Molecular Characterization of *Anogeissus acuminata* Genotypes Employing RAPD Markers

Sanjay Singh, Kanchan Kumari, Shweta Chaturvedi, Nutan Pandey and Ashley Varghese

Abstract

Anogeissus acuminata (Roxb. ex DC.) Wall. ex Guill. & Perr.** is commonly known as button tree and locally Phasi/Pasi. It is the specific kind of tree species with spiritual and cognitive environs of Odisha as its timber is useful in famous Ratha Yatra to make chariot wheels of Lord Jagannatha. Due to its potency and efficient qualities; *Anogeissus* timber is of implicit value, including its medicinal and economical values. The present work was aimed at studying the genetic diversity and relationship existing among the genotypes and discrimination at the inter-specific and intra-specific level, employing RAPD markers. Using 22 primers, 166 distinct bands were generated out of which 106 bands (66.63%) were found to be polymorphic. The amplicons obtained after PCR were run on 1.5% gel and was visualized under gel documentation system. The timber availability has become a challenge during the festival every year. So, it is necessary to conserve this species for future generations. Thus, genetic diversity and molecular characterization assessment through DNA-based molecular markers may be reliable and authentic for the species. In plant breeding programmes the most diverse genotypes shall be used in the hybridization programme.

Keywords

A. acuminata · Genetic diversity · Species conservation · RAPD markers

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Introduction

Biotechnology is the manipulation of living organisms or their components to produce useful and/or usually commercial products, viz. pest resistant crops, new bacterial strains or novel pharmaceuticals. Highly instructive DNA markers have been developed which helped immensely in the identification of genetic polymorphism. In molecular biology techniques, generally polymerase chain reaction (PCR)-based random amplified polymorphic DNA (RAPD) markers are used.

A study conducted by IFP Ranchi has found the no. of Phasi trees is decreasing to a significant extent due to selective logging of promising trees for Ratha Yatra purpose. The timber availability has become a challenge during the festival every year. So it is necessary to conserve this species for future generations. It is also necessary to check whether sufficient genetic diversity is present in natural populations or not. Genetic diversity and molecular characterization assessment through DNA-based molecular markers may be reliable and authentic for the species. To study the genetic diversity and relationship existing among the various genotypes of *Anogeissus acuminata* through RAPD markers is the objective of our present study.

Materials and Methods

Sample Collection

Fresh young leaves of 8 different germplasms of *A. acuminata* were collected and assembled from botanical garden of IFP.

DNA Extraction, Quantification and Gel Electrophoresis

Young leaf tissues that are visually clean and unaffected by pathogens of each genotypes were collected for genomic DNA isolation and analysis. DNA extractions following CTAB method with slight modification was done to remove the interfering polyphenolic compounds and other polysaccharides. Quantification of DNA was accomplished by UV spectrophotometer (Biophotometer plus, Genetics) and by analyzing the DNA on 0.8% agarose gel. DNA was diluted in TE buffer to a concentration of 50 ng/μl for use in PCR amplification.

Twenty-two desultory decamer oligonucleotide primers (RPI series, Bangalore GeNei) were used for RAPD-based PCR amplification of genomic DNA. Table 2 contains information regarding primer sequence. The PCR reaction mix included 50 ng/μl template DNA, 1X Taq Buffer A (Tris with 15 mM $MgCl_2$), deoxynucleotide triphosphates (dNTPs) each of 0.2 mM (Banglore GeNei). In a Thermal cycler namely GeneAmp* PCR 9700; all PCR amplification reactions were performed. The PCR conditions included: the first step as denaturation at 94 °C for 4 min, followed by 40 cycles of 1 min at 94 °C, annealing temperature of 1 min at

30–50 °C (annealing temperature was chosen based on gradient) and extension at 72 °C for 2 min. A final extension of 7 min at 72 °C was used for primer extension. The amplicons obtained were separated on 1.5% Agarose gel in 1X TAE buffer containing 5 µg/ml ethidium bromide. The size of fragments amplified was assessed by comparing to the ΦX174/Hae III digest DNA Ladder (Banglore GeNei) and gel was photographed on Gel Documentation System (Syngene).

Amplicons Assay

Amplified product for RAPD analysis were scored on the basis of presence (taken as 1) or absence (taken as 0) of band for each primer. Obvious and clear bands/fragments were considered as potential RAPD markers. The percentage of polymorphism and monomorphism for each primer of eight genotypes of *A. acuminata* was determined. Without considering the band intensity, the observed bands were taken significantly thus avoiding taxonomic resemblance. The basic measures for population genetic analysis were calculated by subjecting the data to POPGENE Software.

Results and Discussion

Evaluation using genetic diversity and variation is prerequisite for genetic improvement of important plant species to enhance biomass/production, increase oil yield, develop stress tolerance and use of elite accessions/genotypes. The present disquisition was conducted to ascertain the superior genotypes of *Anogeissus acuminata*, a tree species with multiple utilities, through molecular characterization employing the available RAPD markers.

Gel Scoring and Data Analysis

To determine the purity of a DNA solution a comparison of optical density value at various wavelengths was employed. In case of pure DNA, the absorbance ratio

Table 1 Data obtained for quantification of DNA by biophotometer

S. No.	Population	Quantity (ng/µl)	A_{230}	A_{260}	A_{280}	A_{340}	$A_{260/280}$	$A_{260/230}$
1	Pop1	2.9	0.056	0.059	0.047	0.017	1.25	1.05
2	Pop2	2.4	0.050	0.047	0.042	0.016	1.13	0.96
3	Pop3	1.4	0.026	0.028	0.029	0.012	0.97	0.08
4	Pop4	13.4	0.259	0.267	0.269	0.249	0.99	1.03
5	Pop5	1.0	0.020	0.020	0.018	0.006	1.11	0.99
6	Pop6	0.5	0.009	0.009	0.012	0.002	0.73	1.00
7	Pop7	0.4	0.008	0.008	0.012	0.005	0.69	1.00
8	Pop8	63.3	1.361	1.267	1.244	1.173	1.02	0.93

Table 2 Amplified DNA bands and polymorphism generated in *A. acuminata* genotype using 22 RAPD markers

S. No	Primer	Sequence of the primer	Total no. of amplified products	No. of polymorphic products	No. of monomorphic products	Polymorphisms (%)	Tm (°C)	Annealing temperatures (°C)
1	RPI01	AAAGCTGCGG	7	3	4	42.85	32	35
2	RPI02	AACGCGTCGG	5	4	1	80	34	35
3	RPI03	AAGCGACCTG	2	2	0	100	32	40
4	RPI04	AATCGCGCTG	5	2	3	40	32	45
5	RPI05	AATCGGGCTG	4	3	1	75	32	40
6	RPI06	ACACACGCTG	8	6	2	75	32	35
7	RPI07	ACATCGCCCA	8	5	3	62.8	32	45
8	RPI08	ACCACCCACC	10	7	3	70	34	50
9	RPI09	ACCGCCTATG	4	2	2	50	32	35
10	RPI10	AGCATGAGCG	7	4	3	57.14	32	40
11	RPI11	ACGGAAGTGG	6	5	1	83.33	32	30
12	RPI12	ACGGCAACCT	8	3	5	37.5	32	40
13	RPI13	ACGGCAAGGA	15	9	6	60	32	40
14	RPI14	ACTTCGCCAC	9	7	2	77.77	32	40
15	RPI15	ACCTGAAGCC	6	5	1	83.33	32	40
16	RPI16	AGGCGGCAAG	8	8	0	100	34	40
17	RPI17	AGGCGGGAAC	11	8	3	72.72	34	40
18	RPI18	AGGCTGTGTC	11	6	5	54.54	32	40
19	RPI20	AGTCCGCCTC	9	3	6	33.33	34	40
20	RPI21	CACGAACCTC	7	4	3	57.14	32	45
21	RPI24	CCAGCCGAAC	4	3	1	75	34	45
22	RPI25	GAGCGCCTTC	12	7	5	58.33	34	40
Total			166	106	60			
Average			7.54	4.8	2.72	63.66		

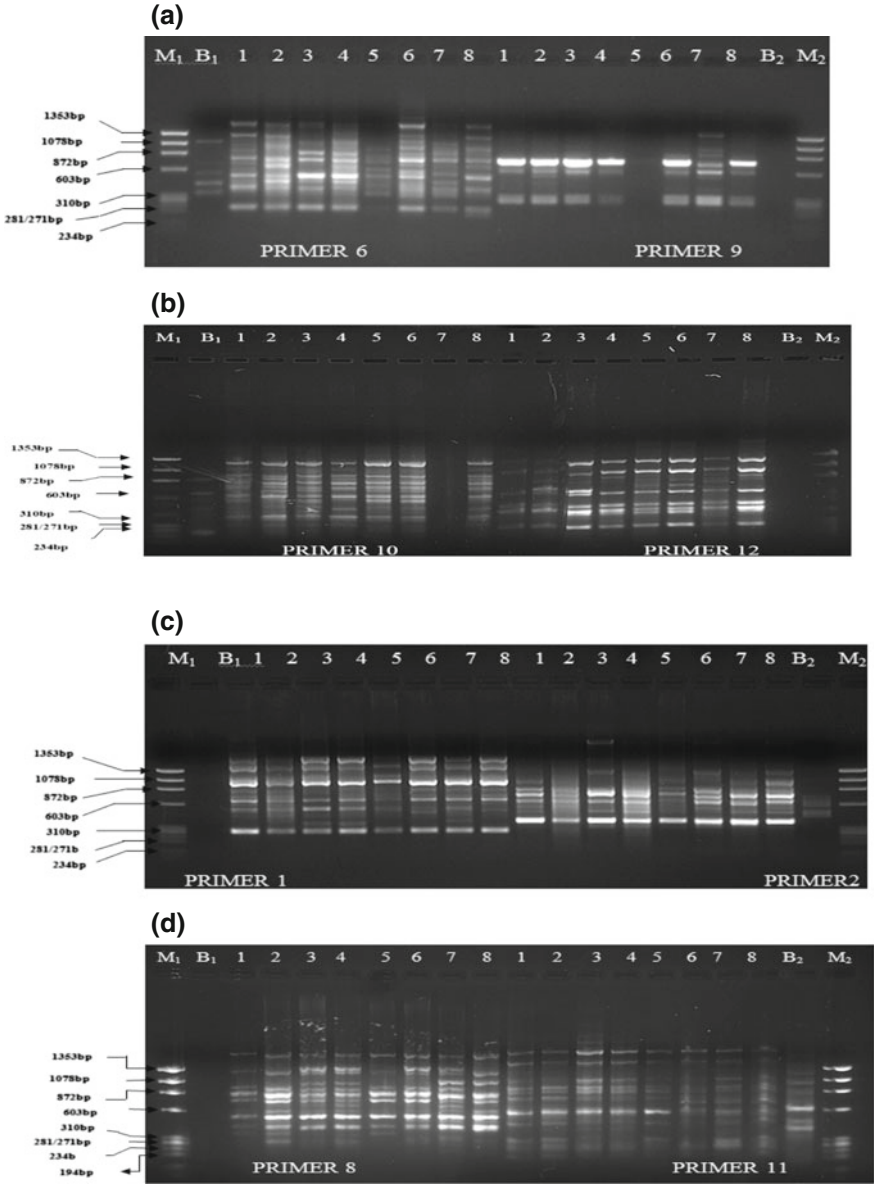

Fig. 1 **a–f** RAPD profile of eight genotypes of *Anogeissus acuminata* using the primer RPI#01, RPI#02, RPI#06, RPI#09, RPI#10, RPI#12, RPI#08, RPI#11, RPI#21, RPI# 24, RPI# 25, RPI#15 and RPI#16. *M* represent molecular weight and size of marker (ΦX174/Hae III digest DNA Ladder). The *numbers* represent different genotypes. M_1 and M_2—100 base pair ladder (B_1 and B_2—blank)

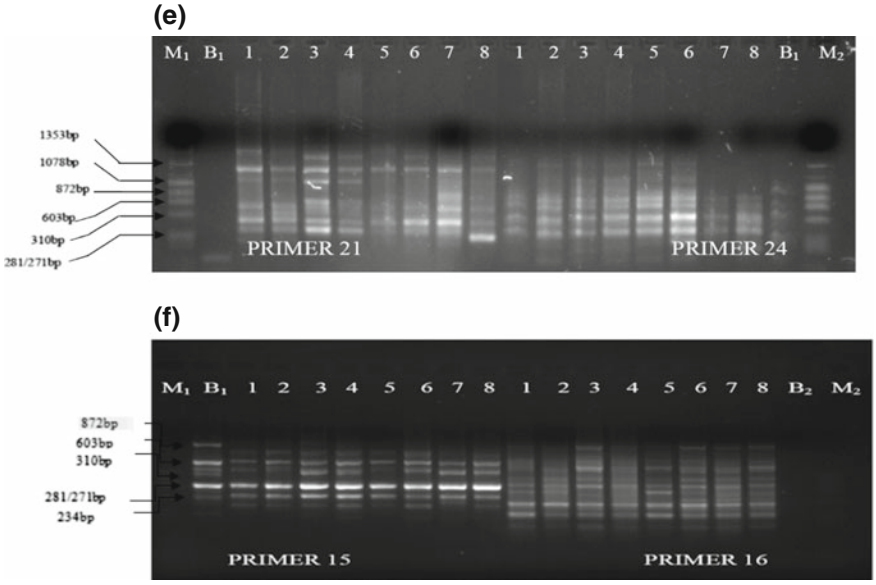

Fig. 1 (continued)

$A_{260/280}$ should be near to 1.8. Generally elevated ratio indicates the presence of RNA. $A_{260/280}$ ratio less than 1.8 often signals the phenol or protein contamination. Alternately, $A_{260/230}$ ratio greater than 0.5 indicates protein and phenol contamination. Phenol contamination is detected with a peak at 270 nm and an $A_{260/280}$ ratio 1.2. DNA having phenol uncontamination will have $A_{260/280}$ of around 2. Absorption at 230 nm can be caused by contamination by phenolate ion, thiocyanate and other organic compounds. Absorption at 330 nm and higher indicates particulate contamination causing light scattering. Thus we can say that in above table population 1 has phenol contamination (Table 1).

Genetic relationships among the eight genotypes have been carried out using RAPD markers. The particulars of the primers yielding amplified DNA bands, their nucleotide sequence, amplicons number, monomorphic/polymorphic bands count, unique bands count and amplified bands range are mounted in Table 2.

Band patterns produced by some of the RAPD primers have been shown in Table 2. The use of RAPD primer generated a total of 166 distinct band/fragments out of which 106 (63.85%) bands were found to be polymorphic and 60 bands were monomorphic. Primers were ranged from 2 to 15 wherein each primer produced an average of 8 bands. The primer RPI16 generated maximum number (8) of polymorphic fragments while RPI04 produced minimum number of polymorphic bands that is 2. The percentage of polymorphism ranged from 33.33 to 100%. Primer RPI03 and RPI16 showed 100% polymorphism where as RPI20 showed 33.33% polymorphism. In their ability to discover polymorphism, various primers showed

Table 3 Nei's genetic identity (above diagonal) and genetic distance (below diagonal)

Pop ID	1	2	3	4	5	6	7	8
1	****	0.7725	0.6826	0.5988	0.5928	0.6886	0.6527	0.6826
2	0.2582	****	0.7305	0.6467	0.6287	0.7365	0.7126	0.7066
3	0.3818	0.3140	****	0.7964	0.6707	0.7904	0.7784	0.7246
4	0.5128	0.4359	0.2276	****	0.6707	0.7425	0.6946	0.7246
5	0.5229	0.4640	0.3995	0.3995	****	0.6407	0.6766	0.6946
6	0.3731	0.3058	0.2352	0.2977	0.4452	****	0.7365	0.8263
7	0.4266	0.3389	0.2505	0.3644	0.3906	0.3058	****	0.7425
8	0.3818	0.3473	0.3222	0.3222	0.3644	0.1907	0.2977	****

variation but all the 22 primers were effective in determining variation among the *acuminata* genotypes (Fig. 1).

The amplified products were analyzed using the software POPGENE. Genetic Distance Nei's (1973) analysis of genetic distance and identity was used to study the genetic distance between the eight populations. Table 3 shows that the highest genetic identity exists between the pop 7 and pop 8, i.e 0.8263 and maximum genetic distance exists between pop 1 and pop 5, i.e. 0.5928. In plant breeding programmes, the most diverse genotypes shall be used in the hybridization programme.

According to the dendrogram (Fig. 2) based on Nei's analysis (1973), the whole genotype was divided into two clusters, cluster I containing pop 1, pop 2, pop 3, pop 4, pop 6, pop 7, pop 8 and the cluster II contain pop 5. Pop 5 stood separate while rests of the population were grouped together. Cluster I was sub-divided 2 groups pop 1 and pop 2 grouped together. Separation from the rest and pop 3, pop 4, pop 6, pop 7 and pop 8 were in another group. This group was further divided into groups where pop 7 remain separately and the rest pop 3, pop 4, pop 6 and pop 8 formed another group. Now the group containing pop 3, pop 4, pop 6 and pop 8 were further divided into two groups where pop 3 and pop 4 remain together and pop 6 and pop 8 remain together.

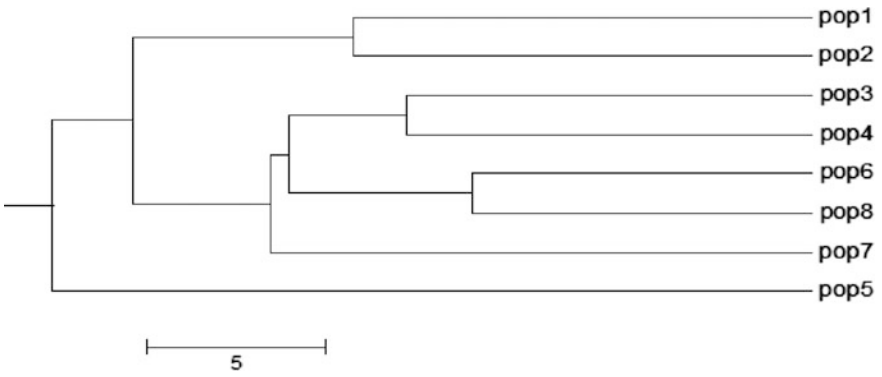

Fig. 2 Dendrogram obtained from RAPD analysis based on Nei's (1973) genetic distance

According to the dendrogram, pop 1 and pop 2 were highly similar to each other with respect to pop 3, pop 4, pop 6, pop 8, pop 7 and pop 5 which seem to be recently separated. Similarly pop 3 and pop 4 are more similar to each other than the pop 6, pop 8, pop 7 as they are separated in the earlier stage. Pop 6 and pop 8 are also highly similar with respect to pop 3 and pop 4 after undergoing further division. The genotype of plant population shows the interaction of a lot of various processes such as in the origin and evolution of the species (e.g. distribution curve and/or species isolation), mutation types, genetic drift in a population, mating system of species, gene migration, inheritance and natural selection. The above factors may be responsible for complex genetic structure within a population. The sustainable development of any plant population relates to its genetic diversity. In RAPD assay polymorphism is seen due to various types of mutations viz. insertion, deletion or substitution of one or more nitrogen bases inside the priming site sequence. However, it is apparent that RAPD markers can be used in a reproducible manner for detection of inter-specific and intra-specific differences.

Reference

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