electrophoresed in 1.5% agarose gels in 0.5× TBE buffer. Amplification with each primer was carried out in two replications.

Table 1 Geographic location and physical characteristics of the populations analysed

Sl. No.	Population	Location	Latitude and longitude	Annual rainfall (mm)	Salinity (ppt)	Soil pH	Dominant soil type
1	Pichavaram	Eastern coast	11°27′N 79°47′E	1300	23–49	6.2–8.0	Clay/fine sand
2	Karaikal	Eastern coast	10°98′N 79° 78′E	1300	12–21	6.1–7.5	Clayey, dry and compact
3	Pondicherry	Eastern coast	12°08′N 79°52′E	1250	10–25	5.8–7.6	Fine sand
4	Muthupet	Eastern coast	10°46′N 79°52′E	1280	6–18	5.9–7.3	Fine sand and clay
5	Bhitarkanika	Eastern coast	20°40′N 86°52′E	E1125	2–21	6.7–7.8	Fine silt/clay
6	Goa	Western coast	17°08′N 73°52′E	890	2–35	6.8–7.7	Lateritic rock and clay
7	Ratnagiri	Western coast	17°08′N 73°19′E	946	10–24	5.2–7.6	Lateritic rock with dark-gray soil
8	Calicut	Western coast	11°55′N 75°81′E	1380	6–18	6.2–7.4	Sandy and clayey soil

Table 2 Intrapopulation polymorphism

Population	RAPD analysis				RFLP analysis		
	Number of plants analysed	Number of primers used	Number of amplification products	Percentage polymorphism	Number of plants analysed	Total no. of RFLP fragments	Percentage polymorphism
1	7	16	78	3.8	5	31	3.2
2	6	16	74	4.1	6	33	9.1
3	6	18	83	4.8	6	29	6.9
4	6	16	72	6.9	6	33	6.1
5	5	16	79	5.6	5	36	8.3
6	6	18	86	6.9	6	27	7.4
7	6	14	69	7.3	6	29	6.8
8	6	15	72	5.6	5	33	6.1

RFLP analysis

Probe preparation

For RFLP studies, either PCR amplification products or genomic clones of *A. ilicifolius* were used as probes. PCR probes were made by amplification of the genomic DNA with 10-mer random primers followed by elution and purification of prominent high-molecular-weight (0.5–2.0 kb) PCR products. For genomic clones, genomic DNA libraries were made from size-fractionated *Pst*I- and *Eco*RI-digested DNA. Fragments with a size of 0.5–2 kb were eluted from the gel and ligated to either pUC18 or pUC19 plasmid vectors. Excised inserts or the whole plasmids were used as probes for the RFLP analyses (Sambrook et al. 1989).

DNA digestion and blotting

Ten micrograms of genomic DNA were digested at 37°C with *Eco* RI, *Eco*RV and *Hin*dIII. The fragments were separated by electrophoresis in 0.8% agarose gels in 1 × TBE (45 m*M* Tris-borate and 1 m*M* EDTA). The DNA from the gels was blotted onto Hybond N^+ (Amersham) membrane by Southern transfer (Southern 1975).

RFLP detection

Non-radioactive labelling of the probes was done using fluorescein 11-dUTPs. The blots were hybridised overnight at 60°C and washed in 0.1 × SSC and 0.1% SDS at 60°C for 30 min. After stringency washes, blocking, incubation and detection were carried out as per the manufacturers' instructions (ECL Random Prime Labelling and Detection Systems, Ver. 2, Amersham).

Data analysis

In both the RFLP and RAPD analysis, the presence or absence of the bands was taken into consideration and the differences in intensity among the bands was ignored. The RAPD analysis represented a consensus of two replicates. Pairwise comparisons were made using Nei's Index (Nei and Li 1979), and cluster analysis was carried out using the UPGMA (unweighted pair group mean average) method (Sokal and Sneath 1973).

Results

Cytology

The mitotic chromosome analysis was carried on samples collected from three populations (Pichavaram, Goa and Calicut) using root-tip meristem cells. The cells were characterized by the presence of 48 chromosomes resolved into 24 homomorphic pairs. The complements were characterized by chromosomes with mostly submedian centromeres. No variation in chromosome number was observed either in the plants belonging to the same or to different populations. Figure 1a and b show the metaphase chromosome complement of a plant from the Pichavaram and Calicut populations.

RAPD analysis

Forty-eight genotypes from eight geographically distinct populations of *A. ilicifolius* were selected for the present analysis. The samples were initially screened for intra-population variation using 15–18 random primers. The number of amplification products ranged from 69 to 86 for the different populations (Table 2), and polymorphism was between 3.8% and 7.3%. RAPD profiles of 7 genotypes of the Pichavaram population obtained for 5 random primers are shown in Fig. 2. The profiles were reproducible, as can be seen from comparisons between lanes 8–14 and lanes 22–27, which were obtained through the use of a single primer in two replicates.

On the basis of the results from RAPD analysis of intra-population variations, one representative sample from each population was selected, and the genomic DNA was amplified for 13 primers to account for inter-population variation. Between 3 (OPA 06) and 11 (OPA 19) amplification products were observed that ranged in size between 0.35 kb and 3.6 kb. A total of 73

amplification products were observed of which 25 were polymorphic in at least one pairwise comparison. Of the 13 primers tested, only 2 (OPA 03, OPA 06) revealed similar profiles for all the genotypes analysed representing all of the eight populations. Although for other primers, the RAPD profiles of these eight populations shared a number of common bands, population-specific profiles could be observed through the use of 1 or 2 primers. Figure 3 depicts the RAPD profile of one sample randomly selected from each population. The profile of each population is distinguishable.

RFLP analysis

Forty-five plants of *A. ilicifolius* representing eight populations were analysed for RFLPs. The level of polymorphism both within and between the populations was assessed using PCR-generated fragments and clones with low-copy sequences from the *Pst* genomic library as probes.

Genomic DNA of 5–6 genotypes from each population was digested with three enzymes and probed with 2 PCR-generated and 3 *Pst* genomic probes. The size of probes used was 0.5–2.0 kb. Table 2 lists the total and polymorphic RFLP fragments observed for each population using 15 probe/enzyme combinations. The size of the fragments ranged from 0.5 to 10 kb for *Eco*RI, 0.9–12 kb for *Eco*RV and 1.0–6.5 kb for *Hin*dIII. Figure 4 shows the intra-population RFLP pattern in 5 individuals from the Pichavaram (population 1) revealed through the use of two restriction enzymes and probed with a *Pst* genomic clone. The polymorphism in RFLP loci ranged narrowly from 3.2% (population 1) to 9.1% (population 2) (Table 2).

As the level of polymorphism at the intra-population level was negligible, we selected five populations (three from the western coast and two from the eastern coast) for RFLP analysis between populations. One genotype was analysed for each population. Here also, 15 probe/enzyme combinations were used. They produced a total number of 96 RFLP fragments of which 44 were polymorphic across the populations in at least one one-pair-wise comparison. The overall size of the fragments produced ranged from 0.8 to 7.0 kb for *Eco*RI, 0.3–9.0 kb for *Eco*RV and 0.35–8.0 kb for *Hin*dIII. Figure 5 shows the RFLP pattern in the five populations using two restriction enzymes and probed with a *Pst* genomic clone.

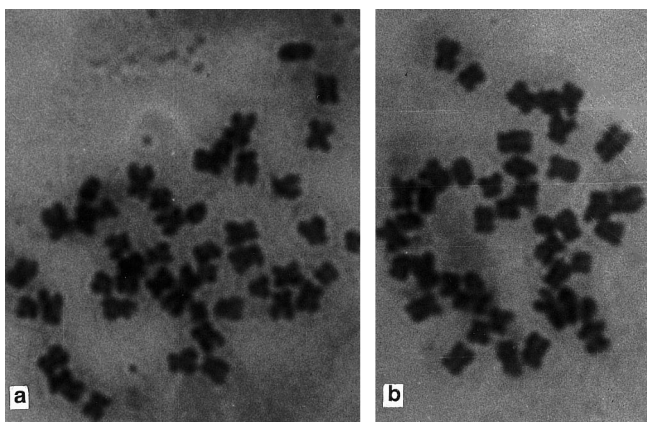

Fig. 1a, b Mitotic metaphase chromosome complements. **a** Pichavaram and **b** Calicut population

Cluster analysis

Pairwise comparisons were made for the RAPD profiles obtained through the use of 13 random primers in the representative samples of all eight populations. The five populations analysed from the eastern coast formed the main cluster separated at a DC (dissimilarity coefficient) of 0.38. In the subsequent analysis, two populations from the eastern coast and three from the western coast were surveyed for both RAPD and RFLP markers. The relationship between the five populations was analysed using 69 RAPD and 96 RFLP fragments of which 22 and 44 fragments, respectively were polymorphic. The two eastern populations

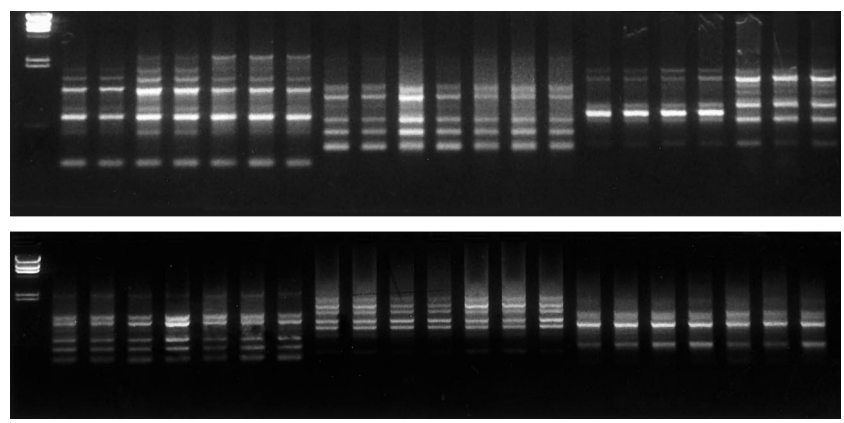

Fig. 2 RAPD profile in seven genotypes of Pichavaram population revealed using five random primers

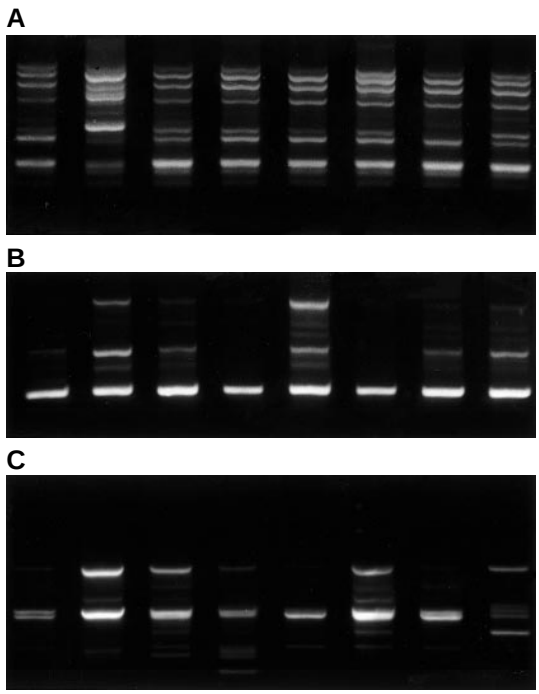

Fig. 3A–C RAPD profile in representative samples of eight populations revealed using three random primers. **A** OPA 08, **B** OPA 18, **C** OPC 12

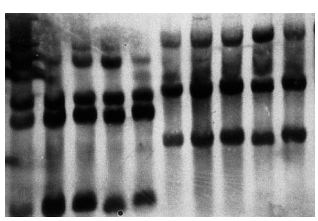

Fig. 4 RFLP pattern in five genotypes belonging to Pichavaram population with *Eco*RI and *Eco*RV following hybridisation with the *Pst* genomic clone of *A. ilicifolius*

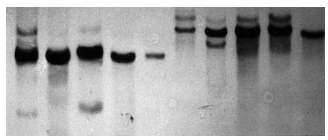

Fig. 5 RFLP pattern in representative samples from five populations with *Eco*RI and *Eco*RV following hybridization with the *Pst* genomic clone of *A. ilicifolius*

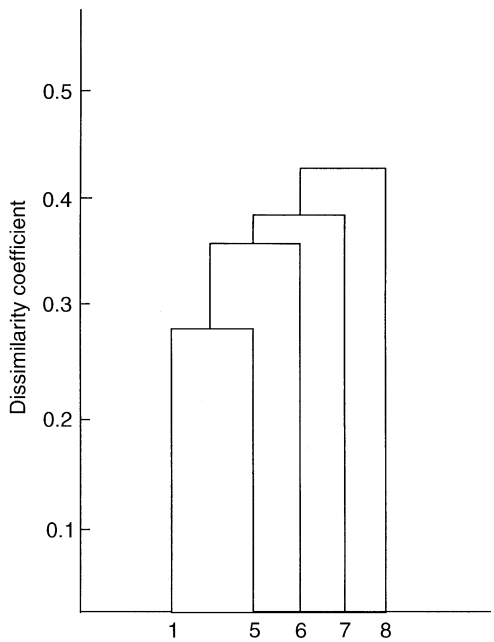

Fig. 6 Dendrogram depicting the inter-population differences

formed a distinct cluster separated at a DC of 0.28, and the three populations from the western coast were separated from this cluster at a DC of 0.36, 0.39 and 0.42 (Fig. 6).

Discussion

Mangroves are recalcitrant plants where both physical and systemic constraints are obvious. Information on the reproductive biology and population genetics of mangrove species, to a large extent, are lacking. Conventional genetic analysis is difficult in mangrove species, and so far no detailed studies have been carried out on this unique group of plants. The information available on the genetic structure of mangrove species, is scanty and largely restricted to some stray reports on chromosome number and isoenzymes (Kumar and Subramanian 1988; McMillan 1968). On the basis of the information avilable, it is not possible to partition the existing variation in mangrove species into environmental and genetic components. To aid us in our on-going conservation programme, we have initiated investigations to document both intra-site and inter-population polymorphism in various mangrove species using molecular markers, with an overall objective of selecting/identifying distinct genotypes for long-term conservation.

The present analysis of 48 genotypes of *A. ilicifolius* representing eight distinct populations of Indian coastline reveals a low level of genetic differences between the genotypes at the intra-population level. Although 4–6% of amplification products in these cases were polymorphic across the genotypes, the RFLP profiles were largely uniform between the individuals of a given population. At the inter-population level, the polymorphism in RAPD loci was about 28%. This was the case with the RFLPs also. The observed low level of

polymorphism at the intra-population level could therefore broadly speak of a stable genetic constitution of the species. In the case of chromosome analysis also, no numerical and/or structural variations were observed.

Mangrove species are constantly subjected to physiological stress caused by fluctuating growing conditions Chapeker (1994). Despite such extremes, they have successfully colonized suitable areas by developing morphological, physiological and reproductive adaptations (Clough et al. 1982; Clough 1994; Saenger 1982). Therefore, depending on the genetic architecture of these species and their edaphic preferences and adaptations, different species are likely to display varying degrees of polymorphism. Present observations on *A. ilicifolius* and our results from studies on other mangrove species (Parani et al. 1996; Parida et al. 1995; Lakshmi et al. unpublished data) do support this presumption. In fact, the extent of polymorphism in *Excoecaria agallocha*, a dioecious mangrove tree species, was of much higher magnitude; intra-population polymorphism ranged from 20.4% to 31.0%, while as much as 65% of the amplification products were polymorphic at the inter-population level (Lakshmi et al. unpublished data).

A. ilicifolius is an opportunistic colonizer of suitable habitats (Clough 1994) and shows wide-spread occurrence in all types of mangrove formations inhabiting swampy sites away from the sea coasts. The observed inter-population differences could be ascribed to the fluctuating microclimatic conditions in the locations where these populations inhabit (Table 1). However, the overall polymorphism in this species is of lower magnitude. Therefore, the collection of samples from geographically and physically distinct populations would be a better option for conserving the diverse gene pools of this species, than selection within a particular population. Based on our present observations, we have selected, distinct genotypes (identified through molecular marker analysis) from each population for conservation. An *in situ* Genetic Resources Centre has been established in the Pichavaram Mangrove Forest for assembling these distinct genotypes for long-term conservation. This is a part of an anticipatory research programme designed for consolidation of genetic material capable of facing the altered growing conditions in the coastal regions that may arise in future from a rise in the sea level due to global warming. In conclusion, our results demonstrate that molecular markers provide an effective tool to assess the existing genetic polymorphism in an otherwise difficult mangrove species and to design the conservation strategy.

Acknowledgements We gratefully acknowledge Prof. M. S. Swaminathan for his valuable suggestions. We appreciate the help received from N. Mathan during the collection of samples. This work was carried out with financial assistance from the Department of Biotechnology, Govt. of India and IPGRI, Rome.

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