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Phylogenetic relationships among the mangrove species of Acanthaceae found in Indian Sundarban, as revealed by RAPD analysis

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ABSTRACT

RAPD markers were successfully used to identify and differentiate all the five species of Acanthaceae found in the mangrove forest of Indian Sundarban, to assess the extent of interspecific genetic diversity among them, to reveal their molecular phylogeny and to throw some light on the systematic position of Avicennia. The dendrogram reveals that the five species under study exhibits an overall similarity of 60.7%. Avicennia alba and A. officinalis (cluster C1) have very close relationship between them and share a common node in the dendrogram at a 73.3% level of similarity. Avicennia marina and Acanthus ilicifolius (cluster C2) also have close relationship between them as evident by a common node in the dendrogram at 71.8% level of similarity. Acanthus volubilis showed 68.1% similarity with cluster C1 and 60.7% similarity with cluster C2. Our study also supported the view of placing Avicennia under Acanthaceae. Regarding the relative position of Avicennia within Acanthaceae, it was shown to be very close to Acanthoideae. 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 in situ conservation programmes. Extent of genetic diversity was the lowest in Acanthus volubilis and therefore demands priority of this species in conservation programme to prevent extinction.

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

INTRODUCTION

The ability to survive in mangrove habitats, characterized by high salt concentrations, low aeration of waterlogged soil, and frequently changing water levels due to tidal cycles, has clearly evolved several times independently within angiosperms [1]. Mangrove flora, a heterogeneous group of independently derived lineages, play an important role in coastline wetland ecosystems by stabilizing shores [2] and buffering the destructiveness of storm [3]. 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. They have many medicinal values as well [4].

Tomlinson (1986) [5] and Duke (1991) [6] classified *Avicennia* L., a genus of mangrove woody trees or shrubs, to eight species. *Avicennia* has always presented a problem to systematists. Like other mangroves, *Avicennia* exhibits a number of conspicuous adaptations to the mangrove habitat of which they are an important constituent. The large number of convergent and autapomorphic characters have made it difficult to classify *Avicennia* within angiosperms. To a large extent, morphological characters in Avicennia appear to be controlled by environmental factors [7], making it a difficult task to trace the evolutionary history of the genus. However, there are also a number of traits, involving stem and root anatomy, pollen morphology, gynoecium anatomy, and embryology, which may or may not be related to the mangrove habit [8]. Studies of these characters have led to a number of different suggestions to classify *Avicennia*.

Bentham & Hooker (1876) [9] placed *Avicennia* in Verbenaceae. Van Tieghem (1898) [10] suggested a relationship with Santalaceae based on unspecified embryological similarities, whereas Moldenke (1960) [11] favoured a place among Dipterocarpaceae apparently because of similarities between the groups reported to him in a letter from Le´on Croizat. Dahlgren (1975) [12] pointed to shared cellular endosperm development in linking *Avicennia* with Celastraceae. However, most authors have placed *Avicennia* either within Verbenaceae ([13], [14]) or as a separate family Avicenniaceae closely related to Verbenaceae ([15], [16], [17], [18]), although synapomorphies linking these two groups have never been identified. Recent molecular analyses using chloroplast DNA suggest that Avicenniaceae is more closely related to Pedaliaceae (represented by *Sesamum* L.) or Acanthaceae than Verbenaceae [19]. More recently and quite surprisingly, a molecular study by Schwarzbach & McDade (2002) [1] using data from both the chloroplast and the nuclear genome, implied that the mangrove genus Avicennia, usually treated as a separate family in Lamiales or as a genus within Verbenaceae, is a part of Acanthaceae. The flowering plant family Acanthaceae (Lamiales) consist of at least 4000 mainly tropical and subtropical species [20]. In their study, *Avicennia* is consistently placed as sister group to Thunbergioideae. Although, Agneta Julia Borg & Jürg Schönenberger (2008) [21] did not find enough evidence to conclude that *Avicennia* was more closely related to Thunbergioideae than to other Acanthaceae. Recent phylogenetic studies have suggested that *Avicennia* is derived from within Acanthaceae, and the genus is included in that family in the Angiosperm Phylogeny Group system. The need to study *Avicennia* in more detail in order to learn more about the taxonomic status and relationships of the genus has been pointed out several times ([22],[1]). In view of these information, systematic determinations need to be reassessed.

Recently, development of molecular methods has provided opportunities to take mangrove research in new directions and to address unresolved issues in mangrove studies [1]. 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 and portray genetic relationships between plant groups [23]. DNA markers provide an opportunity to characterize genotypes and to measure genetic relationships more precisely than other markers [24]. 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 plant taxonomy. Among the various DNA markers, randomly amplified polymorphic DNA (RAPD) has been used extensively in phylogenetic studies of different plant groups including some mangrove species [25].

The goal of the present study was to clarify the systematics of *Avicennia*, specifically addressing the relationship with core Acanthaceae. We included a representative sample of all the species of *Avicennia* found in Indian subcontinent (*Avicennia marina*, *Avicennia offlcinalis* and *Avicennia alba*) and *Acanthus* (*A. ilicifolius* and *A. volubilis*) found in the mangrove forest of Indian Sundarban. We used DNA data to reveal the genetic diversity among the five species and to construct a phylogenetic tree showing evolutionary relationship of these species, using RAPD analysis. This will in turn shed some light to solve the problem on systematics of *Avicennia* which has always presented a problem to taxonomists. Moreover this study will provide important clues in developing conservation strategies to prevent potential extinction of these valuable plants.

MATERIALS AND METHODS

Plant Material

Young, fresh and healthy leaf samples of *Avicennia marina* (Forsk.) Vierh., *Avicennia offlcinalis* L., *Avicennia alba* Blume., *Acanthus ilicifolius* Linn. and *Acanthus volubilis* Wall. 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 except *Acanthus volubilis* (three individuals) 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

Genomic DNA of the *Avicennia* species was extracted from silica gel-dried young leaf tissue following the CTAB method described by Saghai-Maroof *et al*. (1984) [26] with certain modifications, whereas the genomic DNA of the *Acanthus* species was extracted following the method described by Surya *et al*. (2013) [27]. After isolation, the DNA was analyzed spectroscopically to check yield and purity and visualized under a UV light following electrophoresis on a 0.8% (w/v) agarose gel stained by 0.5 µg/ml ethidium bromide to check the integrity. 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 2.0 Unit Taq DNA polymerase (Bangalore Genei Pvt., Ltd., Bangalore India). The thermocycler 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 (locus) 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 [28] 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 [29]. All analyses were done using the computer package NTSYS-PC ver. 2.00 [30].

RESULTS AND DISCUSSION

All of the five species of Acanthaceae (according to Angiosperm Phylogeny Group system) 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 371 bands under 106 loci across five genotypes with an average of 10.6 loci / primer. Of the total 106 loci scored in the five species with different primers, 61 were polymorphic and 9 were unique. Therefore, the family Acanthaceae exhibited an overall 57.55% polymorphism at species level in Indian Sundarban. Different species of Acanthaceae revealed varying degrees of genetic polymorphism in their RAPD profiles. The total number of the amplified polymorphic loci produced by each primer varied from a minimum number of 2 by primer Oligo-01, and Oligo-05 to a maximum of 13 by the primer Oligo-09. The percentage of polymorphism ranged from 20% (primer Oligo-05) to 100% (primer Oligo-08). The size of amplified bands also varied with different primers. Only three out of 10 primers showed 80% or more polymorphism and as many as seven primers showed 50% or more polymorphism whereas three primers showed less than 50% 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 0.568 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	08	02	25
Oligo-02	AGGTGACCGT	10	03	30
Oligo-03	GTGAGGCGTC	10	05	50
Oligo-04	GATGACCGCC	11	07	63.64
Oligo-05	GGAGGGTGTT	10	02	20
Oligo-06	GTTTCGCTCC	11	05	45.45
Oligo-07	ACCGCGAAGG	12	06	50
Oligo-08	GTCGCCGTCA	09	09	100
Oligo-09	GAAACGGGTG	14	13	92.86
Oligo-10	CCCGGCATAA	11	09	81.82

The overall banding pattern and unique bands generated by RAPD fingerprinting allowed us to unambiguously identify and differentiate the closely related species under study, using tissue from any part and at any developmental stage. Authentic identification of genotypes is a prerequisite for many studies in the field of breeding, conservation and pharmacology.

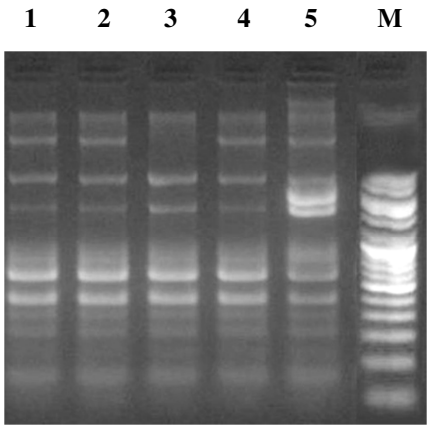

Figure 1a. Ethidium bromide stained 1.5% agarose gel showing PCR-amplified products of the five plant species under study generated by a random primer Oligo-02 (5' AGGTGACCGT 3'). Lane 1 to 5 corresponds to *Avicennia alba, A. marina*, *A. oficinalis, Acanthus ilicifolius and Acanthus. volubilis* respectively. M= Marker, λ DNA digested with *EcoRI* and *Hind-III*;

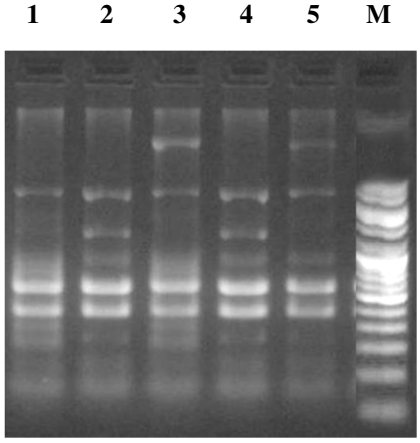

Figure 1b. Ethidium bromide stained 1.5% agarose gel showing PCR-amplified products of the five plant species under study generated by a random primer Oligo-04 (5' GATGACCGCC 3'). Lane 1 to 5 corresponds to *Avicennia alba, A. marina*, *A. oficinalis, Acanthus ilicifolius and A. volubilis* respectively. M= Marker, λ DNA digested with *EcoRI* and *Hind-III*.

The dendrogram reveals that the five species under study exhibits an overall similarity of 60.7%. *Avicennia alba* and *A. officinalis* have very close relationship between them and share a common node in the dendrogram at a 73.3% level of similarity. *Avicennia marina* and *Acanthus ilicifolius* also have close relationship between them as evident by a common node in the dendrogram at 71.8% level of similarity.

The UPGMA cluster analysis of the five species showed a high coefficient of interspecific diversity, forming three distinct classes viz., C1, C2 and C3 with 2, 2 and 1 genotype respectively. Cluster C1 consisted of two genotypes namely *Avicennia alba* and *A. oficinalis*, cluster C2 comprised of 2 genotypes namely *Avicennia marina* and *Acanthus ilicifolius* whereas the cluster C3 comprised of only one genotype namely *Acanthus volubilis* which showed 68.1% similarity with cluster C1 and 60.7% similarity with cluster C2. Distances between the class centroids reveal a genetic distance of 0.503 between cluster C1 and C2, 0.426 between cluster C1 and C3 and 0.589 between cluster C2 and C3. So, cluster C1 is closest to C3 then to C2. Again cluster C3 is farthest to C2.

In a previous study by the authors [31], it was revealed that the 3 species of *Avicennia* exhibit an overall similarity of 58.4%. When we included the 2 mangrove species of *Acanthus*, nearly identical overall similarity of 60.7% was observed. It probably indicates a very close relationship of the two genera and supports the recent views of placing *Avicennia* under Acanthaceae. In recent classification system proposed by Angiosperm Phylogeny Group, Acanthaceae has been divided into four subfamilies namely Acanthoideae, Avicennoideae, Nelsonioideae and Thunbergioideae. In a molecular study by Schwarzbach & McDade (2002) [1], *Avicennia* is consistently placed as sister group to Thunbergioideae. But our study indicates very close relation of *Avicennia* with the species of *Acanthus* as evident by clustering of *Acanthus ilicifolius* with *Avicennia marina* and closeness of *Acanthus volubilis* with the *Avicennia alba-Avicennia oficinalis* cluster. Moreover, Agneta Julia Borg & Jürg Schönenberger (2008) [21] did not find, in their study, enough evidence to conclude that *Avicennia* was more closely related to Thunbergioideae

than to other Acanthaceae. As we did not include any member from Thunbergioideae, we cannot conclude about the relative position of *Avicennia* in Acanthaceae. But the results indicate that the systematic position of *Avicennia* is yet to be settled and we recommend a large scale higher resolution study to deal with the matter.

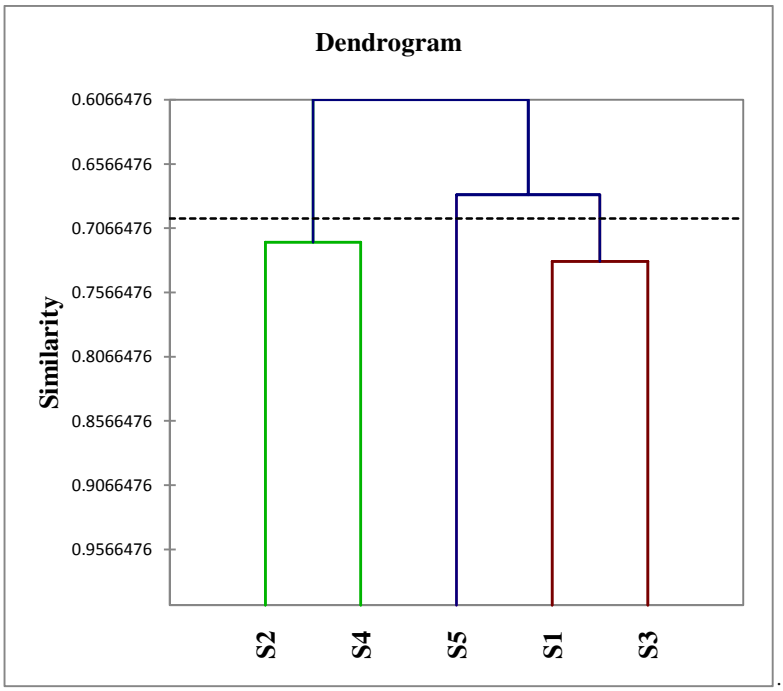

Figure 2. Dendrogram, generated using UPGMA analysis, showing phylogenetic relationships among the 5 species of Acanthaceae. S1 to S5 corresponds to *Avicennia alba, A. marina, A. oficinalis, Acanthus ilicifolius and A. volubilis* respectively

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 *in situ* conservation programmes. Extent of genetic diversity was the lowest in *Acanthus volubilis* and therefore demands priority of this species in conservation programme to prevent extinction. A higher level of genetic diversity results in a greater ability to adapt and evolve. Low genetic diversity can result in reduced adaptability leading to eventual extinction of the species. Genetic diversity at the species level, the product of long-term evolution, is a prerequisite for survival of a species in evolutionary time. The richness of genetic diversity thus provides very important information about the status of a species, an assessment of its conservation value.

CONCLUSION

We used DNA data to reveal the genetic diversity among the five species and to construct a phylogenetic tree showing evolutionary relationship of these species, using RAPD analysis. RAPD fingerprinting allowed us to unambiguously identify and differentiate the closely related species under study. Our study also supported the view of placing *Avicennia* under Acanthaceae. Regarding the relative position of *Avicennia* within Acanthaceae, it was shown to be very close to Acanthoideae. Moreover this study will provide important clues in developing conservation strategies to prevent potential extinction of these ecologically, economically and medicinally valuable plants.

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