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Random amplified polymorphic DNA based genetic characterization of four important species of Bamboo, found in Raigad district, Maharashtra State, India

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Bamboos are arborescent grasses belonging to the family *Poaceae* and are grouped under the subfamily *Bambusoideae*. *Bambusoideae* are differentiated from other members of the family by the presence of petiolate blades with parallel venation and stamens are three, four, six or more, gynoecium is with single style with one to three stigmas and caryopsis. Bamboo is regarded as the major resource that meets the need of common man and also a poverty alleviator due to its multipurpose uses. The Raigad district, Maharashtra, India, is a good pocket of wild as well as cultivated bamboo. This district is rich in Bamboo flora specially four species *viz*. *Bambusa bambos*; *Pseudoxytenanthera ritcheyi*; *Pseudoxytenanthera stocksii* and *Dendrocalamus strictus.* To study the genetics of bamboo, one has to face enormous difficulties simply because of rare flowering and we cannot make hybridizations as easily as like other angiosperms. The randomly amplified polymorphic DNA (RAPD) method is used for genetic characterization of bamboo. This method of DNA profiling is proven excellent for identification, phylogenetic analysis, population studies and genetic linkage mapping of many plant species. Establishing multilocus markers RAPD analysis of genomes provides a versatile and rapid technology requiring low infrastructural input and use of arbitrary primers for developing plant DNA fingerprinting. In the present study, a comparative genetic analysis of four species of bamboo *B. bambus, P. ritcheyi*, *P. stocksii* and *D. strictus* collected from different locations of Raigad district of Maharashtra, India had been carried out using PCR-RAPD analysis. The present results warrant an extensive survey of genetic variation between the given four species of bamboo. The results would be helpful in designing sequence characterized amplified regions (SCAR) marker for each species. These SCAR markers can be used as diagnostic marker to authenticate samples of given four species.

Key words: Randomly amplified polymorphic DNA (RAPD), DNA-fingerprinting, sequence characterized amplified regions (SCAR), Genetic characterization, ritcheyi, stocksii, Raigad.

INTRODUCTION

Bamboo is a tribe of flowering perennial evergreen plants in the grass family Poaceae, subfamily Bambusoideae, tribe Bambuseae. Bamboos are some of the fastest-growing plants in the world, due to a unique rhizome-dependent system. The way bamboo grows and its wide distribution throughout the world makes it an important natural resource for hundreds of millions of people across the globe (INBAR Strategy, 2006). Bamboos are arborescent grasses belonging to the family *Poaceae* and are grouped under the subfamily *Bambusoideae*. *Bambusoi-*

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deae are differentiated from other members of the family by the presence of petiolate blades with parallel venation and stamens are three, four, six or more, gynoecium is with a one style, one to three stig-mas and caryopsis (Prasad and Gadgil, 1981). Bamboos are monocotyledonous plants which produce primary shoots without any later secondary growth (Seethalakshmi et al., 1998). The peculiarity of bamboo is that most species flower very infrequently, with intervals as long as 60 to 120 years. These species exhibit 'mass flowering' where all plants in the population flower at the same time. Bamboo is regarded as the major resource that meets the need of common man and also a poverty alleviator due to its multipurpose uses.

The Raigad district, Maharashtra, India, is a good pocket of wild as well as cultivated bamboo (Nagi, 1998). The Raigad district of Mahrashtra state, India is rich in bamboo flora including four important species *viz*. *Bambusa bambos*; *Pseudoxytenanthera ritcheyi*; *Pseudoxytenanthera stocksii* and *Dendrocalamus strictus.* The availability of bamboo offers many opportu-nities to address rural poverty in the Konkan and surrounding regions (Rao et al., 2009).

Due to the unusually long sexual cycle and unavailability of any other diagnostic tool, identification of bamboo is mainly dependent on vegetative descriptors such as culm morphology, and the morphology of the culm-sheath including ligule and auricle (Ohrnberger et al., 1986). Polymerase chain reaction (PCR)-based genetic markers are now well documented for species/cultivar identification (Khasa and Dancik, 1996). A number of PCR-based methods, including randomly amplified polymorphic DNA (RAPD) are available that do not require previous sequence information of the genome to be studied. For genetic characterization of bamboo, the RAPD method is used. This method of DNA profiling is proven excellent for identification, phylogenetic analysis, population studies and genetic linkage mapping of many plant species (Williams et al., 1990). Establishing multilocus markers RAPD analysis of genomes provides a versatile and rapid technology requiring low infrastructural input and use of arbitrary primers for developing plant DNA fingerprinting patterns that is useful in authentication of wide populations of closely related genotypes and in the study of the genetic variability of species or natural populations (Karp et al., 1997). In the present study, a comparative genetic analysis of four species of bamboo (*B. bambus, P. ritcheyi*, *P. stocksii* and *D. strictus*) collected from Raigad district of Maharashtra, India had been carried out using PCR-RAPD analysis.

MATERIALS AND METHODS

Plant materials for RAPD analysis

The plant materials used in the present study were *B. bambus*, (Sample code A-1)-from Adivli village (Land mark, Karjat- Kondivade road) of Tq.Karjat, district Raigad, India, on 14-04-2012, *P. ritcheyi*, (Sample code A-2) from Mutkyachi Wadi (Land mark, near Kondivade) of Tq. Karjat, district Raigad, India, on 14-04-2012, *P. stocksii*, (Sample code A-3)- from Chochichi Wadi (Land mark, near Kondivade) of Tq. Karjat, district Raigad, India on 14-04-2012 and *D. strictus*, (Sample code A-4) from Beed Village of Tq. Karjat, district Raigad, India, on 14-04-2012.

RAPD-PCR amplification

RAPDs are polymorphic DNA sequences that can be amplified using PCR; the resultant products can be separated using electrophoresis as discrete bands on an agarose gel. RAPDs are polymorphic DNA sequences that can be amplified using PCR; the resultant products can be separated using electrophoresis as discrete bands 2 on an agarose gel. Decamers (10 nucleotide sequence) are generally used as primer in a PCR system to amplify a locus of a polymorphic template DNA. The five primers have been designed primarily by Operon Technologies (Alameda, California, USA). These five primers with 10 nucleotide sequence were used in this RAPD PCR amplification (Sherif et al., 2009; Yuyu et al., 2010): 1. OPA-01: 5'-CAGGCCCTTC-3'; 2. OPA-13: 5'-CAGCACCCAC-3'; 3. OPD-08: 5'-GTGTGCCCCA-3'; 4. OPD-20: 5'-ACCCGGTCAC-3'; 5. OPN-05: 5'-ACTGAACGCC-3'.

DNA extraction and quantification

The leaf tissue of four bamboo samples were used for DNA extraction carried out using cetyltrimethyl-ammonium bromide-polyvinylpyrrolidone (CTAB-PVP) method (Doyle and Doyle, 1987). Approximately 0.5 x 0.5 cm tissue (washed) was ground in 0.5 ml extraction buffer (10% 1M Tris-HCl, 10% 200 mM EDTA, 40% 5 M NaCl, 3% CTAB and 1g PVP) (Chemical used was Tris-HCl from SD Fine Chemicals; EDTA also from SD Fine Chemicals; NaCl, Qualigen; CTAB, Himedia; PVP: Himedia) and incubated for 15 min at 65°C. 100 µl of sodium deodecyl sulfate (SDS) (20%) was added and re-incubated for 30 min. 150 µl of 7.5 M ammonium acetate was then added to the above solution and mixed by inverting. The tubes were incubated at 4°C for 15 min. DNA was extracted using 1 volume of chloroform: isoamyl alcohol mixture (24:1) and centrifuging at 10000 rpm for 5 min. Double volume of ethanol (96-100%) was added to the aqueous phase in a new tube, inverted twice and allowed to stand at 4°C for 30 min. The mixture was then centrifuged at 10000 rpm for 15 min. After drying for few seconds, pellet was dissolved in 50 µl elution buffer (10 mM Tris-HCl, 1 mM EDTA). 5 µl of RNase (Sigma) was added to the elute and incubated at room temperature overnight. Concentration of DNA was determined using UV-1800 spectrophotometer (Schimadzu Corporation). The DNA was stored at 4°C till further use.

Polymerase chain reaction (PCR) amplification

The DNA isolated from four samples was subjected to (PCR) amplification using 5 random 10-mer primers. PCR amplification was performed using Biometra thermal cycler with 2.5 µl of 10X buffer, 1 µl of each primer, 2.5 µl of 2.5 mM of each dNTP (dATP, dTTP, dGTP and dCTP), 2.5 Units of *Taq* DNA polymerase (Bangalore Genei) and 1 µl Template DNA and 9.5 µl nuclease free water. The PCR amplification cycle consist of, a cycle of 5 min at 94°C; 35 cycles of 1 min at 94°C, 1 min at 34°C, 2 min at 72°C; and additionally 1 cycle of 7 min at 72°C.

Agarose gel electrophoresis

Agarose gel electrophoresis is the easiest and commonest way of

separating and analyzing DNA. The purpose of the gel might be to look at the DNA, to quantify it or to isolate a particular band. 0.3 g agarose was dissolved into 25 ml TAE (Tris Acetic acid Electrophoresis) buffer, 2 µl of SYBR-safe (DNA staining dye) was added to it, mixed properly, heated for 1 to 2 min and cooled before pouring into the gel plates. The solution was poured into a gel casting tray containing the comb and allowed to set for 45 min. The Comb was carefully removed to avoid damage to the wells. About 300 ml of 1X solution of TAE buffer was used total for 2 gels. 5 µl of sample in the wells gel was mixed with DNA sample and to added to 100 bp DNA marker (Bangalore genei) into one well as standard. SYBR® Safe (Invitrogen, USA) was used for staining DNA and visualized in Green view illuminator (Chromus Biotech, India). The gel electrophoresis was carried out using *Tarsons* Electrophoresis unit. The current was set at 85 mA and voltage adjusted to 180 V; the gels were allowed to run for 30-45 min from negative to positive. The gel was put to the green view illuminator. The DNA band pattern was observed and photographed using digital camera (SONY® Cyber-Shot).

RESULTS

RAPD pattern was obtained by agarose gel electrophoresis and photographed. Lane 1 and 6 in each image are markers and lane 2, 3, 4, 5 for the samples of each species collected from different locations in the particular 3 set. Agarose gel electrophoresis was performed to separate and analyze the DNA, by isolating particular band(s), by using five primers.

Agarose gel electrophoresis for RAPD pattern was obtained in the gel image for *B. bambos* (A1)*; P. ritcheyi* (A2)*; P. stocksii* (A3) and *D. strictus* (A4), which were collected from Raigad district by using primer OPA-01. RAPD data were scored for presence (1), absence (0) or as a missing band. These bands were considered as polymorphic when they were absent in one among those four samples in a frequency greater than 1% and changes in band intensity were not considered as polymorphism. The band size generated by primers ranged from 200 to 800bp. At the band size of 800bp, in A1 sample the band (Figure 1a) was present and it was absent in A2, A3 and A4, while at 700 bp level the band was present in A4, but was absent in A1, A2 and A3. At 550 bp level the band was present in A1 sample and it was absent in A2, A3 and A4. Bands were present in all these four samples: A1, A2, A3 and A4. At 450, 250 and 200 pb level. Bands were absent in A1 and A4 and were present in A2 and A4 at 400 bp, while at 350 bp, the band was missing in A1 sample and it was present in A2, A3 and A4 (Figure 1a and Table 1).

AGE for RAPD pattern was obtained for Set A (A1, A2, A3 and A4) samples which were collected from Raigad district by using primer OPA-13. The band size generated by this primer ranged from 120 to 450 bp. At the band size of 450 bp, bands were present in A2 and A4 and were absent in A1 and A3, while at 320 bp level, the band was present only in A1. At 280 and 220 bp, the bands were present in these entire four samples, while at 120bp, band was missing only in A1 (Figure 1b and Table 2). AGE for RAPD pattern was obtained for Set A samples (A1, A2, A3 and A4) which were collected from Raigad district by using primer OPD-08. The band size generated by this primer ranged from 580 to 200 bp. At the band size of 580 and 500 bp, bands were present in A2, A3 and A4 and were absent in A1, while at 480 bp level the band was present only in A1. At 400 bp level, band was found only in A4 and at 320 bp position it was present in all the samples of set-A. At the band size of 250 band was present in A1, A2 and A3 and was absent in A4, while at 200 bp level the band was missing only in A1 (Figure 1c and Table 3).

AGE for RAPD pattern by using primer OPD-20 was obtained for Set A (A1, A2, A3 and A4) samples which were collected from Raigad district. The band size generated by this primer ranged from 180 to 480 bp. At the band size of 480, 420 and 180 bp, bands were present in A2 and A3 and were missing in A1 and A4, while at 400 bp level; the band was missing only in A4. At 320, 300, 270 and 220 bp level, bands were present in all the samples of set-A, while at 180 bp level, the bands were missing in A1 and A4 and were present in A2 and A3 (Figure 1d and Table 4).

RAPD pattern was generated by AGE, using the primer OPN-05 and was obtained for Set A samples (A1, A2, A3 and A4) which were collected from Raigad district. The band size generated by this primer was in the range of 200 to 700 bp. At the band size of 700 bp, the band was present only in A4, while it was present in A1 at 650 bp level. The presence of band was found in A1 and A3 at 550 bp. The band was present only in A3 at 420 bp size. And bands were present in A2 and A3 and were missing in A1 and A4 at 350 bp level. At 450, 280 and 250 bp, bands were present in all the samples of set-A. The band was missing in A4 and was absent in A1 at the level of 220 and 200 bp respectively (Figure 1e and Table 5).

Comparative RAPD analysis of four samples of Bamboo

RAPD analysis based on the use of short random sequence of five primers each having 10 bases in length was performed between the four species. Based on 0-1 data generated, the genetic polymorphism was studied. RAPD pattern for four species of bamboo viz., *B. bambos; P. ritcheyi; P. stocksii* and *D. strictus,* was obtained by using five primers. Out of 38 loci, 25 are polymorphic, that is, 65.79% (Table 6).

DISCUSSION

Due to the lack of valuable breeding systems in bamboo, genetic mapping is very restricted and back-crossing is almost impossible. To our knowledge, nothing along these lines has been done. Some approaches are use-

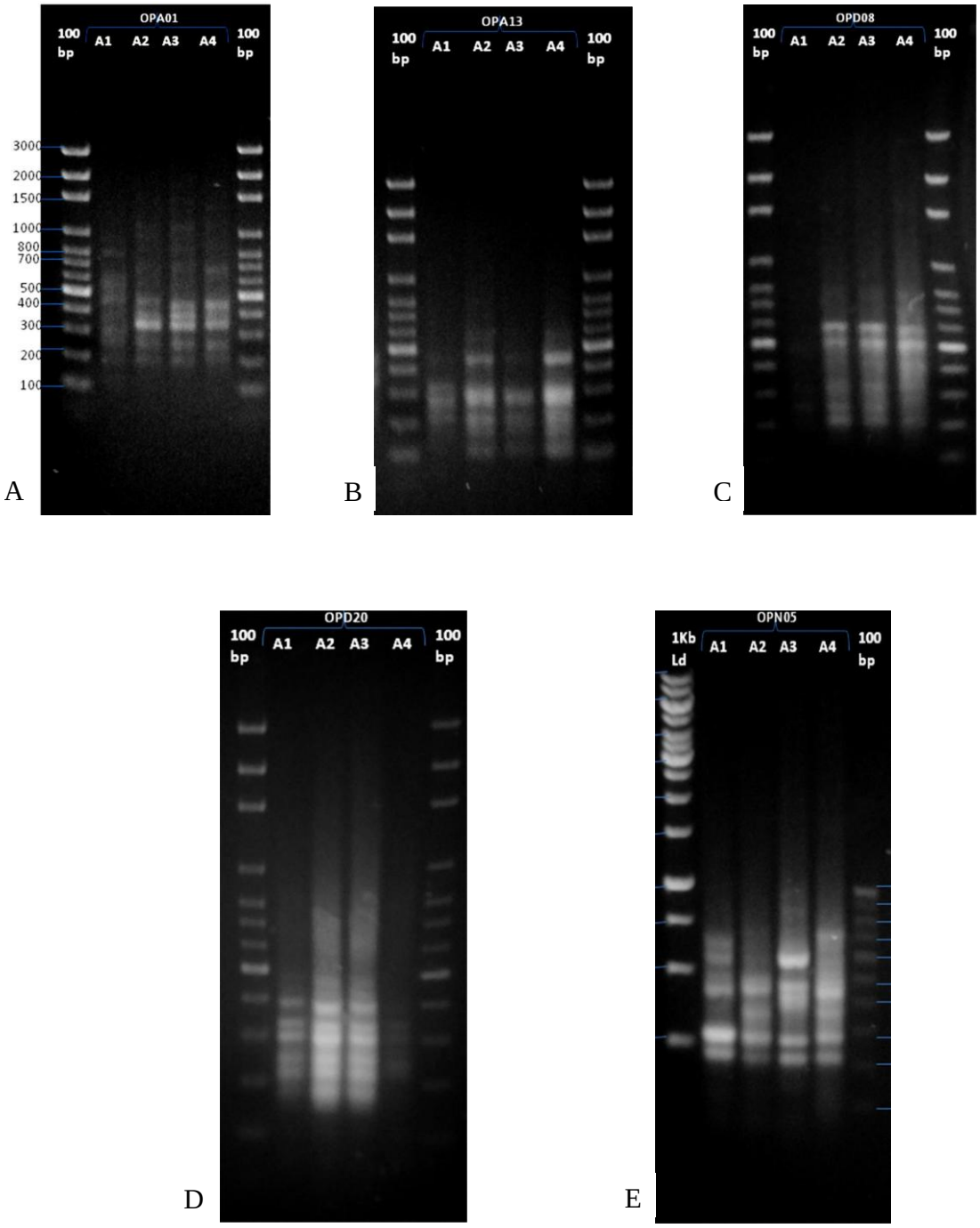

Figure 1. RAPD pattern for four species of bamboo, collected from Raigad dist. by using primers. **a)** OPA - 01; .**b)** OPA-13; **c)** OPD-08; **d)** OPD-20 and **e)** OPN-05 (Samples: A1: *B. bambos*; A2: *P. ritcheyi*; A3: *P. stocksii* and A4: *D. strictus*). Lane 1: 100 bp DNA marker (3000-100 bp); lane 2-5, PCR product of A1-A4; Lane, 6: 100 bp DNA maker (3000-100 bp).

ful for at least attempting to identify genetic traits in bamboo or to construct genetic maps of bamboo. The use of molecular markers was introduced to complement other well-developed techniques such as micropropagation and to develop objective tools for genotype identification (Gielis, 1995).

RAPD markers are proved to be an efficient and inexpensive way to provide molecular data. They have been used successfully in determining genetic relationship and used for DNA fingerprinting (Moreno et al., 1998). The first application of RAPD markers to characterize the genetic diversity of *Dendrocalamus asper* in Indonesia

Table 1. RAPD data by using OPA-01 primer.

Band Size bp	A1	A2	A3	A
800	1	0	0	0
700	0	0	0	1
550	1	0	0	0
450	1	1	1	1
400	0	1	1	0
350	0	1	1	1
250	1	1	1	1
200	1	1	1	1

Table 2. RAPD data by using OPA-13 primer.

Band size (bp)	A1	A2	A3	A4
450	0	1	0	1
320	1	0	0	0
280	1	1	1	1
220	1	1	1	1
120	0	1	1	1

Table 3. RAPD data by using OPD-08 primer.

Band size (bp)	A1	A2	A3	A4
580	0	1	1	1
500	0	1	1	1
480	1	0	0	0
400	0	0	0	1
320	1	1	1	1
250	1	1	1	0
200	0	1	1	1

Table 4. RAPD data by using OPD-20 primer.

Band size (bp)	A1	A2	A3	A4
480	0	1	1	0
420	0	1	1	0
400	1	1	1	0
420	1	1	1	1
300	1	1	1	1
270	1	1	1	1
220	1	1	1	1
180	0	1	1	0

revealed its results indicating the RAPD technique is useful tool for analyzing genetic diversity of the bamboo species. Given the asexual reproductive system of the species, the low genetic diversity is normal (Lee et al., 2000; Hamrick et al., 1992). The RAPD technique produced high polymorphism information content. The polymorphic RAPD profiles are reasonably in agreement with one of the most reliable and orthodox systems of bamboo classification proposed by Gamble (1896). Thus, the present investigation demonstrated the reliability of RAPD technique in determining the relationships in this plant group with high commercial importance and diagnostic fingerprinting pattern of four bamboo species each obtained from four different geographic locations.

Table 5. RAPD data by using OPN-05 primer.

Band size (bp)	A1	A2	A3	A4
700	0	0	0	1
650	1	0	0	0
550	1	0	1	0
450	1	1	1	1
420	0	0	1	0
350	0	1	0	1
280	1	1	1	1
250	1	1	1	1
220	1	1	1	0
200	0	1	1	1

Table 6. Comparative RAPD data for *B. bambos* (A1), *P. ritchey* (A2), P. stocksii (A3) and *D. strictus* (A4).

Primer name	Sample	Total loci	Polymorphic loci	% Polymorphism
OPA-01	A1, A2, A3, A4	08	05	62.50
OPA-13	A1, A2, A3, A4	05	03	60.00
OPD-08	A1, A2, A3, A4	07	06	85.71
OPD-20	A1, A2, A3, A4	08	04	50.00
OPN-05	A1, A2, A3, A4	10	07	70.00

Due to unusual flowering cycle, the identification of bamboo is based on vegetative characters (Muktesh Kumar et al., 2001). The present efforts of this research work may add an important molecular data in support to the vegetative characters. The *B. bambos* (A1) and *D. strictus* (A4), shares maximum number of similar bands (Table 1, 4 and 5), and these similarities are convening with the similar vegetative characters of these species. *P. ritcheyi* (A2) and *P. stocksii* (A3) shared many common bands (Tables 1, 2, 3 and 4); this may be because of very less genetic distance as these species belongs to the same genus, that is, *Pseudoxytenanthera.* The primer OPA-01 reveals 62.50% polymorphism, while OPA-13 reveals 60%. OPD-08, OPD-20 and OPN-05 revealed 85.71, 50 and 70% polymorphism, respectively (Table 6). The comparative RAPD analysis in the four species of Bamboo *viz. B. bambos, P. ritcheyi*, *P. stocksii* and *D. strictus,* collected from Raigad district showed 65.79% polymorphic loci (Table 6).

The present results warrant an extensive survey of genetic variation between the given four species of bamboo. The results would be helpful in designing SCAR marker for each species. These SCAR markers can be used as diagnostic marker to authenticate samples of the given four species (Paran et al., 1993).

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