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Studies on genetic relatedness of *Acacia* tree species using RAPD markers

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Studies were undertaken for identification and genetic relationships in six tree species of *Acacia* through Random amplified polymorphic DNAs (RAPD) markers. A total of two hundred and fifty three distinct DNA fragments (bands), ranging from 0.1–3.1 kb were amplified by using selected 17 random 10-mer primers. The genetic similarity analysis was conducted on the basis of presence or absence of bands which revealed a wide range of variability within the species. The cluster analysis clearly showed that there was high degree of diversity ($\sim$ 70%) within the six tree species of *Acacia*. Three major clusters were obtained belonging to 6 species of *Acacia*. First and second clusters were represented by only one species each i.e. *Acacia mollissima* and *A. arabica* respectively whereas 3rd cluster was represented by four species i.e. *A. farnesiana*, *A. catechu*, *A. auriculiformis*, *A. concinna*. *A. farnesiana* and *A. catechu* were the closest member sharing about 30% similarity. *A. auriculiformis* and *A. concinna* shared about 28% and 18% similarity, respectively, with the cluster formed by *A. farnesiana* and *A. catechu*. The closest genetic distance existed within populations of different *Acacia* tree species. Thus, these RAPD markers have the potential for conservation of identified clones and characterization of genetic relatedness among the species. It is also helpful in tree breeding programs and provide an important input into conservation biology.

Key words: *Acacia*, phylogenetic relationships, RAPD analysis, legume tree.

Introduction

The genus *Acacia* belongs to family Leguminosea: Mimosoideae. This genus represents both trees and herbs distributed in the tropical and subtropical regions of the world. Most of the tree species of the genus *Acacia* have high timber quality, medicinal as well as economic value (Anonymous, 1989). The *Acacia* trees are growing rapidly, can adapt to a wide range of degraded lands, and have high pulp quality and yield. Most of the *Acacia* trees have become the most important plantation species in southeast Asia and Australia. The trees are reported to have medicinal properties i.e. antiseptic, anti-dysenteric and antifever. The seeds are astringent, and given in piles, diarrhea and other maladies (Anonymous, 1989). However, these valuable timber species face extinction as germplasm conservation is difficult because of high intrafruit seed abortion and inbreed-

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ing depression. The conservation of plant populations and species is mostly concerned with the number of genetic individuals present in populations in order to assess factors such as genetic drift, inbreeding depression and lack of mates in self-compatible species (BARRETT & KOHN, 1991). The breeders need to be able to estimate the degree of relatedness between the existing material. Germplasm characterization and evolutionary process in viable populations are important links between the conservation and utilization of plant genetic resources. Conventionally, morphological characters like growth habit, leaf type, floral morphology and fruit characters have been used to define the plant name. Molecular techniques help researchers not only to identify the genotypes, but also in assessing and exploiting the genetic variability through molecular markers (WHITKUS et al., 1994). Insights into the relative genetic diversity within the *Acacia* species would be useful in plant breeding and also for the development of strategies for *ex situ* conservation of plant genetic resources. DNA finger printing of all the genetic resources of the timber yielding tree plants, is a necessity for generating a molecular database to catalogue as well as to utilize the information in a systematic manner. Moreover, the commercial value associated with identifying useful traits creates a direct value on gene banks ensuring long-term preservation of a collection. The genetic relationship and markers can be useful for designing strategies for gene introgression and breeding programs to produce desired recombinant hybrid genotypes with both timber quality and biomass yields. Management decisions for the conservation of taxa ideally necessitate an understanding of their biology and other factors, including genetic variability, that influence their survival. Conservation management decisions for rare taxa, however, often have to be made quickly without adequate ecological data or information on their genetic variability (GASTON & KUNIN, 1997). Random amplified polymorphic DNA (RAPD) analysis has proved useful for estimating genetic diversity particularly to assist in the conservation of rare species and plant genetic resources (ANDERSON & FAIRBANKS, 1990). RAPD analysis in particular has proven to be a rapid and efficient means of genome mapping (WILLIAMS et al., 1990) and is well suited for genetic resource characterization (ANDERSON & FAIRBANKS, 1990). The DNA fingerprinting generated by the polymerase chain reaction (PCR) using arbitrary primers, has provided a new tool for the detection of DNA polymorphism in number of tree species (CARLSON et al., 1991; BINELLI & BUCCI, 1994; BRADSHAW et al., 1994; BYRNE et al., 1995; VERHAEGEN & PLOMIO, 1996). In this context, information on the genetic relationships and diversity of available germplasm is essential for the identification of potential germplasm and conservation biology. In the present investigation, we report on genetic relationships and genetic variability within the population of *Acacia* tree species based on RAPD markers.

Material and methods

Plant materials

Leaf material was collected from 20 individuals of Acacia namely *Acacia auriculiformis* A. CUMM, *A. concinna* DC, *A. catechu* WILLD., *A. farnesiana* WILLD., *A. arabica* WILLD. and *A. mollissima* WILLD. from natural stand grown at Chandaka Reserve Forest, Bhubaneswar. Mature seeds were collected and grown in the 10 inches earthen pots under net house condition. The leaf materials were used for DNA isolation.

DNA isolation

DNA was extracted from young leaves using the N-cetyl-N,N,N-trimethylammonium bromide (CTAB) method described by DOYLE & DOYLE (1990) with modifications. Two grams of fresh leaf material were washed in distilled water and subsequently ringed with 80% (v/v) ethanol and then grounded in liquid nitrogen. Ten milliliters of preheated extraction buffer (2% CTAB (w/v), 0.2% β-mercaptoethanol (v/v), 100 mM Tris-HCl pH 8.0, 2 mM EDTA, 1.4 M NaCl) were then added per 2g of powder material and incubated for two hours at 60 °C. The DNA pellet was resuspended in 200 μL to 300 μl of Tris-EDTA (10 mM – 1mM). DNA quantification was performed by visualizing under UV light, after electrophoresis on 0.8% agarose gel. The resuspended DNA was then diluted in sterile distilled water to 5 ng/μL concentration for use in polymerase chain reaction amplification reactions.

Primer screening

Forty 10-mer primers, corresponding to kits A,B, D and N from Operon Technologies (Alameda, California) were initially screened using six species (five sample each) to determine the suitability of each primer for the study. Primers were selected for further analysis based on their ability to detect distinct, clearly resolved and polymorphic amplified products within the species of *Acacia*. To ensure reproducibility, the primers generating no, weak, or complex patterns were discarded.

Polymerase Chain Reactions (PCR) amplifications

A set of forty random decamer oligonucleotides purchased from Operon Technologies Inc. (Alameda, California, USA) was used providing single primers for the amplification of RAPD fragments. Polymerase Chain Reactions (PCR) were carried out in a final volume of 25 μL containing 20 ng template DNA, 100 μM each deoxynucleotide triphosphate, 20 ng of decanucleotide

Table 1. Total number of amplified fragments and number of polymorphic fragments generated by PCR using selected random decamers in six *Acacia* tree species.

Name of primer	Sequence of the primer	Total No. amplification products	No. of polymorphic products	Size range (Kbp)
OPA 01	5'-CAGGCCCTTC-3'	10	10	0.8–2.9
OPA 02	5'-TGCCGAGCTG-3'	10	10	0.2–2.4
OPA 09	5'-GGGTAACGCC-3'	13	13	0.3–1.8
OPA 11	5'-CAATCGCCGT-3'	8	8	0.5–1.7
OPA 14	5'-TCTGTGCTGG-3'	13	13	0.3–1.7
OPA 16	5'-AGCCAGCGAA-3'	11	11	0.2–1.9
OPA 20	5'-GTTGCGATCC-3'	10	10	0.6–2.0
OPB 17	5'-AGGGAACGAG-3'	10	10	0.5–1.1
OPB 20	5'-GGACCCTTAC-3'	20	20	0.2–2.5
OPN 05	5'-ACTGAACGCC-3'	20	20	0.3–2.2
OPN 08	5'-ACCTCAGCTC-3'	15	15	0.2–2.0
OPN 09	5'-TGCCGGCTTG-3'	22	22	0.2–2.0
OPN 10	5'-ACAACTGGGG-3'	15	15	0.2–2.0
OPN 13	5'-AGCGTCACTC-3'	19	19	0.2–2.0
OPN 14	5'-TCGTGCGGGT-3'	15	15	0.4–3.1
OPN 15	5'-CAGCGACTGT-3'	19	19	0.4–2.6
OPN 16	5'-AAGCGACCTG-3'	19	19	0.1–2.1

primers (M/S Operon Technology, Inc., Alameda, CA 94501, USA), 1.5 mM $MgCl_2$, 1 × Taq buffer (10 mM Tris-HCl pH 9.0, 50 mM KCl, 0.01% gelatin), and 0.5 U Taq DNA polymerase (M/S Bangalore Genei, India). Amplification was achieved in a PTC 100 thermal cycler (M J Research, USA) programmed for a preliminary 4 min denaturation step at 94 °C, followed by 45 cycles of denaturation at 94 °C for 1 min, annealing at 37 °C for 1 min and extension at 72 °C for 2 min, finally at 72 °C for 10 min. Amplification products were separated alongside a molecular weight marker (1kb ladder, MBI Fermentas, USA) by electrophoresis on 1.2% agarose gels run in 0.5X TAE (Tris Acetate EDTA) buffer, stained with ethidium bromide and visualized under UV light. Gel photographs were scanned through Gel Doc System (Gel Doc. 2000, BioRad, USA) and the amplification product sizes were evaluated using the software Quantity one (BioRad, USA). In another set of experiment, PCR amplification was made in 20 individuals in each species.

Data analysis

Data were recorded as presence (1) or absence (0) of band products from the examination of photographic negatives. Each amplification fragment was named by the source of the primer (Operon, Advanced Biotechnologies), the kit letter or number, the primer number and its approximate size in base pairs. Bands with similar mobility to those detected in the negative control, if any, were not scored. Similarity index was estimated using the Nei & Li coefficient of similarity (Nei & Li, 1979). Cluster analyses were carried out on similarity estimates using the unweighted pair-group method arithmetic average (UPGMA) using NTSYS-PC, version 1.80 (Rohlf, 1995).

Results and discussion

The primer screening step resulted in seventeen 10-mer primers which showed polymorphisms within the six species of *Acacia* used. Fourteen primers showed polymorphism, but could not distinguish within the species, and 9 primers gave no amplification products. The reproducibility of the amplification product was tested on template DNA from three independent extractions of the five selected clones using leaf samples from different seasons. Most of the amplification reactions were duplicated. Only bands that were consistently reproduced across amplifications were considered for the analysis. Bands with the same mobility were considered as identical fragments, receiving equal values, regardless of their staining intensity. When multiple bands in a region were difficult to resolve, data for that region of the gel was not included in the analysis. The results indicate that seventeen informative primers were selected and used to evaluate the degree of polymorphism within all the tree species of *Acacia*. The selected primers generated distinctive products in the range of 0.1–3.1 Kbp. Maximum numbers of bands were produced by the primer OPN-09 (22) and minimum by primer OPA-11 (8) (Tab. 1). A total of two hundred and fifty three amplified fragments were scored across six species for the seventeen selected primers, and were used to estimate genetic relationships within the species.

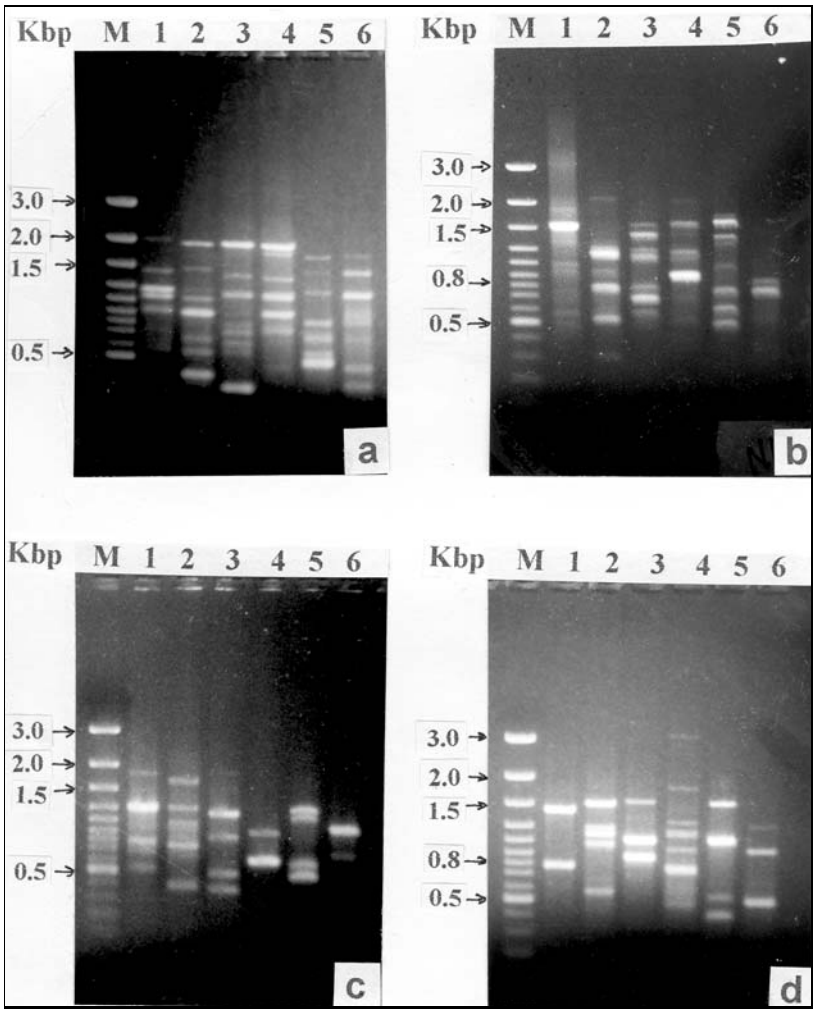

Fig. 1. RAPD patterns of six species of *Acacia* generated by primer: **a** – OPN-09 (5'-TGCCGGCTTG-3'), **b** – OPN-15 (5'-CAGCGACTGT-3'), **c** – OPA-09 (5'-GGGTAACGCC-3'), **d** – OPN-14 (5'-TCGTGCGGGT-3'). M – Kbp molecular weight ladder. 1– *Acacia mollissima,* 2 – *Acacia auriculiformis,* 3 –*Acacia farnesiana,* 4 – *Acacia catechu,* 5 –*Acacia concinna,* 6 – *Acacia arabica.*

Pattern of RAPD fragments produced by the 10-mer primer OPN-09, OPN-15, OPA-09 and OPN-14 are shown in Figs 1a-d. Further analysis of these RAPD profiles for band similarity indices could clearly differentiate all the tree species of *Acacia* i.e. *A. mollissima*, *A. concinna*, *A. auriculiformis*, *A. arabica, A. catechu* and *A. farnesiana*. The similarity matrix obtained after multivariant analysis using "Nei & Li" coefficient (Nei & Li, 1979) is presented in Table 2. The results of the genetic similarity matrix coefficient indicate that *A. catechu* had about 11%, 29% and 31% similarity with *A. mollissima, A. arabica* and *A. farensiana,* respectively. The cluster analysis indicate that six species of *Acacia* formed two major clusters (Fig. 2). The first major cluster represented by only one species each i.e. d *A. arabica*. Second major cluster was represented by five species i.e. *A. catechu*, *A. farnesiana*, *A. auriculiformis*, *A. concinna* and *A. mollissima*. *A. farnesiana* and *A. catechu* representing a minor cluster with about 30% similarity. *A. auriculiformis* shares about 28% similarity with *A. farnesiana* and *A. catechu*. *A. mollissima* shares about 18% similarity with *A. arabica*. The dendrogram represents the close distances among the species occurring in adjacent tips of the classification as per the numerical taxonomy (Sneath & Sokal,

Table 2. Similarity matrix for Nei & Li's coefficient of six different tree species of *Acacia*.

	Am	Aa	Af	Ac	Aco	Aar
Am	1.0					
Aa	0.18	1.0				
Af	0.12	0.3	1.0			
Ac	0.11	0.29	0.31	1.0		
Aco	0.19	0.23	0.23	0.17	1.0	
Aar	0.12	0.16	0.10	0.09	0.15	1.0

Am – *A. mollissima*, Aa – *A. auriculiformis* Af – *A. farnesiana* Ac – *A. catechu,* Aco – *A. concinna,* Aar– *A. arabica.*

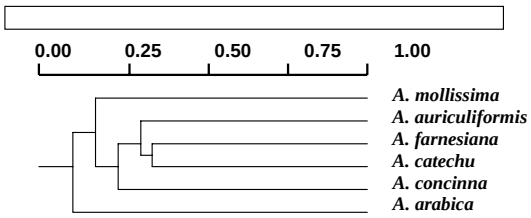

Fig. 2. Dendrogram of cluster analysis of RAPD markers illustrating the genetic relationships among the six species of *Acacia*.

1973). One minor cluster comprises *A. farensiana* and *A. catechu* which were about 30% similarity because of the close association with regards to growth habit and taxonomical classification.

The results indicate that the mean levels of genetic variation was low among the individuals of different *Acacia* tree species (data not shown). The present study of DNA profiling in tree *Acacia* species clearly showed that it was possible to analyse the RAPD patterns for correlating their similarity and distance between species by which one could predict the origin of the species to a great extent. Further, the taxa-specific bands can be utilized for defining the uniqueness, which will be helpful in species and taxa identification. From the results of the RAPD profiling, it was observed that some species performed good number of amplified bands but few species showed less number of amplified bands on same primer. Similarly, unique patterns were observed differentiating all six tree species from each other by using seventeen 10-mer random primers which can be concluded as RAPD markers for differentiating *Acacia* tree species and its help for conservation of germplasm (Virk et al., 1995), breeding programs and management of genetic resources (Bretting & Widrelechner, 1995). Miller & Bayer (2001) reported that the genus *Acacia* is subdivided into three subgenera: subg. *Acacia*, subg. *Aculeiferum*, and the predominantly Australian subg. *Phyllodineae*. Morphological studies have suggested the tribe Acacieae and genus *Acacia* are artificial and have a close affinity to the tribe Ingeae. Robinson & Harris (2000) studied the plastid DNA phylogeny of the genus *Acacia*. They concluded that the tribe *Acacieae* and genus *Acacia* are not monophyletic. Furthermore subgenera *Acacia* and *Aculeiferum* are sister taxa and neither of them appears closely related to subgenus *Phyllodineae*. Bukhari et al. (1999) noted that *A. nilotica* and *A. farnesiana* are sister species, though *A. nilotica* is Afro-Asiatic and *A. farnesiana* is of American origin.

In this investigation we provide information on genetic relatedness within species for conservation. An understanding of the level and partitioning of genetic variation within the species would provide an important input into determining appropriate management strategies.

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