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Phylogenetic relationships among three species of the mangrove genus *Avicennia* found in Indian Sundarban, as revealed by RAPD analysis

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ABSTRACT

Random amplified polymorphic DNA (RAPD) markers were successfully used to identify and differentiate all the three species of Avicennia found in Indian Sundarban, to assess the pattern of interspecific genetic diversity among them and to reveal their molecular phylogeny. Ten primers amplified a total number of 221 bands under 100 loci across three genotypes with an average of 10 loci / primer. Of the total 100 loci scored, 50 were polymorphic. Thus the average percentage of polymorphism was 50. Different primers yielded a total of 7 unique bands for A. alba, 14 for A. marina and 8 for A. officinalis. The distinct RAPD bands could be cloned and used as markers for species identification. The dendrogram reveals that A. alba and A. officinalis have closer relationship between them and share a common node in the dendrogram at a 73.3% level of similarity. In comparison to other species, A. marina showed most genetic variability, suggesting utilization of this species over others in breeding programmes. Genetic distance was less between A. marina and A. alba than between A. marina and A. officinalis. Phylogenetic tree shows that A. marina is more closely related to A. alba than to A. officinalis. Extent of polymorphism and uniqueness reveals lowest genetic diversity in A. alba and therefore, demands priority of this species in conservation programme to prevent extinction.

Key words: *Avicennia;* Genetic Diversity; Indian Sundarban; Molecular Phylogeny; RAPD.

INTRODUCTION

The Sundarbans, shared between India and Bangladesh is the largest mangrove forest in the world. Mangrove plants comprise a heterogeneous group of independently derived lineages that are defined ecologically by their occurrence in tidal zones along shorelines and physiologically by their ability to withstand high salt concentrations [1]. Tomlinson [2] (1986) and Duke [3] (1991) classified *Avicennia* L., a genus of mangrove woody trees or shrubs, to eight species. In the Indian Sundarban, the genus is represented by three species, namely *Avicennia marina* (Forsk.) Vierh., *Avicennia offlcinalis* L., and *Avicennia alba* Blume [4] .

Avicennia plays an important role in coastline wetland ecosystems. They stabilize shores and prevent excessive shifting of coastlines and soil erosion resulting from tidal currents [5]. They also buffer the destructiveness of wind and storm tides during hurricane and typhoons [6]. In addition to their ecological importance, they also provide many forest products, such as firewood, timber, materials for making boats and paper, and feeding grounds for fish, prawns and shellfish. *Avicennia* leaves make a superior fodder due to their high salt and iodine content [7]. They have been used as traditional medicine in South Asian countries including India [8].

Overexploitation of mangroves for their economical and medicinal values has resulted in the loss of genetic diversity [9]. With global warming, environmental change and human activities, mangroves are being destroyed at an alarming rate [10]. More importantly, habitat destruction has remained the major threat for mangrove plants [11]. To overcome these losses, conservation and sustainable management is, thus, a major priority in the coastal areas of many countries. However, the genetic structure of plant species within the mangrove ecosystem is poorly

understood. Studying the genetic diversity of mangrove plants is important in taking effective measures to protect these species [12].

Molecular markers, unlike morphological markers, are not prone to environmental influences and have been found to be very useful to quantify accurately the extent of interspecific genetic diversity [9] and portray genetic relationships between plant groups [13]. DNA markers provide an opportunity to characterize genotypes and to measure genetic relationships more precisely than other markers [14]. DNA based genetic markers have been recently integrated into the study of several plant systems and are expected to play a very important role in the future of molecular genetics and plant taxonomy. Among the various DNA markers, randomly amplified polymorphic DNA (RAPD) has been used extensively for a variety of purposes including large scale genetic diversity studies [15]. It has widespread application due to technical simplicity and the availability of large numbers of arbitrary primers that saturate the genome. PCR based RAPD markers have extensively been used to quantify accurately the inter- and intra- specific and inter-generic variability in different plant groups including some mangrove species [16].

The research work on genetic variation among *Avicenniaceae* family using molecular methods is inadequate. Duke et al. (1998) [17] examined the genetic structure and evolution of species of *Avicennia* based on allozyme analysis. However, the allozyme study did not include *A . officinalis*. Parani *et al*., (1997) [18] reported an intra and inter-specific variation in *Avicennia* genus, revealed by molecular markers from other locations of India, except Sundarban. Kader *et al.,* (2012) [16] reported on the genetic diversity of *Avicenniaceae* family but did not shed any light on phylogeny.

The objective of this work was to reveal the genetic diversity among all the three species of *Avicennia* present in Sundarban using RAPD analysis and to construct a phylogenetic tree showing evolutionary relationship among them. The fingerprint generated would also help in the identification and differentiation of these three closely related species. Moreover this study will provide important clues in developing effective conservation strategies for the sustainable management of the genetic resources in order to prevent potential extinction.

MATERIALS AND METHODS

Plant Materials

Young, fresh and healthy leaf samples of *Avicennia marina* (Forsk.) Vierh., *Avicennia offlcinalis* L., and *Avicennia alba* Blume. were collected from various sites in the mangrove forest of Sundarban, West Bengal and stored with silica gel in separate zip-lock plastic bags. From each of the sites, seven individuals of each species were randomly selected and leaf samples of small quantity were harvested. Leaves were collected and bulked from different plants for each species and replicated three times for DNA isolation. Leaf material was stored at –20°C for later analysis. 1 gm of leaf tissue from each species was subsequently used for each DNA isolation experiment.

Genomic DNA Extraction and RAPD-PCR Reaction

Total DNA was extracted from silica gel-dried young leaf tissue following the CTAB method described by Saghai-Maroof *et al*. [19] (1984) with minor modifications. After purification, it was quantified spectroscopically and visualized under a UV light after electrophoresis on a 0.8% (w/v) agarose gel stained by 0.5 µg/ml ethidium bromide. The resuspended DNA was stored in autoclaved ddH_2O. A total of 22 RAPD primers (Bangalore Genei Pvt. Ltd., Bangalore, India) were initially screened to amplify genomic DNA in order to identify potential primers that produced a higher number of polymorphic and reproducible fragments. PCR amplifications were carried out in a thermal cycler (Perkin Elmer, Gene Amp thermal cycler 2400) in a final volume of 25 µl, containing 25 ng template DNA, 200 µM each of the four dNTPs, 10 picomoles of primers, 3 mM MgCl2, 2.5 µl Taq buffer (10 mM Tris HCl pH 9.0, 50 mM KCl) and 0.2 Unit Taq DNA polymerase (Bangalore Genei Pvt., Ltd., Bangalore India). The thermo cycler was programmed for an initial denaturation at 94^{o}C for 4 minutes followed by 36 cycles at 94^{o}C for 1 min, annealing at 38^{0}C for 1 minute and extension at 72^{o}C for 2 minutes, followed by one final extension at 72^{o}C for 6 minutes and at last the hold temperature was of 4^{o}C. 10 µl of amplified PCR amplified product was separated by gel electrophoresis on a 1.5% agarose gel stained by ethidium bromide (0.5 μg/ml of gel solution) and photographed with a gel documentation system (Uvi Tec, UK). For each experiment the reproducibility of the amplification products was tested twice using similar reaction conditions at different times. Only those amplification products that consistently appeared in two replications (consensus products) were scored for further analysis.

RAPD Data Scoring and Analysis

In RAPD analysis, the presence or absence of the bands was taken into consideration and the difference in the intensity of the band was ignored. RAPD is a dominant marker, and all bands amplified by the same primer with identical electrophoretic mobility were homologous. A particular DNA band which is generated from the genome of one species, but absent of a second species represents a polymorphism. The banding patterns obtained from RAPD

gel were used to assign loci for each primer and scored as present (1) or absent (0). The data obtained from the markers were pooled for different analyses. Jaccard's similarity coefficient values [20] were calculated for each pair wise comparison between genotypes and similarity matrix was constructed. To illustrate the genetic relationships among the species, a dendrogram was constructed based on the similarity matrix using unweighted pair group method with arithmetic average (UPGMA) cluster analysis [21]. All analyses were done using the computer package NTSYS-PC ver. 2.00 [22].

RESULTS AND DISCUSSION

All of the three species of *Avicennia* found in the mangrove forest of Indian Sundarban were fingerprinted using molecular markers. We used RAPD markers to analyze the genetic variability and establish phylogenetic relationships among them. A total number of 10 RAPD primers (Bangalore Genei Pvt. Ltd., Bangalore, India) (Table 1) that produced polymorphic and reproducible fragments were selected to amplify genomic DNA of the plant species under investigation. Ten primers amplified a total number of 221 bands under 100 loci across three genotypes with an average of 10 loci / primer. Of the total 100 loci (Figure 1a and Figure 1b) scored in the 3 species with different primers, 50 were polymorphic and 29 were unique. Therefore, the genus *Avicennia* exhibited an overall 50% polymorphism at species level in Indian Sundarban. Different species of *Avicennia* revealed varying degrees of genetic polymorphism in their RAPD profiles. Polymorphism and uniqueness were highest in *A. marina* followed by *A. officinalis and A. alba*, respectively. The total number of the amplified loci produced by each primer varied from a minimum number of 1 by primer Oligo-01, Oligo-02 and Oligo-05 to a maximum of 10 by the primer Oligo-09. The percentage of polymorphism ranged from 10% (primer Oligo-05) to 88.89% (primer Oligo-08). The size of amplified bands also varied with different primers. Only three out of 10 primers showed 70% or more polymorphism and as many as seven primers showed 45% or more polymorphism whereas three primers showed less than 45% polymorphism. In general, the extent of polymorphism found was moderately high. The data obtained was subjected to UPGMA analysis to find out the relationship among the species being analyzed. The value of Jacaard's similarity coefficient ranged from 0.584 to 0.733.

Table 1. List of RAPD primers and their sequences along with some of the characteristics of the PCR-amplified products

Primer Code	Primer Sequence (5' to 3')	Total No. of Amplified Loci	Total No. of Polymorphic Loci	% of Polymorphism
Oligo-01	CCAGGAGGAC	07	01	14.29
Oligo-02	AGGTGACCGT	08	01	12.50
Oligo-03	GTGAGGCGTC	10	05	50
Oligo-04	GATGACCGCC	11	07	63.64
Oligo-05	GGAGGGTGTT	10	01	10
Oligo-06	GTTTCGCTCC	11	05	45.46
Oligo-07	ACCGCGAAGG	11	05	45.46
Oligo-08	GTCGCCGTCA	09	08	88.89
Oligo-09	GAAACGGGTG	13	10	76.92
Oligo-10	CCCGGCATAA	10	07	70

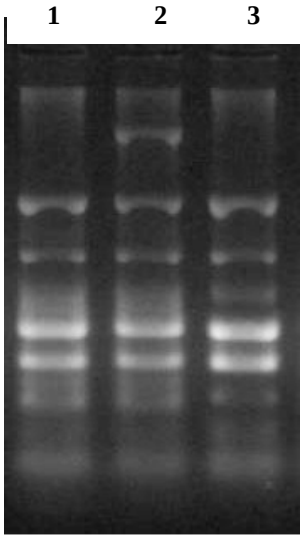

Figure 1a. Ethidium bromide stained 1.5% agarose gel showing PCR-amplified products of three species of *Avicennia* generated by a random primer Oligo-03 (5' GTGAGGCGTC 3')

Lane 1 to 3 corresponds to A. alba, A. marina and A. officinalis, respectively

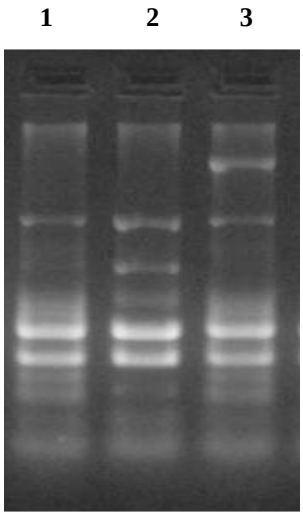

Figure 1b. Ethidium bromide stained 1.5% agarose gel showing PCR-amplified products of three species of *Avicennia* generated by a random primer Oligo-04 (5' GATGACCGCC 3')
Lane 1 to 3 corresponds to A. alba, A. marina and A. officinalis, respectively

The dendrogram (Figure 2) reveals that *A. alba* and *A. officinalis* have closer relationship between them and share a common node in the phylogenetic tree at 73.3% level of similarity. In comparison to other species, *A. marina* showed most genetic variability, suggesting utilization of this species over others for breeding programme and as source material in conservation programmes. Thus, greater efforts should be made in taking its provenance when screening germplasm, improving genetics, and introducing and transplanting. Also, it should be a priority to protect its germplasm resources. Genetic distance was less between *A. marina* and *A. alba* than between *A. marina* and *A. officinalis*. Phylogenetic tree shows that widely distributed *A. marina* is more closely related to *A. alba* than to *A. officinalis*.

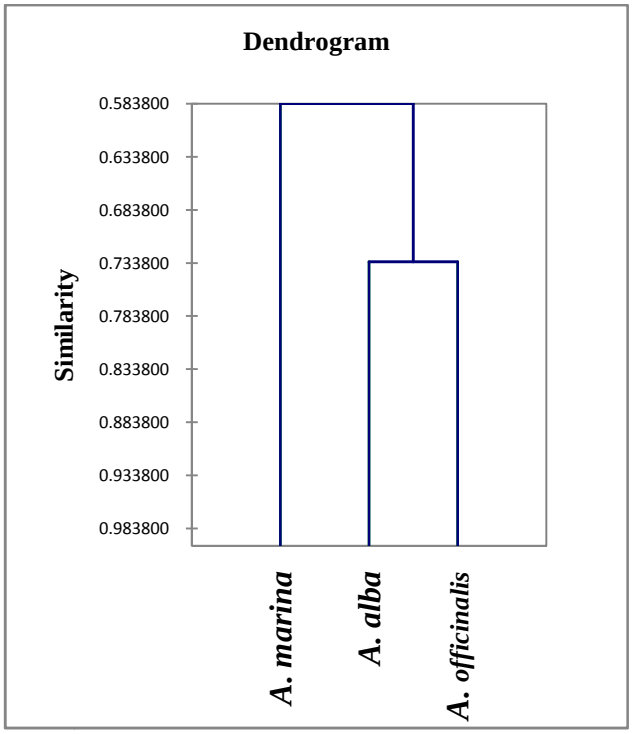

Figure 2. Dendrogram, generated using UPGMA analysis, showing phylogenetic relationships among the 3 species of *Avicennia* based on RAPD markers

The UPGMA cluster analysis of the three species showed a high coefficient of interspecific diversity, forming three distinct classes each represented by one species. Distances between the class centroids reveal a genetic distance of 0.378 between *A. alba* and *A. officinalis*, 0.604 between *A. alba* and *A. marina*, and 0.611 between *A. marina* and *A.*

officinalis. Extent of polymorphism and uniqueness reveals lowest genetic diversity in *A. alba* and therefore demands priority of this species in conservation programme to prevent extinction.

Genetic diversity at the species level, the product of long-term evolution, is a prerequisite for survival. Studying the genetic diversity and genetic structure of a species is the basis of exploring its adaptability and viability [23]. From the genetic perspective, a higher level of genetic diversity results in a greater ability to adapt and evolve [12]. Low genetic diversity can result in reduced adaptability and increased occurrence of less beneficial genes, leading to eventual extinction of the species. The richness of genetic diversity thus provides very important information about the status of a species, an assessment of its conservation value, and *ex situ* conservation. The percentage of polymorphic loci can be used as indicator to measure the level of genetic diversity.

Since introduction [24], the RAPD marker is being extensively used for measuring inter- and intra-specific genetic relationship in several plants [15]. Being a DNA based diagnostic assay RAPD is able to identify genotypes directly and can therefore diminish the complications arising from earlier morphological, cytological and biochemical studies [25]. RAPD is an efficient marker system because of the capacity to reveal several informative bands in a single amplification and also for its simplicity and low expenses. Such studies contribute to create phylogenetic tree and to develop conservation strategies by identifying units for conservation.

Different primers yielded a total of 7 unique bands for *A. alba*, 14 for *A. marina* and 8 for *A. officinalis*. Unique bands differentiate one species from the others. The distinct RAPD bands could be cloned and used as markers for species identification. The successful identification of these three species using species-specific nuclear markers and only a limited set of primers proved to be promising for species detection. The DNA amplification fingerprinting technique allowed us to unambiguously differentiate *A. marina*, *A. officinalis and A. alba*. Although these three species have many similar morphologies (leaf morphology, leaf length, the colour of the petiole, the colour of the midrib), their RAPD fingerprinting differed markedly. For management and conservation purposes, the identification of *A. marina*, *A. officinalis and A. alba*. and their putative hybrids or intermediates could be accomplished using RAPD markers, even at an early stage of their life cycle, e.g. seedling stage.

However, it may be mentioned here that the interspecific relationship reported in the present study has the limitation that they were represented by only one population. The detailed analysis of inter-specific variation in *Avicennia* using molecular markers indicated that *A. alba* where genetic erosion is of greater magnitude should be given priority for *in situ* conservation as these species also contribute substantially to the overall genetic variation in the genus, and for the same reason, other populations should be included in genetic sampling to represent the species in any *ex situ* conservation programme to withstand the anticipated rise in sea level that is imminent due to global warming.

CONCLUSION

In conclusion, this study demonstrates that decanucleotide DNA fingerprinting can be an effective and useful method to successfully distinguish and identify all the three species of *Avicennia* found in Indian Sundarban. The nature and extent of genetic variation among these species as well as their phylogenetic relationships were also revealed. Moreover this study has provided important clues in understanding genotype relationship, which may further assist in developing breeding strategies for the sustainable management of the genetic resources and conservation. An efficient conservation strategy will lead to preserve the genetic variation and the evolutionary process in viable populations of ecologically valuable genotypes, in order to prevent potential extinction of this important group of plants.

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