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Genetic differentiation between *Bruguiera gymnorhiza* and *B. sexangula* in Sri Lanka

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Abstract

The identification of populations of *Bruguiera sexangula*, *Bruguiera gymnorhiza* and their putative hybrids in the field is difficult using only morphological and phenological characters. Using a PCR based technique, RAPD (Random Amplified Polymorphic DNA), the genetic variation of *Bruguiera* populations was studied from contrasting climatic and geographic regions along the southwest coastal region of Sri Lanka. Out of 45 primers screened, 20 primers allowed us to observe polymorphism, not only between species (interspecific) but also within the species (intraspecific). Analysis of RAPD data appears to be helpful in determining the genetic relationship among populations of *B. gymnorhiza* and *B. sexangula*. RAPD markers revealed that the two species are well separated without any hybrid position between the two taxa though they occur in mixed stands. Although sampling sizes of populations of this study were small, genetic variation among *B. gymnorhiza* and *B. sexangula* populations could be observed. For *B. sexangula*, it was possible to differentiate each of the three populations, even when using a small number of primers.

Introduction

Mangroves or mangals are tropical and subtropical forests developed in the intertidal zone (Arulchelvam, 1968; Lindén & Jernelöv, 1980; De Silva & Balasubramaniam, 1985; Tomlinson, 1986; Amarasinghe & Balasubramaniam, 1992; McKee, 1995; Pemadasa, 1996). Though mangroves occur in tropical and subtropical areas of the world, the most luxuriant mangrove forests are found in Southeast Asia (Lindén & Jernelöv, 1980). The present extent of mangroves in Sri Lanka has been estimated at 4000 ha (Pemadasa, 1996). Sri Lankan mangroves include approximately 23 mangrove species (Jayawardene, 1985) from nine genera (*Rhizophora*, *Bruguiera*, *Ceriops*, *Avicennia*, *Xylocarpus*, *Excoecaria*, *Lumnitzera*, *Aegiceras* and *Nypa*: definition according to Tomlinson, 1986).

Mangroves are ecologically important components of the coastal ecosystems because they have developed on sheltered shores of lagoons and estuaries, and because they prevent sea erosion and act as a land building system. Physiologically and morphologically, they have various features for extremely harsh and hostile dry atmospheric conditions and wet, anoxic and saline root environments.

Economically, the mangroves are providing suitable habitats for crustaceans and fish, and they deliver plant products such as tannins and dyes, timber, resources for alcoholic beverages and food (Lindén & Jernelöv, 1980; De Silva & Balasubramaniam, 1985; Jayawardene, 1985; Tomlinson, 1986; Pemadasa, 1996; Hellier, 1988). As a result of human activities, extensive mangrove areas are being lost at an alarming rate (Lindén & Jernelöv, 1980; Jayawardene, 1985; Hellier, 1988; Pemadasa, 1996).

Despite the great interest in their conservation, in management of mangrove species (Jayawardene, 1985; Dodd et al., 1995; Farnsworth & Ellison, 1997)

and in their intrinsic value as a genetic resource, relatively few genetic studies have been conducted. Some studies have been reported on the genetics of flowering time and morphological characters (Duke, 1984; Duke, 1995). Isozymes (McMillan, 1986; Goodall & Stoddart, 1989) have been used rarely for a systematic assessment of genetic variability in mangroves. Albino genotypes occurred by either nuclear or plastid genome mutations of *Rhizophora mangle* L. and have been fingerprinted (Corredor et al., 1995; Klekowski et al., 1996). Variation among *Rhizophora* had been studied using epicuticular wax composition, hydrocarbons and triterpenoids (Dodd et al., 1995), using RAPD (Random Amplified Polymorphic DNA) and restriction fragment length polymorphism (Parani et al., 1997b). Traditional measurements of genetic diversity of plants rely upon the ability to differentiate morphological, physiological or biochemical characters (Khasa & Dancik, 1996). Though *Bruguiera* species can usually be distinguished by morphological (Arulchelvam, 1968) and phenological traits (P. L. Hettiarachchi, pers. com. University of Sri Jayawadenepura, Sri Lanka), most of the morphological characters used as descriptors can only be assessed at maturity. Particular phenological traits can only be measured at certain times of the year, while other morphological traits require extensive observation during the whole growing season of the plant. In the field, hybridisation and introgression (putative hybrids or intermediates) between *B. sexangula* and *B. gymnorhiza* can be observed, when using morphological criteria.

RAPD has been shown to be a very useful technique for the construction of genetic maps (Tingey & Del Tufo, 1993), DNA fingerprinting (Welsh & McClelland, 1990; Williams et al., 1990), the identification of genetic polymorphism and parentage determination (Welsh et al., 1991).

Despite the great interest in management and conservation of tropical tree species, a very limited number of researches have been conducted on the population genetics of tropical tree species (Dawson et al., 1996; Parani et al., 1997a,b; Schierenbeck et al., 1997). The identification of a species, information on the geographic structure and precise understanding of the organisation of the existing genetic diversity within and among populations are very important for the conservation of genetic resources (Triest, 1991; Aldrich et al., 1992; Russell et al., 1993; Dawson et al., 1995, 1996). For management and conservation purposes, the identification of *B. sexangula* and *B. gymnorhiza* and their putative hybrids or introgressed intermediates, even at the very young stage of their life cycle e.g. seedling stage, could be accomplished using RAPD markers.

The objective of this paper was to evaluate the use of RAPD to genetically differentiate the two sympatric *Bruguiera* species which originate from different geographic and climatic areas in Sri Lanka. Consistent DNA amplification products should be selected to differentiate *B. sexangula* and *B. gymnorhiza* and assess the possible occurrence of hybrids or intermediates.

Materials and methods

Sample collection

Leaf material was collected from populations of *B. sexangula* and *B. gymnorhiza* (nomenclature according to Tomlinson, 1986 except for *B. gymnorhiza*) in order to cover the contrasting climatic zones (wet zone, dry zone and intermediate zone) and different geographical areas of the island (Figure 1). A total of six localities were chosen from the western to the southeastern coastal region of Sri Lanka (Table 1). Climatically the intermediate zone covers the sampling sites in Pambala (P) and Rekawa (R, disjunct along the coast) which are well separated geographic locations in the intermediate zone of the island. Merawala (M) is located in the dry zone of the island. Dondra (D) and Kudawella (K) are geographically close in the wet zone, while Negombo (N) is at the limit of the wet zone (Müller-Dombois, 1968). These locations were selected in order to resolve the maximal geographic variation on the island. During collection, morphological criteria were applied to classify populations as either *B. sexangula* or *B. gymnorhiza* (Arulchelvam, 1968).

DNA extraction and amplification

Total genomic plant DNA was extracted from young frozen leaf samples. Leaf tissue was ground to a powder in liquid nitrogen using a mortar and pestle, and then 5 ml of extraction buffer was added. The extraction buffer consisted of 3% of CTAB (hexadecyl trimethyl ammonium bromide), 2 M NaCl, 100 mM Tris–HCl pH 8.0, 1% β-mercaptoethanol, 20 mM EDTA and 1% PVP (polyvinyl polypyrrolidone). The homogenate was incubated at 65 °C for 30 min, 5 ml of chloroform/isoamyl alcohol (24:1) was added and the mixture was centrifuged at 3000 g for 10 min. The supernatant was transferred to a new 50 ml

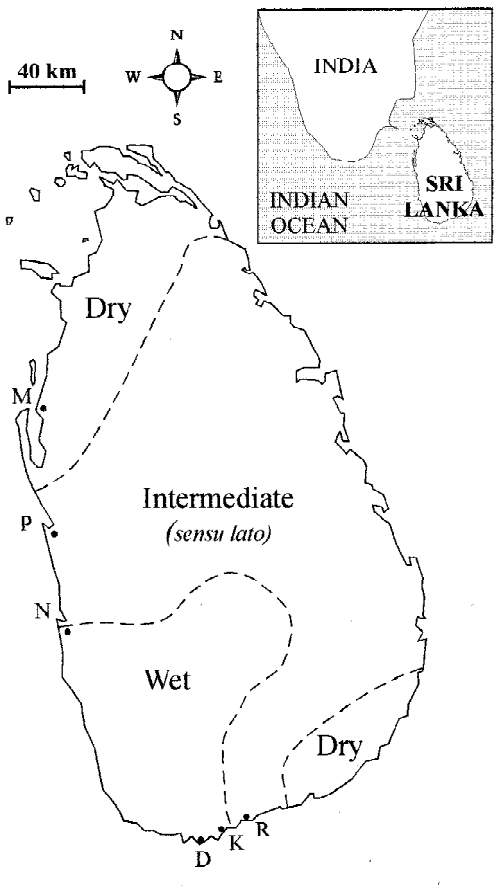

Figure 1. Geographical distribution of sampling locations for *B. gymnorhiza* and *B. sexangula* in Sri Lanka: Merawala (M), Pambala (P), Negombo (N), Dondra (D), Kudawella (K) and Rekawa (R). Adapted from Dahdouh-Guebas et al.

Table 1. The location, climatic zones and number of individuals of *B. gymnorhiza* and *B. sexangula* investigated for RAPD (climatic zones according to Müller-Dombois, 1968)

Species		Climatic zone	No. of individuals
B. gymnorhiza	Merawala (M)	Dry	02
	Negombo (N)	Wet	10
	Pambala (P)	Intermediate	01
	Rekawa (R)	Intermediate	04
B. sexangula	Rekawa (R)	Intermediate	06
	Kudawella (K)	Wet	06
	Dondra (D)	Wet	04

centrifuge tube (Greiner) which was filled with chloroform/isoamyl alcohol (24:1) and centrifuged again for 10 min. Once again, the supernatant was transferred to a new 50 ml tube. DNA was precipitated by mixing with 0.6 volume ice-cold isopropanol, then kept at room temperature for 15 min and pelleted by centrifugation at 7700 g for 15 min. The supernatant was removed, washed with 70% ethanol to get rid of salts, air dried and dissolved in sterile water. This was treated with 10 mg/ml RNase A (Sigma). The quality of the DNA was monitored by scanning with a UV spectrophotometer from 200 nm to 300 nm. The concentration of the DNA samples was estimated by gel electrophoresis against a standard DNA (lambda-DNA digested with Pst 1). Amplification was carried out according to Williams et al. (1990) and amplification products were size-fractionated on a 1% agarose gel containing 10 μg/ml of ethidium bromide. They were visualized under UV light and photographed for further analysis.

Forty five decanucleotide primers were screened using the template DNA from eight individuals from *B. sexangula* and *B. gymnorhiza* to identify informative primers that generated polymorphic patterns of PCR (Polymerase Chain Reaction) products. Fragments were considered polymorphic when both absence and presence of the same fragment in the same species was observed and monomorphic when a fragment was present among all individuals within the species. Reproducibility of the amplified products was tested repeating the same reaction at least twice. No amplification was found in the control sample in which no DNA was added to the reaction mixture indicating that all the fragments observed in other samples are not artefacts, but due to the amplification of genomic DNA (Williams et al., 1990). A similarity matrix using the simple matching coefficient and a Principal Coordinate analysis (PCOORDA) were performed using NTSYS-pc (Rohlf, 1993).

Results and discussion

Primer screening and discrimination of B. sexangula *and* B. gymnorhiza

The sizes of the amplification products were 0.5–2.8 kb. According to the patterns of the amplified products, primers were categorized into three groups which were interspecific, intraspecific and both (Table 2). Out of 45 primers, 20 informative primers

Table 2. The number of primers used for amplification of genomic DNA from *B. gymnorhiza* (Bg) or *B. sexangula* (Bs) and the number of amplified products (loci) generated for each primer

Primer	Sequence	No of polymorphic band		
		Bg	Bs	Both
OPA-14	TCTGTGCTGG	01	–	02
OPA-15	TTCCGAACCC	01	–	01
OPE-07	AGATGCAGCC	02	–	02
OPE-14	TGCGGCTGAG	–	–01	
OPE-15	ACGCACAACC	02	–	01
OPE-16	GGTGACTGTG	01	02	–
OPJ-07	CCTCTCGACA	–	0101	
OPJ-11	ACTCTCGACA	01	0101	
OPJ-12	GGGTGTGTAG	–	–	02
OPJ-20	AAGCGGCCTC	–	0102	
OP0-15	TGGCGTCCTT	02	—-	
OPT-06	CAAGGGCAGA	01	0101	
Total	12	11	06	14

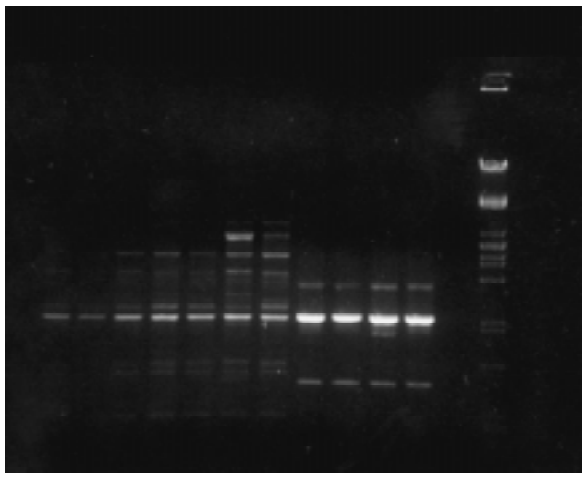

Figure 2. PCR amplification of genomic DNA from *B. gymnorhiza* and *B. sexangula*, with primer OPE-16 showing 3 RAPD markers. DNA. Lane 1–7 is *B. gymnorhiza*, lane 8–11 is *B. sexangula*, and lane 13 is the molecular weight marker (L-Lambda DNA digested with Pst I). The negative control (lane 12) contained all reagents except template. The 0.9 kb fragment is present in both *B. gymnorhiza* and *B. sexangula* while 1.6 kb and 0.7 kb fragments are present only in *B. sexangula*.

revealed simple, unique, easily resolved bands and one or more RAPD markers. Six primers showed amplification without detection of polymorphism while two primers gave a smear pattern. Eleven primers produced complex banding patterns that were difficult to interpret and were eliminated from the analysis. The remainder (six primers) failed to amplify. The individuals showed the same amplification pattern throughout the repeats.

In this study, with 12 primers, 31 RAPD bands (loci) were obtained for both *B. gymnorhiza* and *B. sexangula*. Out of 31 markers, 14 marker alleles were present in both species (Table 2). As an example, the decanucleotide primer OPE-16 primed the amplification of eight and three fragments of *B. gymnorhiza* and *B. sexangula*, respectively. One fragment, (0.9 kb) was present in both *B. gymnorhiza* and *B. sexangula*, while the amplified fragments of 1.6 kb and 0.7 kb could only be observed in samples of *B. sexangula* (Figure 2). Amplification with primer OPO-15 showed a diagnostic and unique banding pattern for *B. gymnorhiza* and *B. sexangula* also. These results indicated that RAPD markers allow us to differentiate *B. gymnorhiza* and *B. sexangula* unambiguously and provided a useful technique for studying genetic variation among populations of *B. gymnorhiza* and *B. sexangula*.

Genetic differentiation of B. gymnorhiza *and* B. sexangula

In order to assess the inter and intraspecific variation of *Bruguiera* populations, all 31 diagnostic RAPD markers were analyzed. The computation of the simple matching coefficient using 31 RAPD loci and subsequently PCOORDA revealed variation between the two species, and without exception *B. sexangula* and *B. gymnorhiza* were separated into two distinct groups along the first coordinate axis which represents 65% of the total variation (Figure 3). Though populations displaying morphological characters of both species were observed, no genetic hybrids or intermediates of *B. sexangula* and *B. gymnorhiza* could be observed in this study, indicating that these sympatric species must have a separate gene pool. RAPD analysis and tentative morphological identifications are correlated, however an evaluation of intra and interspecific variations of *Bruguiera* populations in the field is difficult and morphological descriptors may have a utility in describing complex genetic relationships.

The genetic similarity values for *B. sexangula* ranging from about 69% to 92% indicates a possible narrow genetic base for *B. sexangula*. Multivariate analysis on RAPD markers separated the *B. sexangula* populations along the second coordinate axis into groups according to their different geographical

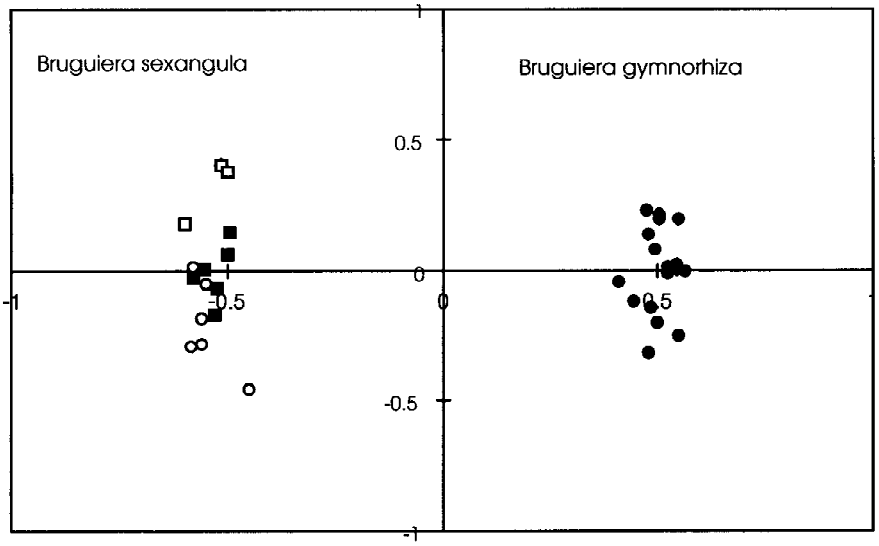

Figure 3. Principal coordinate analysis of first two components based on 31 RAPD markers for *B. gymnorhiza* and *B. sexangula* from Sri Lanka. Each symbol represents one individual. *B. sexangula*: open squares, Dondra; solid squares, Rekawa and open circle, Kudawella and solid circles, all *B. gymnorhiza* individuals.

regions. Two *B. sexangula* individuals from Dondra (small population) were identical. Genetic variation within each of the populations of *Bruguiera* may vary with the population size. Therefore, we propose to compare the small forest area at Dondra with the large population area of Negombo.

Although the present study was an initial geographical survey at species level, RAPD markers indicated possible variability among and within populations of *B. sexangula* and *B. gymnorhiza* e.g. 10 different genotypes were observed among the 10 Negombo samples which originated from the wet climatic zone. The major differences between populations may reflect the geographic differences. A next working hypothesis, therefore, would be to check whether genetic differentiation among populations of *Bruguiera* species are connected to the geographical regions that differ in their climatic regions.

Our results showed that geographical variation of populations of *B. sexangula* which originated from different locations along a coastal belt of Sri Lanka could be observed using only a few random primers. Unfortunately, there are no reports on *Bruguiera* species to compare genetic variation with our results. However, based on isozyme studies on *Avicennia germinans* (L) L. (McMillan, 1986), genetic variation has been observed on large scale geographic areas, while for an Australian mangrove, *Rhizophora stylosa* Griff., little isozyme variation had been observed. Studies with RFLP (Restriction Fragment Length Polymorphism) and RAPD of different *Avicennia* species originating from India revealed low genetic variation among populations (Parani et al., 1997a).

In conclusion, this study demonstrated that decanucleotide DNA fingerprinting was an effective method to select markers at species level and to assess the possibility of hybridisation among species. An additional working hypothesis is to get a better picture of genetic diversity within the genus based on the size of the populations with a broader array of samples and based on their origin in different climatic regions.

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