Use of RAPDs to Evaluate the Genetic Diversity in Micropropagated Plants of *Dendrocalamus giganteus* and in a Population of the Same Species in the Royal Botanic Gardens, Peradeniya

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ABSTRACT

***Dendrocalamus giganteus* is an economically important bamboo and can be micro propagated on a large scale by tissue culture techniques. The detection of variation among such clones is important for large-scale plantation. Molecular methods of DNA techniques can be applied to detect genomic variation, which are not detected phenotipically. Therefore, RAPD technique was used to detect genetic diversity in 50 micropropagated plants of *D.giganteus* and twenty-three clumps of the same species growing in the Royal Botanic Garden, Peradeniya. Modified Doly and Doyle method was used for the extraction of DNA. The random primers OPF4, OPF14 and OPH4 were used in the Polymerase Chain Reaction. However it was not possible to generate DNA fragments possibly due to poor quality of DNA. Protein, phenol and certain polysaccharides may inhibit the RAPD reaction. In *D.giganteus* population in Royal Botanic Garden the genetic diversity was low (0.00-0.12) and clumps were closely related. It may have been propagated mainly by vegetative methods after its introduction to Sri Lanka approximately about 150 years ago.**

KEY WORDS: *Dendrocalamus giganteus*, Genetic diversity, RAPD markers, Royal Botanic Garden

INTRODUCTION

Germplasm characterization is an important for the conservation and utilization of plant genetic resources. Traditionally, morphological characters like growth habit, leaf type and floral characters are used to define taxa (Nayak *et al*, 2003). In the past, plant taxa were mostly defined by morphological features only. Morphological features can be affected by environmental factors and therefore not always dependable in taxonomic studies. DNA is not affected in this manner and 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 (Nayak *et al.*, 2003).

Bamboo, which belongs to sub family Bambusoidae of the family Poaceae, is a woody grass of tropical, sub tropical and temperature regions. It is used for food, fodder, building material and raw material for production of paper (Das and Rout, 1994). Therefore, demand for bamboo has increased rapidly. Traditionally bamboo is propagated by seed, offset and culms cuttings. The difficulty, which arises in utilizing seed, is their low viability, poor storage characteristics and inborn microbial infestation and limited availability. Vegetative propagation by conventional methods also has proved to be of limited use and cannot cater in the required scale. An alternative method is the use of *invitro* techniques of micropropagation. Micropropagated bamboo clones are morphologically similar. However, during rapid propagation encountered in tissue culture techniques, genetic variation may be possible. These cannot be Detected unless the phenotype is affected. DNA markers are able to detect such genetic variation.

The most common types of markers used today are Restriction fragment Length Polymorphism (RFLP), Random Amplified polymorphic DNA (RAPD), Simple Sequence Repeat (SSR), Variable Number of Random Repeat (VNTR) and Amplified fragment Length polymorphism (AFLP). But RAPD assay is the cheapest method for identifying genotypes within a short period and requires only a limited amount of DNA. The development of RAPD markers, generated by the polymerase chain reactions (PCR) using arbitrary primers, has provided a 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.*, 1995, Kolliker *et al.*, 1999, Nair *et al.*, 1999). The primary drawback in using RAPD markers is that repeatability is sometimes not possible and do not permit the scoring of heterozygous individuals.

In this study, an attempt was made to detect genetic diversity in 50 plants of *D.giganteus* raised by micropropagation.The genetic diversity in a population of twenty-three clumps of *D.giganteus* in the Royal Botanic Garden, Peradeniya was also investigated using RAPD data collected earlier in the Plant Biotechnology Project of the IFS.

MATERIALS AND METHODS

The experiments were carried out at the Plant Biotechnology Project of the Institute of Fundamental Studies, Hanthana Road, Kandy during a period of 24 weeks.

Plant Establishment and Maintenance

In vitro rooted axillary shoots of *D. giganteus* were selected for the study. After acclimatization they were planted in a coir dust medium in hardening trays. After 3-4 weeks plants were transferred to soil in polythene bags. Lawn grass fertilizer (CIC) was provided at two-week intervals and plants were watered regularly.

DNA Extraction

After three months, DNA was extracted from young leaves of micropropagated *D. giganteus* using a modified CTAB extraction protocol (Doly and Doyle, 1990). The solution of CTAB buffer (2% CTAB, 1.4M NaCl, 20mM Na_2EDTA), mortar and pestle were kept at -4°C for 1 hour. 0.5 g of leaf tissues from the base of unexpanded leaves were placed in the cooled mortar and pestle and ground with 1.5ml cold extraction buffer. The ground mixture was placed in tubes containing CTAB extraction buffer to which 4.5µl β-mercaptoethanol was added and maintained at 65°C in a water bath. The suspension was incubated at 65 °C in a water bath for 30 minutes with occasional gentle shaking. Next an equal volume of chloroform: isoamylalchohol (24:1) was added and thoroughly mixed. This was repeated using the top aqueous layer that separated after centrifugation at 5000 rpm 5 minutes for the removal of protein. The crude DNA in the top aqueous layer was precipitated in 0.6 volume of isopropanol and washed in a solution 76% ethanol and 10 mM ammonium acetate. RNA was removed by incubating the precipitate with RNAase at 37°C and polysaccharide removed in 7.5M ammonium acetate. The DNA was precipitated in absolute ethanol, washed in 70% ethanol and finally suspended in 50µl TE (10 mM Tris-HCL: 1 mM EDTA). The DNA extraction was stored at -20 °C.

Purity Analysis of Extracted DNA Samples

2µl DNA sample was mixed with 2µl of gel loading buffer and 6 µl of distilled water. The mixture was loaded on a 0.8% agarose gel for electrophoresis using 0.5xTBE buffer. The DNA was visualized by ethilium bromide staining on a UV transilluminator. Light absorbance was also measured using 1µl sample of DNA with 1 ml 0.1xTE buffer using spectrophotometer at 260nm and 280nm.

DNA was further purified to avoid contamination. Chloroform: isoamylalchol were used to remove the proteins. Then DNA was precipitated using two volumes of absolute ethanol and 1/10 volume of 7.5 M ammonium acetate. Then washed in 70% ethanol and precipitated DNA as a pellet. Finally DNA was dissolved 0.1 M TE buffer.

PCR Amplification

Amplification was carried out in a 25µl reaction volume with 5µl DNA, 5pM random 10-mer primer (operon Technologies California), 125µM each of dATP, dTTP, dGTP, and dCTP (Promega Coorporation, USA), 0.2 units Taq DNA polymerase in 1x polymerase buffer, 2.5 mM $MgCl_2$ (Promega Coorporation, USA) and overlaid with 25µl mineral oil. Amplification was performed in a Sanyo DNA thermal cycle (MIR-D30) programmed for 45 cycles with the first cycle at 94°C for 4 min, 36°C for 1min and 72°C for 2min, fallowed by 44 cycles at 94°C for 1min, 36°Cfor 1min and 72°C for 2 min. A serial dilution of DNA was also carried out to identify the optimum concentration of DNA, in the reaction mixture.

The amplification products were separated by electrophoresis in 1.5% agarose gels in 0.5xTEB (45mM Tris Borate, 1 mM EDTA) buffer. The gels were stained with ethilium bromide and photographed on Polaroid 665 Film under UV light.

Data Analysis

Amplified products (bands) were recorded as present (1) or absent (0) by examination of photograph of stained gels. The genetic distance and the dendrogram were computed using the RAPDistance Version 1.04, freeware package (Armstrong *et al.*, 1994).

RESULTS AND DISCUSSION

In the present study, unfolded tender leaves were selected as source material to facilitate easy cell disruption for DNA extraction. CTAB is a cationic detergent, which solubilizes membranes and forms a complex with DNA. These techniques capitalize on the remarkable ability of CTAB to bind with DNA and RNA when salt (NaCl) concentration is 0.7 M or above and precipitate the nucleic acids when the salt concentration is below 0.4 M (Everard, 1983). In this study, a modified CTAB protocol was used. Because it is easy as well as reliable and cheap.

According to the gel photograph (fig. 1) of extracted DNA, most of the DNA was degraded. The ratios of A_{260}/A_{280} of the micropropagated *D. giganteus* plant DNA sample were estimated (Table 1.).

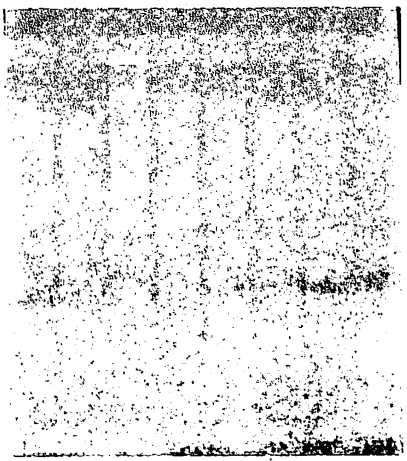

Figure 1-Quantity and Quality assessment of extracted DNA using gel electrophoresis:

Table1-Yield and Quantity of genomic DNA extracted from *Dendrocalamus giganteus:*

DNA Samples	1	2	3	4	5
A_{260}/A_{280}	1.5	1.29	1.31	1.14	1.12
DNA yield µg/mg	0.3	1.1	0.85	0.4	0.9

The ratio A_{260}/A_{280} measurement should be about 1.8 for pure DNA. A ratio lowers than 1.6 indicates significant contamination with protein or phenol. High concentrations of β-mercaptoehanol inhibit the polyphenol oxidation. The remaining of polyphenols, which are powerful oxidizing agent present in many plant species, can reduce the DNA yield and purity by making it unsuitable for most research application (Katterman and Shattack, 1983, Peterson *et al.*, 1997, Porebski *et al.*, 1997). CTAB is soluble in ethanol and residual amounts are removed in the subsequent ethanol washes (Fang *et al.*, 1992).

DNA was further purified to avoid contamination. But DNA was present as smears in the gel after electrophoresis though it was better than unpurified DNA. A sample with a better absorbance value (sample 1) was selected for PCR. RAPD technique has been used for the analysis of diversity and identification of duplicated samples within a large germplasm population (Virk *et al.*, 1995). Phylogenetic relationship (Millan *et al.*, 1996), rational designing of breeding programs (Powell *et al.*, 1996) and management of genetic resource (Bretting and Widrelechner, 1995). Evidently RAPD technology is a rapid and sensitive technique, which can be used to estimate relationship between closely and more distantly related species and groups of bamboo (Nayak, 2003). OPF9, OPF14 and OPH4 were used for PCR amplification. However it was not possible to detect clear bands after electrophoresis. The amount of added DNA is critical for achieving a quantitative assay. Too much DNA will saturate the replication machinery in terms of not only specific products generated, but also nonspecific products. Even after a serial dilution of DNA, bands could not still be detected.

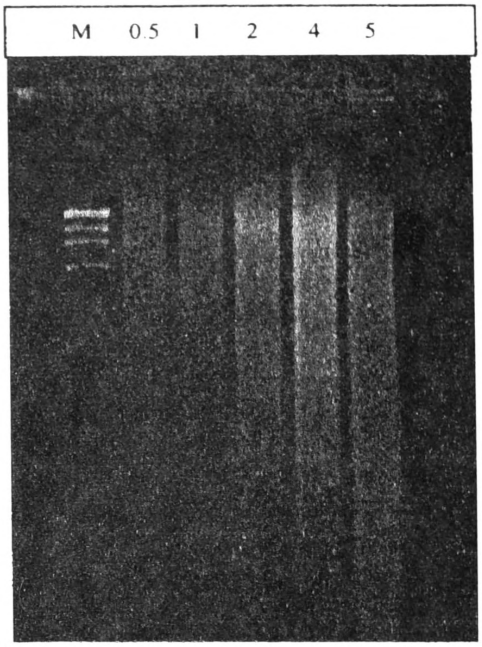

Figure 2- Gel photograph after the PCR:

This problem may due to the poor quality of DNA. Certain polysaccharides are known to inhibit RAPD reactions. They distort the results in many analytical applications (Kotchoni *et al.*, 2003). The problem may also be due to partial denaturing of Taq DNA polymerase used in this study.

RAPD profiles (photographs) of DNA from 23 clumps of *D.giganteus* in the Royal Botanic gardens Peradeniya that was also scored for presence or absence of bands from six primers (Table 2.) generalized a distance matrix and a dendrogram (figure 4.).

Table 2 - Total number of amplified fragments and polymorphic fragment generated by PCR using selected random decamers.

Name of Primer	Sequence of primer	No. of polymorphic product
OPF 9	5'CCAAGCTTCC 3'	12
OPF14	5'TGGTGCAGGT 3'	8
OPH 4	5'GGAAGTCGCC 3'	10
OPI 4	5'CCGCCTAGTC 3'	10
OPJ 20	5'AAGCGGCCTC 3'	10
OPG19	5'GTCAGGGCAA 3'	8

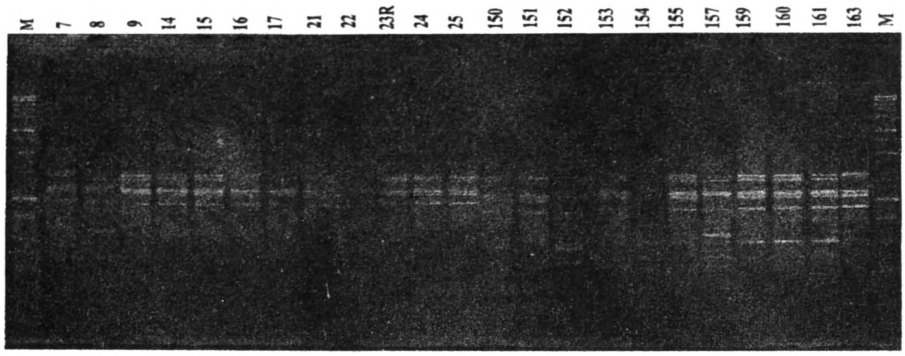

Figure 3 - Polymorphism detected by Primer OPH4:

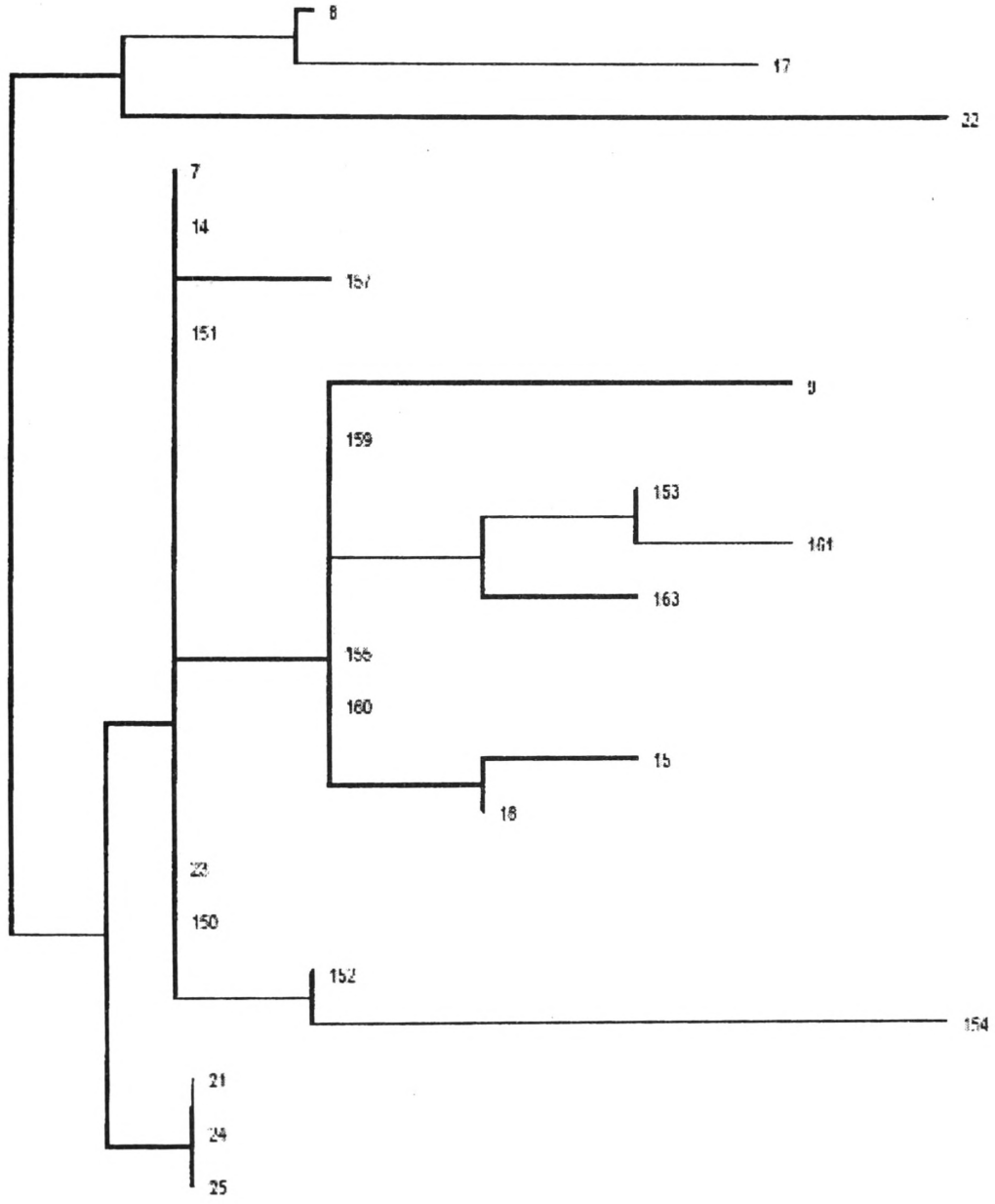

Figure 4 - The dendrogram of population of *Dendrocalamus giganteus* (23 clumps) in the Royal Botanic Garden, Peradeniya:

The range in genetic distance of 0.00-0.12 shows that the 23 clumps of *D.giganteus* in the Royal Botanic Gardens, genetic diversity were low and they were closely related. Clump number 21, 24, 25 and 7, 14, 151, 23,150 had a genetic distance of zero among them indicating they are clones. Some of them were

spatially close and may have separated from a single clump after a long period of vegetative growth. Clumps of 22 had the highest mean genetic distance of 0.077 from all other clumps. This plant may be a mutated of a seed raised plant. The species was introduced to Sri Lanka relatively recently about 150 years ago. It may have been propagated by mainly by vegetative methods as seeds as rare. This may be the reason for the low genetic diversity observed in this investigation.

CONCLUSION

The ratio of 260A/280A obtain from the spectrophotometer meter reading of DNA samples are less than 1.6 due to contaminate with phenol and the protein. DNA can be contaminated with polysaccharide due to dissolving in the ethanol residuals. Therefore, poor quality of DNA affect at the PCR reaction to yield RAPD fragments. According to the results, 23 clumps of *D.giganteus* in Royal Botanic Garden indicate that the RAPD technique is a useful tool for the germplasm characterization analysis of and genetic relationship within same species of bamboo clumps. The relatively low polymorphism detected due to close relationship among the *D.giganteus* clumps. Other analysis method can be used to get more accurate results. Furthermore, such an approach might be helpful in the genetic improvement programmed.

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REFERANCES

Armstrong, J.S., A.J.Gibbs, R.Peakall and G.Weiller (1994)."TheRAPDistancePackage".fttp://life.anu.edu.au/pub/software/RAPDistance or http://life.anu.edu.an/molecular/software/rapd.html

Bretting, P.K., M.P.Widrelechner (1995). "Genetic markers and horticultural germplasm management".Hort.Sci, 30:1349-1356.

Das, P., G.R.Rout (1994). "Analysis of current methods and approaches on the Micropropagation of bamboo."pro.nat.Acad.Sci, India.64:235-246.

Doyle, J.J. and Doyle, J.L (1990). "Isolation plant DNA from fresh tissue". Focus 12, 13-15.

Everard, J.M.D.T.(1983).Course Notes.CRI.Lunuwila

Fang, G., Hammers, and R.Grumet (1992). "A Quick and inexpensive method for removing polysaccharides from plant genome DNA".Biotechniques.13:52-57.

Gunter, L.E., G.A.Tuskan, S.D.wullschleger (1996). "Diversity among populations of switchgrass based on RAPD markers". Crop Sci., 36:1017-1022.

Huff, D.R, R.Peakall, P.E.Smouse (1993). "RAPD variation within and among natural populations of outcrossing bufferlograss [*Buchloe dactyloides* (Nutt.)Engelm]".Theor.Appl.Genet.86:927-934.

Katterman, F.R.H., V.I.Shattuck (1983). "An effective method of DNA isolation from the mature leaves of *Gossypium* species that contain large amounts of phenolic terpenoids and tannins" prep.Biochemestry.13:347-359.

Kolliker, R., F.J.Stadelmann, B.Reidy, J.Nosber (1999). "Genetic Variability of forage grass cultivars". A comparison of *Festuca pratensis* Huds, *Llium perenne* L., and *Dactylis glomerata* L.Euphytica, 106:261-270.

Kotchoni, S.O., E.W.Gachomo, E.Betiku, O.O.Shonukan (2003).a home made kit for plasmid DNA mini-preparation.Afr.J.Biotechnology.2:88-90.

Millan, T., F.Osuna, S.Cobos, A.M.Torres, J.J.Cubero (1996). "Using RAPDs to study phylogenetic relationships in *Rosa"*.Theor.Appl.Genet.92:273-277.

Nair, N.V., S.Nair, T.V.Sreenivasan, M.Mohan (1999). "Analysis of genetic diversity and phylogeny in *Saccharum* and related genera using RAPD markers".Genet.Res.Crop Evol, 46:73-79

Nayak, S., G.R.Rout, P.Das,(2003)."Evaluation of the genetic variability in bamboo using RAPD markers". Plant Soil erosion 49(1).Regional Plant Resourcecenter.Bhubaneswar.Orissa.India:24-28.

Peterson, D.G., K.S.Boehm, S.M.Stack (1997). "Isolation of milligram quantities of nuclear DNA from tomato (*Lycopersicon esculentam*) a plant containing high levels of polyphenol compounds". Plant Mol.Biot.Rep.15:148-153.

Porebski, S., L.G.Baily, B.R.Baum (1997). "Modification of a CTAB DNA extraction protocol for plant containing high polysaccharide and polyphenol compound". Plant Mol.Biot.Rep.15:815.

Powell,W.,M.Morgante,C.Andre,M.Hanafey,J.Vogel, S.Tigey and A.Rafalski (1996). "The comparison of RFLP, RAPD, AFLP and SSR (micro satellite) markers for germplasm analysis".Mol.Breed. 2:225-238

Virk, P.S., B.V.Ford-Lloyd, M.T.Jackson, H.J.Newbury (1995). "Use of RAPD for the study of diversity within plant germplasum collection".Heredity.74:170-179.

Williams, J.G.K., A.R.Kubelik, K.J.Livak, J.ARafalski, S.V.Tingey (1990)."DNA polymorphisms amplified by arbitrary primers are useful as genetic markers".nucl.Acid.Res.18: 6531-653