Evaluation of the genetic variability in bamboo using RAPD markers

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ABSTRACT

Classical taxonomic studies of the bamboos are based on floral morphology and growth habit, which can cause problems in identification due to erratic flowering. Identification and genetic relationships in 12 species of bamboo were investigated using random amplified polymorphic DNAs (RAPD) technique. Analysis started by using thirty 10-mer primers that allowed us to distinguish 12 species and to select a reduced set of primers. The selected primers were used for identification and for establishing a profiling system to estimate genetic diversity. A total of one hundred thirty seven distinct polymorphic DNA fragments (bands), ranging from 0.4–3.3 kb were amplified by using 10 selected primers. The genetic similar 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 12 species of bamboo. Two major clusters were further divided into three minor clusters. The species of *Bambusa vulgaris* and *Bambusa vulgaris* var. striata were the most closely related and formed the first minor cluster along with *Bambusa ventricosa*. The variety of *Bambusa multiplex* var. Silver stripe and *Bambusa multiplex* were very closely related and there was no variation with *Bambusa ventricosa*. Another minor cluster was obtained between *Bambusa arundinacea*, *Cephalostachyum pergracil* and *Bambusa balcooa*. The RAPD technique has the potential for use in species identification and genetic relationships between taxa and species of bamboo for breeding program.

Keywords: bamboo; genetic variability; phylogenetic relationships; RAPD analysis

Germplasm characterization is an important link between the conservation and utilization of plant genetic resources. Traditionally, morphological characters like growth habit, leaf type, floral morphology have been used to define taxa. Majority of plant taxa were defined morphologically and in most cases, this is still poor taxonomic description. Molecular DNA techniques allow researchers to identify genotypes at the taxonomic level, assess the relative diversity within and among the species and locate diverse accessions for breeding purposes. Moreover, the commercial value associated with identifying useful traits creates a direct value on gene banks ensuring long-term preservation of a collection. RAPD assay is the cheapest method for identifying the genotypes within a short period and also requires only limited amount of DNA. The development of randomly amplified polymorphic DNA (RAPD) markers, generated by the polymerase chain reaction (PCR) using arbitrary primers, has provided a new tool for the detection of DNA polymorphism (Williams et al. 1990). RAPD analysis has been used to study genetic relationship in a number of grasses (Huff et al. 1993, Gunter et al. 1996, Kolliker et al. 1999, Nair et al. 1999).

Bamboo is an important member of the grass family in the tribe Bambuseae, distributed in the tropical and subtropical regions of the world. It is used for food, fodder, building material and a raw material for production of paper (Das and Rout 1994). Breeding process was slow due to irregular flowering. To enlarge this genetic base, we need to utilize the genetic variability found in different species and genera of the *Bambusa* complex. In this context, information on the phylogeny and genetic diversity of available germplasm is essential for the identification of potential germplasm groups and for optimising hybridisation and selection procedures. In this investigation, we used RAPD markers to study the genetic variation among different species and to determine the genetic similarities between species.

MATERIAL AND METHODS

Plant materials. Twelve taxa of bamboo namely *Bambusa vulgaris* Schrader ex Wendl, *Bambusa vulgaris* var. striata Schrad ex Wendl, *Bambusa ventricosa* Maclure, *Bambusa multiplex* var. Silver stripe, *Bambusa multiplex* (Lour.) Raeushel ex. Schult & Sehult.f, *Bambusa arundinacea* Willd., *Bambusa balcooa* Roxb., *Dendrocalamus giganteus* Munro, *Dinocloa m'Clellandi* Kurz., *Cephalostachyum pergracil* Munro, *Dendrocalamus strictus* and *Sasa* species Makino & Shibata were collected from the Bambusetum established at Regional Plant Resource Centre, Bhubaneswar. The leaf materials were used for DNA isolation with three replications.

DNA isolation. DNA was extracted from young leaves using the N-cetyl-N,N,N-trimethylammonium bromide (CTAB) method described by Doyle and Doyle (1990) with modifications. 2 g of fresh leaf material were washed in 80% (v/v) ethanol and then ground in liquid nitrogen. 10 ml of preheated extraction buffer [2% CTAB (w/v),

0.2% β-mercaptoethanol (v/v), 100mM Tris-HCl (pH 8.0), 2mM EDTA, 1.4M NaCl] were then added per 2 g of fresh material. The DNA pellet was resuspended in 200–300 µl of Tris-EDTA (10–1mM). DNA quantifications were performed by visualising 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 amplification reactions.

PCR amplifications. A set of thirty random decamer oligonucleotides purchased from Operon Technologies Inc. (Alameda, California, USA) was used as 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, 100µM each deoxynucleotide triphosphate, 20 ng of decanucleotide primers, 1.5mM $MgCl_2$, 1 × taq buffer [10mM Tris-HCl (pH 9.0), 50mM 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 (MJ 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 (1 kb 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).

Data analysis. Data were recorded as presence (1) or absence (0) of amplified products from the examination of photographic negatives. Each amplification fragment was named by the source of the primer, 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 Dice coefficient of similarity (Nei and Li 1979). Cluster analyses were carried out on similarity estimates using the unweighted pair-group

method with arithmetic average (UPGMA) using NTSYS-PC, version 1.80 (Rohlf 1995).

RESULTS AND DISCUSSION

Identification and genetic relationships 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 traditional method of identifying species by morphological characters is now gradually being replaced by protein that is more reliable or DNA profiling largely because of several limitations of morphological data. In recent years, DNA profiling through RAPD technique has been used for the analysis of diversity and identification of duplicates within the large germplasm populations (Virk et al. 1995), phylogenetic relationship (Millan et al. 1996), rational designing of breeding programs (Powell et al. 1996), and management of genetic resources (Bretting and Widrelechner 1995). 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. The primer-screening step resulted in 10 decamer primers which, detected good polymorphisms (Table 1) and 20 other random primers, which did not give any amplification products. The reproducibility of the amplification products was tested on template DNA from three independent extractions of two initial clumps, using leaf samples in different seasons. The amplification profiles of total genomic DNA from twelve bamboo species with ten random primers produced a total of one hundred thirty seven fragments ranging in size from 0.4–3.3 kb, out of which all were polymorphic (Table 2). The number of fragments produced by a primer ranged from 10 (OPN 9) to 21 (OPA 11). Pattern of RAPD fragments produced by the random primer OPA 11 and OPA 04 are shown in Figure 1. Figure 1 also depicts that monomorphic fragments (bands) occur between *Bambusa vulgaris*, *Bambusa vul-*

Table 1. Total number of amplified fragments and number of polymorphic fragments generated by PCR using selected random decamers

Name of primer	Sequence of the primer	Total number of amplification products	No. of polymorphic products	Size range (kb)
OPA 04	5'-AATCGGGCTG-3'	18	18	0.4 −2.6
OPA 11	5'-CAATCGCCGT-3'	21	21	0.6−3.0
OPA 19	5'-TCTGTGCTGG-3'	16	16	0.4−2.8
OPA 17	5'-GACCGCTTGT-3'	10	10	0.4−3.3
OPA 20	5'-GTTGCGATCC-3'	14	14	0.5−3.2
OPN 11	5'-TCGCCGCAAA-3'	14	14	0.4−2.7
OPN 13	5'-AGCGTCACTC-3'	11	11	0.7−2.7
OPN 19	5'-GTCCGTACTG-3'	10	10	0.4−1.9
OPN 20	5'-GGTGCTCCGT-3'	13	13	0.4−2.3
OPN 04	5'-GACCGACCCA-3'	10	10	0.5−2.5

Table 2. Similarity matrix for Nei and Li's coefficient of twelve different taxa of bamboo

	1	2	3	4	5	6	7	8	9	10	11	12
1	1											
2	0.82	1										
3	0.62	0.69	1									
4	0.22	0.20	0.21	1								
5	0.18	0.19	0.16	0.68	1							
6	0.26	0.32	0.20	0.27	0.18	1						
7	0.20	0.25	0.24	0.16	0.20	0.27	1					
8	0.20	0.20	0.11	0.20	0.25	0.29	0.27	1				
9	0.21	0.23	0.20	0.20	0.16	0.25	0.24	0.29	1			
10	0.26	0.25	0.23	0.31	0.33	0.20	0.19	0.25	0.12	1		
11	0.26	0.27	0.23	0.15	0.13	0.23	0.22	0.14	0.33	0.18	1	
12	0.12	0.15	0.18	0.37	0.34	0.22	0.23	0.21	0.17	0.22	0.17	1

1 – *Bambusa vulgaris*, 2 – *Bambusa vulgaris* var. striata, 3 – *Bambusa ventricosa*, 4 – *Bambusa multiplex* var. Silver stripe, 5 – *Bambusa multiplex*, 6 – *Bambusa arundinacea*, 7 – *Bambusa balcooa*, 8 – *Cephalostachyum pergracil*, 9 – *Dendrocalamus giganteus*, 10 – *Dendrocalamus strictus*, 11 – *Dinocloa m'Clellandi*, 12 – *Sasa* species

garis var. striata and *Bambusa ventricosa*. The similarity matrix obtained using Nei and Li's coefficient (Nei and Li 1979) is shown in Table 2. Similarity coefficients ranged from 0.11–0.82 in twelve species of bamboo tested in the present experiment. These similarity coefficients were used to generate a tree for cluster analysis using UPG-MA method (Figure 2). Two types of comparisons were carried out to evaluate the degree of genetic diversity and relationships in the bamboo germplasm collection i.e. among groups (which represent mostly groups of taxa), as well as between bamboo species. The cluster analysis indicates that 12 taxa belonging to different genera of bamboo formed two major clusters based on similarity indices. Both major clusters were further divided into
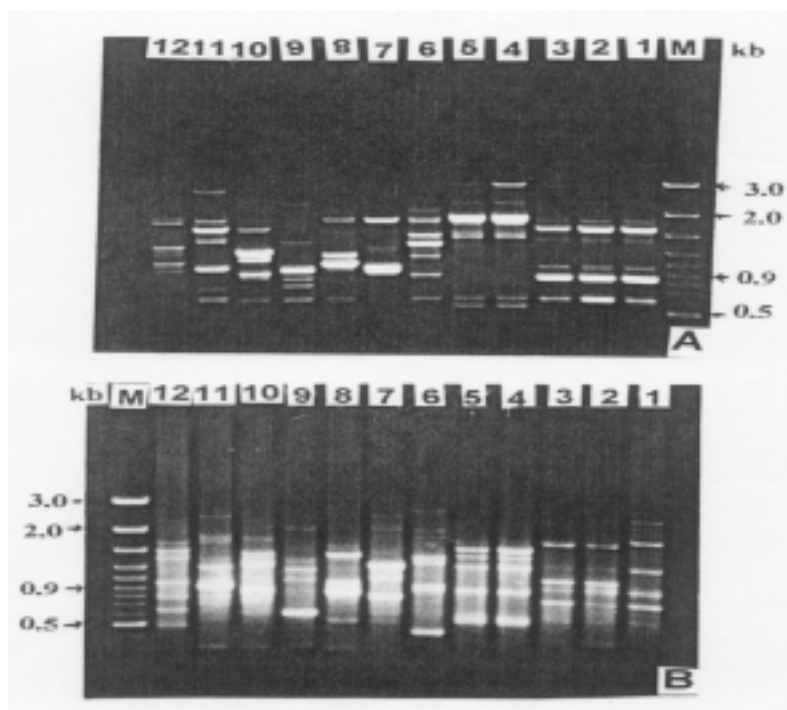

Figure 1. RAPD patterns of 12 taxa of bamboo generated by primer OPA 11 (5'-TCGCCGCAAA-3') (A), and OPA 04 (5'-AATCGG-GCTG-3') (B); M – kb molecular weight ladder; 1 – *Bambusa vulgaris*, 2 – *Bambusa vulgaris* var. striata, 3 – *Bambusa ventricosa*, 4 – *Bambusa multiplex* var. Silver stripe, 5 – *Bambusa multiplex*, 6 – *Bambusa arundinacea*, 7 – *Bambusa balcooa*, 8 – *Cephalostachyum pergracil*, 9 – *Dendrocalamus giganteus*, 10 – *Dendrocalamus strictus*, 11 – *Dinocloa m'Clellandi*, 12 – *Sasa* species
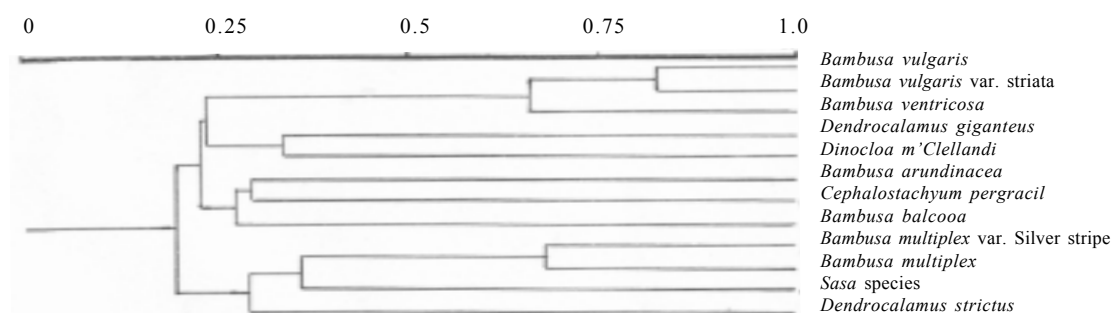
Figure 2. UPGMA dendrogram of cluster analysis of RAPD markers illustrating the genetic relationships among the 12 species of bamboo

three minor clusters. First minor cluster comprises *B. vulgaris*, *B. vulgaris* var. striata, *B. ventricosa*, *Dendrocalamus giganteus* and *Dinocloa m'Clellandi*. *Bambusa vulgaris* and *B. vulgaris* var. striata exhibit about 80% similarity because of two varieties. As per the cluster analysis, *B. ventricosa* was closely related (about 60% similarity) with *B. vulgaris* var. striata. It is clearly evident from the Figure 2 that the monomorphic bands appeared between these two species. Chua et al. (1996) reported that *B. ventricosa* was only a cultivated variety of a *B. vulgaris*. The second minor cluster was formed by *B. arundinacea*, *B. balcoa* and *Cephalostachyum pergracil*. *Bambusa arundinacea* showed about 20% similarity with *Bambusa vulgaris* and about 30% with *Bambusa ventricosa*. Third minor cluster comprises with four taxa i.e. *B. multiplex*, *B. multiplex* var. Silver stripe, *Sasa* sps and *Dendrocalamus strictus*. Among these, *B. multiplex* and *B. multiplex* var. Silver stripe were two varieties and very close to each other. Interestingly, it was noted that two species of *Dendrocalamu*s were not grouped into a single cluster. While *D. giganteus* was present in the first cluster along with climbing bamboo *Dinocloa*. *Dendrocalamus strictus* was associated with *Sasa* sps and *B. multiplex* group. The RAPD analysis indicate that *Dendrocalamus giganteus* showed about 34% similarity to *Bambusa ventricosa*, and 20% similarity to *Bambusa vulgaris* var. striata and *B. vulgaris*. Interestingly, the species of *Dendrocalamus strictus* showed about 70% similarity with *Sasa* sps. Our results show that *B. vulgaris*, *B. vulgaris* var. striata were a more closely related group in the genus *Bambusa*, from which *D. strictus* and *D. giganteus* were having considerable divergence. These results are in close correspondence with the earlier phylogenetic studies in *Phyllostachys* (Poaceae), based on RFLP's (Friar and Kochert 1994) and RAPD (Gielis et al. 1997). Besides, the divergence was higher between the genus *Sasa* and *Bambusa*, *Bambusa* and *Dendrocalamus*. *Bambusa vulgaris* showed a relatively higher level of divergence with *Sasa* sps and a relatively lower level of divergence with *Dendrocalamus* sps, suggesting a genetic progression of the taxa. The matrix analysis showed two different cluster groups of *Dendrocalamus strictus* and *D. giganteus*, though it belongs to same genus. Loh et al (2000) reported that two

species of *Dendrocalamus* i.e. *D. giganteus* and *D. brandissi* were grouped into two different clusters. The appearance of two different cluster groups was due to growth habit and morphological characteristics. In summary, the results from this study indicate that the RAPD technique is a useful tool for the identification of germplasm analysis and genetic relationships between and within the bamboo species. The relatively large number of polymorphisms obtained seems due to large phylogenetic distance among these taxa. It would allow a more quantitative assessment of genetic distances between species. Such an analysis, together with data from other classical methods, could thus be used to make a more accurate reconstruction of the bamboo evolution. Furthermore, such an approach might be helpful in identifying taxa of potential value in genetic improvement programmes.

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ABSTRAKT

Hodnocení genetické variability u bambusu pomocí markerů RAPD

Tradiční taxonomické studie bambusů vycházejí z květní morfologie a charakteristického růstu, což může kvůli nepravidelnému kvetení působit obtíže při identifikaci druhů. Zabývali jsme se identifikací a genetickou příbuzností u 12 druhů bambusu za použití metody náhodně amplifikovaných polymorfních DNK (RAPD). Analýza začala u 30 primerů o 10 merech, která umožnila rozlišení 12 druhů a výběr menšího souboru primerů. Vybrané primery jsme použili k identifikaci a založení profilového systému k vyhodnocení genetické rozmanitosti. Pomocí 10 vybraných primerů jsme provedli amplifikaci celkem 137 rozdílných polymorfních fragmentů (pruhů) DNK v rozsahu 0,4–3,3 kb. Obdobnou genetickou analýzu jsme provedli na základě přítomnosti nebo nepřítomnosti pruhů, která poukázala na široký rozsah variability mezi jednotlivými druhy. Klastrová analýza jednoznačně naznačila dva hlavní klastry, které náležely 12 druhům bambusu. Dva hlavní klastry jsme dále rozdělili na tři vedlejší klastry. Nejtěsnější příbuznost jsme zjistili mezi druhy *Bambusa vulgaris* a *Bambusa vulgaris* var. striata, které představovaly první vedlejší klastr spolu s *Bambusa ventricosa*. Blízce příbuzné byly odrůdy *Bambusa multiplex* var. Silver stripe a *Bambusa multiplex* a nijak se nelišily od *Bambusa ventricosa*. Další vedlejší klastr jsme získali mezi *Bambusa arundinacea*, *Cephalostachyum pergracil* and *Bambusa balcooa*. Existuje potenciální možnost využití metody RAPD ve šlechtitelských programech k identifikaci druhů a genetických příbuzností mezi taxony a druhy bambusu.

Klíčová slova: bambus; genetická variabilita; fylogenetická příbuznost; analýza RAPD

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