Bulletin of Environment, Pharmacology and Life Sciences
Bull. Env. Pharmacol. Life Sci., Vol 2 (8) July 2013: 78-83

Online ISSN 2277-1808
Journal's URL:http://www.bepls.com

ORIGINAL ARTICLE

Evaluation of Genetic Diversity in Bamboo through DNA Marker and Study of Association with Morphological Traits

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ABSTRACT
The taxonomic studies of the bamboos are based on morphological and growth habit, which can cause problems in identification of genetic relatedness. Identification of genetic relationships in 10 species of bamboo was investigated using random amplified polymorphic DNA (RAPD) technique and their association with morphological characters was studied. Analysis started by using twenty five markers (Twenty one RAPD and four ISSR) that allowed us to distinguish 10 species. Out of twenty five, ten primers were used for identification and for establishing a profiling system to estimate genetic diversity. A total of three hundred ninety alleles were amplified out of this hundred distinct polymorphic DNA fragments (bands), ranging from 1.0–0.2 kb were amplified by 10 selected primers. On the basis of morphological traits, Bambusa tulda and Bambusa balcoa were very similar to each other for all four characters. While Guadia angustifolia showed very different types of character in all four traits but it showed highest number of new culms as compared to others. The genetic similarity analysis was conducted based on presence or absence of bands, which revealed a wide range of variability among the species. Cluster analysis clearly showed two major clusters belonging to 10 species of bamboo. Major Cluster1 was further subdivided into three minor clusters. The species of Bambusa tulda and Bambusa balcoa were the most closely related and formed the third minor cluster along with Bambusa nutans and Dendrocalamus strictus. The variety Guadia angustifolia was very distinct and showed as an out group in the dendrogram that was single species in cluster B. Thus, the morphological characterization was confirmed by the genetic diversity analysis.
Key words: *Bamboo, RAPD, ISSR, Morphological traits, Genetic diversity, Association*

Received 21/05/2013 Accepted 30/06/2013

INTRODUCTION

India has the second largest reserve of bamboo populations in the world. The fibres of bamboo are mainly used in the pulp, paper and charcoal industries, while the culms have several other uses as 'poor man's timber'. The total annual bamboo demand in India has been estimated to be approx. 5 million tonnes, of which about 3-5 million tonnes are required by the paper and pulp industry alone [1]. *Bambusa balcoa* is one of the strongest species and is thus preferred for construction purposes; it is also used for paper pulp production [2]. On the other hand, *B. tulda* is sturdy, tall and one of the five quick growing (70 cm d_1) species of bamboo [3]. The species is suitable for the production of quality paper due to its long fibres [4] and also for producing furniture. Among the anatomical characteristics of bamboo culm, fibre length is important for technical evaluation, especially in the pulp and fibre-based industries. Fibre wall thickness of the culm predetermines the pulping characteristics, paper quality, permeability and strength relationships [5]. *Bambusa balcooa* and *B. tulda* are two abundant tropical species that are recognized as priority bamboo species by the FAO (www.unep-wcmc.org, ww.inbar.int) amongst eighteen other bamboo species found globally.

Basic knowledge of the biology and genetics of bamboo is severely lacking. This is a direct result of the unusual life cycle of bamboo. Among bamboo species, the vegetative growth phase varies from 1 year to as long as 120 years, and some species have never been known to flower [6]. Identification of sterile plants is therefore problematic as taxonomic studies of bamboos have traditionally depended heavily on inflorescence and floral morphology because: (1) vegetative characters are often environmentally influenced, which makes them less constant for systematic purposes [7]; (2) characters that delimit species may be more subtle and not available for study; and (3) bamboo clones found in Asia are selected for economic value and are widely distributed without proper identification at the species level.

The application of modern molecular techniques is therefore of great assistance in species identification. PCR-based genetic markers are now well documented for species/cultivar identification.[8-10] A number

of PCR-based methods, including randomly amplified polymorphic DNA (RAPD; [11-12] and amplified fragment length polymorphism (AFLP) [13] are available that do not require previous sequence information of the genome to be studied. In a RAPD assay, a short, usually ten nucleotides long, arbitrary primer is used, which generally anneals with multiple sites in different regions of the genome and amplifies several genetic loci simultaneously. In contrast to RAPD, the AFLP technique generates relatively complex patterns. Random amplified polymorphism DNA (RAPD) markers have been used to characterize identities and relationships of various crops [18, 19] showed that these markers could be of great value in genetic resources management as a quick, cost effective and reliable method for identification, measurement of variation, and determination of similarity at the intra-specific level. Genomic investigation using morphological and isozyme markers have some limitations, which include problems of phenotypic penetrance or heritability, and low map resolution [20]. The RAPD technique is simple, relatively inexpensive and has been employed to analyze the intra and inter-generic genetic diversity of bamboo. [14-17]

MATERIALS AND METHODS

Plant Materials: Samples from fully expanded leaves of 10 bamboo genotypes were collected from the Research field of Aditya Biotech Lab & Research Pvt Ltd. Raipur. The leaf samples were collected in triplicates to account for experimental precision (Table 1).

Table: 1 List of genotypes used in this study

Code for genotypes	Name of Genotype	Site of collection
A	*Bambusa tulda*	Department of forestry, AP
B	*Bambusa nutans*	Department of forestry, AP
C	*Bambusa balcoa*	Department of forestry, AP
D	*Dendrocalamus strictus*	Local
E	*Dendrocalamus giganteus*	FRI, Dehradun
F	*Dendrocalamus hamiltonii*	IHBT, Palampur
G	*Bambusa vulgaris*	IHBT, Palampur
H	*Bambusa bamboos*	IHBT, Palampur
I	*Dendrocalamus asper*	IHBT, Palampur
J	*Guadia angustifolia*	IHBT, Palampur

Genomic DNA extraction: The Genomic DNA of 10 different genotypes of Bamboo was extracted by the method described by Dellaporta *et al.,* (1983) [21] The genomic DNA was quantified on 0.8% agarose gel, diluted and used in the PCR.

RAPD analysis: RAPD analysis was carried out following Williams *et al.,* 1990. [12] Several parameters were studied and altered to get reproducible amplifications. The reaction was carried out in a 20 µL reaction cocktail consisting of 20 ng template DNA, 10X Taq polymerase Buffer (10mM Tris-Hcl pH 8.0, 50mM KCl, 0.1%w/v Trition X 100), 3.2 mM MgCl2, 0.4 µM Primer, 250 µM of each dNTP and 1U Taq DNA Polymerase (Qiagen). The PCR reaction was carried out in a thermocycler programmed for an initial denaturation of 94ºC for 4 minutes followed by 35 cycles of denaturation at 94ºC for 1 minute, primer annealing at 55ºC for 1 minute, extension at 72ºC for 2 minutes and final extension at 72ºC for 10 minutes. The amplified products were electrophoresed on 1.8% agarose gel run in 1X TAE buffer at constant current of 120 V and documented using Gel Documentation system.

Data Analysis and Scoring: The image profiles of banding patterns were recorded and molecular weight of each band was determined by running 1 kb ladder along with the amplicons. The banding pattern was scored based on the presence or absence of clear, visible and reproducible bands (1 for present and 0 for absent). The binary data (1/0) was used to generate a similarity coefficient. Genetic similarity(S), between genotypes was estimated by using Jaccard's coefficient. Pairwise comparisons based on the similarity matrix generated by this analysis were used to generate dendrograms of genetic relatedness by Unweighted pair group method with airthematic averages (UPGMA) using NTSYS-pc (Numerical taxonomy system, applied bio-statistics, Inc., New York, USA, software version 2.02e)

RESULT AND DISCUSSION

Identification of genetic relationships or genetic divergence in bamboo is very difficult because of the lack of morphological differences and erratic flowering. Authentic identification of taxa is necessary both for breeders to ensure protection of intellectual property right and also for propagators and consumers. The most traditional method of identifying species by phenotypic characters is now replaced by protein that is

more reliable and authentic or DNA profiling largely because of several limitations of morphological data. In a current scenario, DNA profiling through RAPD technique has been used for the analysis of diversity and identification of duplicates within the large germplasm populations [22], phylogenetic relationship [23], rational designing of breeding programs [24] and management of genetic resources [25]. Evidently, RAPD technology is a rapid and sensitive technique, which can be used to estimate relationships between closely, and more distantly related species and groups of bamboo.

Table 3:- Morphological observation of ten genotypes

S. No	Name of Genotype	Height of mature culms (mt) Mean±SE	Internode distance (mt) Mean±SE	No. of new culms	Circumference(mt) Mean±SE
1	*Bambusa tulda*	1250.66±2.31	44.66±0.44	50	22.33±0.79
2	*Bambusa nutans*	657.73±5.15	29.33±1.66	45	17.66±1.01
3	*Bambusa balcoa*	1232.66±4.37	39.86±1.63	35	18±0.81
4	*Dendrocalamus strictus*	842.66±2.15	24.16±1.33	36	14.66±0.62
5	*Dendrocalamus giganteus*	1251.5±6.93	39.83±1.60	25	37.33±0.71
6	*Dendrocalamus hamiltonii*	1247±5.27	38±0.71	45	18±0.71
7	*Bambusa vulgaris*	698.33±5.54	22.83±0.44	36	20.66±0.54
8	*Bambusa bamboos*	743.33±6.67	26.3±0.62	35	13.66±0.72
9	*Dendrocalamus asper*	827±2.19	36±0.66	25	20.66±0.87
10	*Guadia angustifolia*	542±2.86	18.33±0.60	58	12.33±0.28

In this study we have taken morphological observation of all the genotypes and co-related them with molecular data. In the morphological observations we included four observations i.e. Height of mature culms, Inter-node distance, Number of new culms and Circumference (Table 3). The observations taken mean of the three consecutive years. It was observed that *Bambusa tulda* and *Bambusa balcoa* were very similar to each other for all four characters. While *Guadia angustifolia* showed very different type's characters in all four traits but it showed highest number of new culms as compared to others. Similarity of structures in closely related species is called Homology, a biological concept more frequently used while describing animals than plants. The unique characters that bamboos display show strong tendencies of homology, especially in relation to culm sheath, arrangement of axillary buds, leaf arrangement, branching and others. Some of these characters are very identical in the closely related bamboo species and one has to be very critical in distinguishing and differentiating the variations among the characters that help to prepare the taxonomic keys and identify the species. The genetic basis of homology is not well established in plants to explain inter and intra specific relationships. [26] A very close study of developmental characteristics in bamboo species will be very interesting to understand the role of ecological and genetical factors that determine the genotypic and phenotypic characters.

Table 2:- List of primers that showed polymorphism

S. No	Name of the Primers	Sequences
1	OPG4	5'-AGCGTGTCTG- 3'
2	OPE1	5'-CCCAAGGTCC- 3'
3	OPD1	5'-ACCGCGAAGG- 3'
4	OPB4	5'-GGACTGGAGT- 3'
5	OPA12	5'-TCGGCGATAG- 3'
6	OPY11	5'-AGACGATGGG- 3'
7	A17898	5'-CACACACACACAAC- 3'
8	B17898	5' -CACACACACACAGG- 3'
9	B17899	5'- CTCTCTCTCTCTCTCTTG- 3'

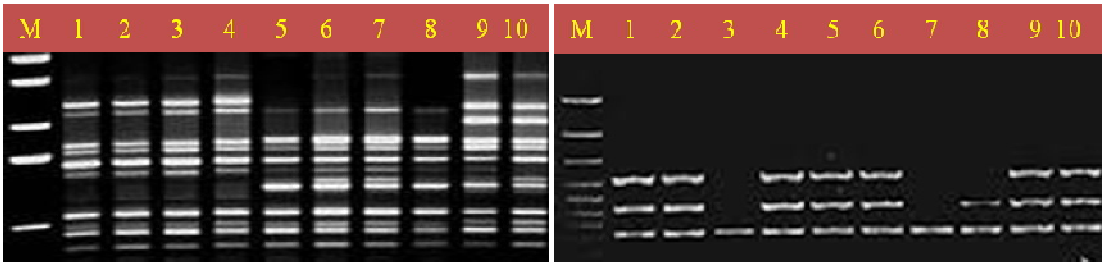

Fig. 1 Gel picture of RAPD marker OPA-12 and OPY-11

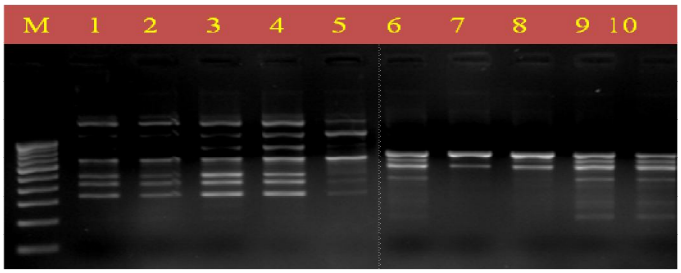

Fig. 2 Gel picture of ISSR marker B-17898

After morphological characterization we evaluated the genetic diversity by using DNA primers. Twenty five primers were used for the genetic diversity analysis but out of twenty five seven random primers and two ISSR markers which detected good polymorphisms (Table 2) and 25 other random primers, which gave poor amplification products. A total of three hundred ninety alleles were amplified out of this hundred distinct polymorphic DNA fragments (bands), ranging from 1.0–0.2 kb were amplified by 10 selected primers. From the seven RAPD maker we gave gel photo of two i.e. OPA-12 and OPY-11 and from ISSR B-17898 (Fig 1 and Fig 2). After scoring the molecular data of the polymorphic marker we generated dendrogram (Fig 3).

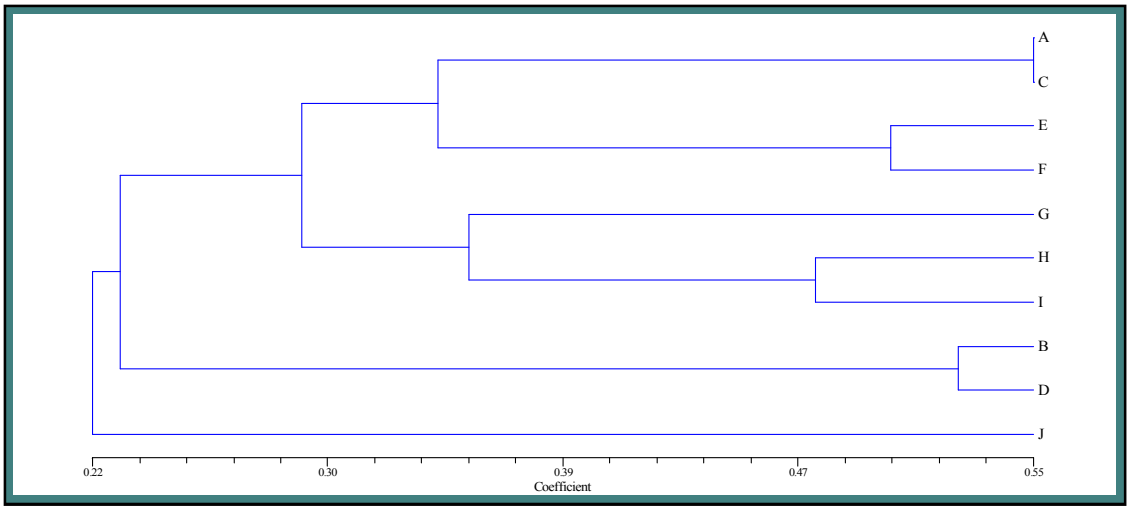

Fig. 3 Dendrogram of the ten genotypes of bamboo.

The ten different cultivar of bamboo were divided into two clusters A and B with 22% similarity. Cluster A has nine cultivars and it further sub divided into three sub-clusters A1, A2 and A3. The *Bambusa tulda* and *Bambusa balcoa* showed maximum similarity at genetic level which also observed and confirmed by the morphological traits basis. Both the subcluster A1 and A2 have three cultivars and showed 34 and 35% similarity respectively. The *Guadia angustifolia* showed as an out group in dendrogram from the rest of the cultivars. It was showed at morphological level also.

In randomly amplified polymorphic DNA (RAPD) technology, a single and short arbitrary primer is used. RAPD was utilized to assess phylogenetic relationships among 73 genotypes of Phyllostachys. [14] The resultant phylogeny neither supported the existence of two distinct sections in the Phyllostachys-species-complex nor did the placement of *P. nigra* under Phyllostachys, hence deviate from the previous proposal by Friar and Kochert, 1994. [27] However, based on a combined application of RAPD and morphometry, it was confirmed that *P. nigra* belongs to the section Phyllostachys [15] and it was also confirmed by AFLP and ITS sequence data that two distinct sections, Phyllostachys and Heteroclada, do exist in the Phyllostachys species pool. The utility of RAPD was extended to the tropical group as well. *B. ventricosa* was found close to *B. vulgaris* var. striata [17] and was supported by a previous finding that B. ventricosa is a cultivated variety of *B. vulgaris.* [28] Bamboo taxonomy like all other plant groups has traditionally been built upon various morphological features and several classification systems have been proposed to date. However, the taxonomic importance of rhizome, at least for the old world bamboo at genera or supra-generic level, is well recognized. [29] Conflicts persisted and in many instances the taxonomic delineation based on the anatomical features was not fully supported by the morphological features. A majority of bamboos belong to the third category (gregarious flowering) where the intermast period may

range from 3 to 120 years. [6] Kellogg and Campbell (1987) [30] considered Bambuseae as monophyletic based on the presence of woody culms and the herbaceous bamboos as either monophyletic or paraphyletic to Bambuseae. Molecular data sets can provide useful information for addressing various aspects of plant taxonomy. Nonetheless, morphology based identification keys are very useful for quick identification at the field, yet it needs further precision as morphological features are often influenced by environment due to the event of true parallelism.

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Citation of this Article
A.Shalini, Rakesh K Meena, Shubhankar Tarafdar and Sanket Thakur. Evaluation of Genetic Diversity in Bamboo through DNA Marker and Study of Association with Morphological Traits. Bull. Env. Pharmacol. Life Sci., Vol 2 (8) July 2013: 78-83