AFLP-PCR MOLECULAR SCREENING OF COBALT-60 GAMMA RADIATION-INDUCED VARIANT OF *Murraya exotica* AND *Dracaena sanderiana*

INTRODUCTION

The International Atomic Energy Agency (IAEA) Technical Cooperation (TC) Program on Mutation Breeding on Selected Ornamental Crops at the Philippine Nuclear Research Institute (PNRI) is made up of two projects. One project involves the development of improved varieties of ornamentals through gamma irradiation *in-vitro* culture techniques and the other is molecular screening of polymorphism in gamma irradiated variant ornamentals and foliage plants. The objectives of this program are twofolds: ❶ to produce variant forms of ornamental and foliage plants with enhanced ornamental qualities through gamma radiation-induced mutation for export purposes and ❷ to generate computer database of cobalt-60 gamma radiation-induced variants of ornamentals and foliage plants for selective propagation by end-users or growers.

Plant breeders use various techniques to enhance the qualities of plants. One of these methods is to induce mutation using radiation source. At PNRI, omamental and foliage plants are irradiated with cobalt-60 source at its Multipurpose Irriadiation Facility. Irradiated plants are grown vegetatively until morphological changes in variant plants become stable. There are 3 objectives why this study is undertaken, namely, ❶ to develop the AFLP-PCR technique as a screening method for polymorphism in cobalt-60 gamma radiation-induced variants, ❷ to demonstrate polymorphism in gamma radiation-induced variants in sequencing gel DNA profile and ❸ to use the results of the study in production of computer database for cobalt-60 radiation-induced variants of ornamental and foliage plants.

Variations found in irradiated ornamentals and foliage plants are due to mutation(s) in the plant DNA. Changes in DNA sequence causing polymorphisms are identified using polymerase chain reaction technology (PCR). This technique amplifies DNA a thousand times producing enough materials for study. PCR technology coupled to DNA fingerprinting technique, amplified fragment length polymorphism (AFLP), is a very powerful tool in demonstrating polymorphism and has been an accepted methodology for fingerprinting plants and microorganisms. In this study we have adapted and modified the AFLP-PCR technology of Life Technologies, Inc. to show gamma radiation-induced polymorphism in *Murraya exotica* and *Dracaena sanderiana*. AFLP markers present in variant plants are documented against their morphological characteristics and encoded in the database for foliage plants, *Murraya exotica* and *Dracaena sanderiana*.

METHODOLOGY

A schematic diagram of the protocol used in our laboratory to demonstrate DNA fingerprints of gamma irradiated variants of *Murraya exotica* and *Dracaena sanderiana* is shown in Fig. 1.

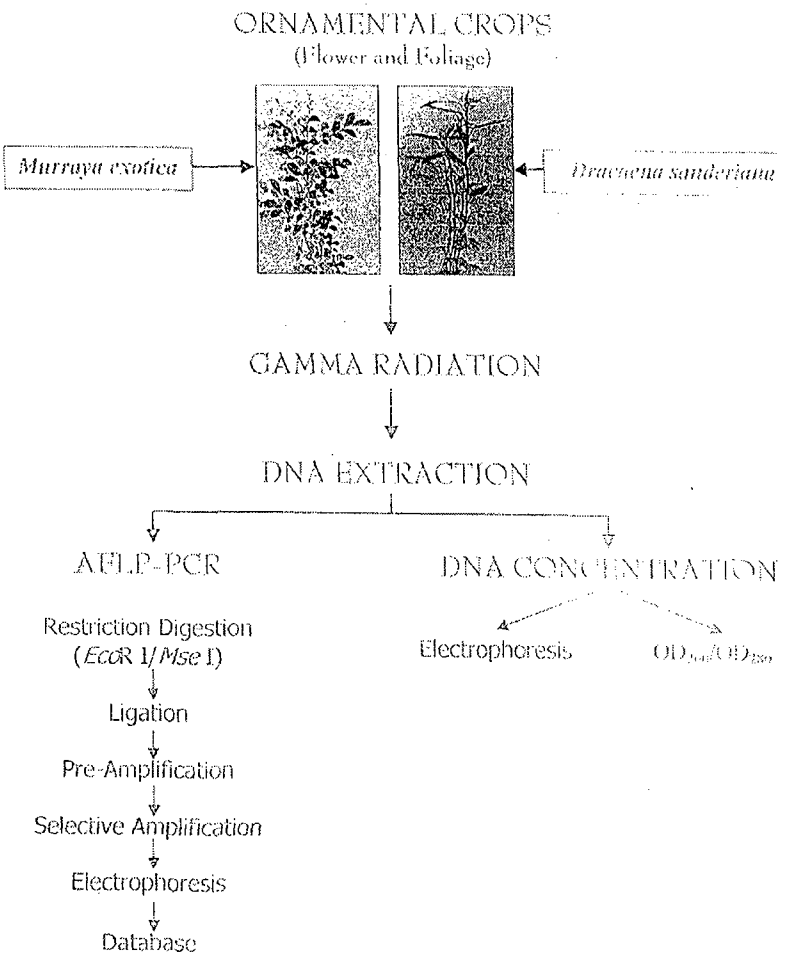


Figure 1. Protocol for DNA Amplification

Gamma Irradiation

Foliage plants *(Murraya exotica* and *Dracaena sanderiana)* were irradiated with cobalt-60 source at the PNRI Multipurpose Irradiation Facility at doses of 10, 20 and 30 Gy. These were vegetatively grown to plant stage M_1V_6 and M_1V_3.

Genomic DNA Extraction

Cobalt 60 gamma irradiated (10 and 30 Gy) foliage plants (*Murraya exotica* and *Dracaena sanderiana)* with prominent phenotypic variations were selected for genomic DNA extraction. Genomic DNA was

extracted from dark-adapted leaves of *Murraya* and *Dracaena* using a miniprep technique (Liscum and Oeller, 1998, Ausubel, 1989). Leaves were dark-adapted for 3-4 days in the cold. These were frozen in liquid nitrogen, ground in buffer and spun down using an Eppendorf microfuge to pellet DNA. The pellet was washed with ethanol, dried and suspended in TE buffer. Genomic DNA extracts were also prepared from larger amounts of plant leaves of both *Murraya* and *Dracaena* which were pelleted using Beckman ultracentrifuge. Cleaner preparation was obtained using the ultracentrifuge in addition to higher DNA concentration compared to DNA extract prepared from the microfuge.

DNA Concentration

Extracted DNA was quantitatively analyzed by UV spectrophotometric measurements using absorbance ratio of A_{260} nm/A_{280} nm (Nucleic Acid purification Guide, 1996). Agarose gel electrophoresis was also used to look at concentration and purity of the sample.

AFLP-PCR

Amplification of genomic DNA was performed with 2 enzyme system, EcoR 1 and Mse 1, and AFLP primers with one selective nucleotide for pre-amplification and three selective nucleotides for selective amplification (AFLP Analysis System I). Pre-amplification step was done with 20 amplification cycles and selective amplification with 23 cycles. AFLP- PCR protocol was adopted from AFLP technology of Life Technologies, Inc. (Lin and Kuo, 1995, GIBCOBRL Instruction Manual).

Electrophoresis

Agarose gel (2%) was used to electrophorese AFLP-PCR amplified DNA products. Ethidium bromide at 5% concentration was added to the gel after electrophoresis to visualize DNA bands (Ausubel, 1989).

Documentation

A Polaroid camera was used to document polymorphic bands. Visualization was done with a UV transilluminator and the data were documented as Polaroid hard copies.

Database

Morphological and molecular variations observed in gamma irradiated variant forms of *Murraya exotica* and *Dracaena sanderiana* were recorded for database of plant polymorphisms. Included in the database are actual photographs of plants irradiated at various radiation doses.

RESULTS AND DISCUSSION

Cobalt-60 gamma irradiated (10 and 30 Gy) foliage plants (*Murraya exotica* at M_1V_6 and *Dracaena sanderiana* at M_1V_3) with prominent phenotypic variations were selected for genomic DNA extraction. Dramatic phenotypic changes are observed in *Murraya exotica* after exposure to cobalt-60 gamma radiation doses of 10 and 30 Gy. At these doses, dwarfism was generated in *Murraya* plant at M_1V_6 as shown in Fig. 2. With *Dracaena sanderiana*, (M_1V_3) dwarfism developed only after exposure of plant to 30 Gy gamma radiation. Cobalt-60 gamma radiation at a dose of 10 Gy did not affect the growth of the foliage plant (Fig. 3). At this dose, the plant grew to its normal height and did not differ much from the unirradiated control plant.

Figure 2. *Murraya exotica*

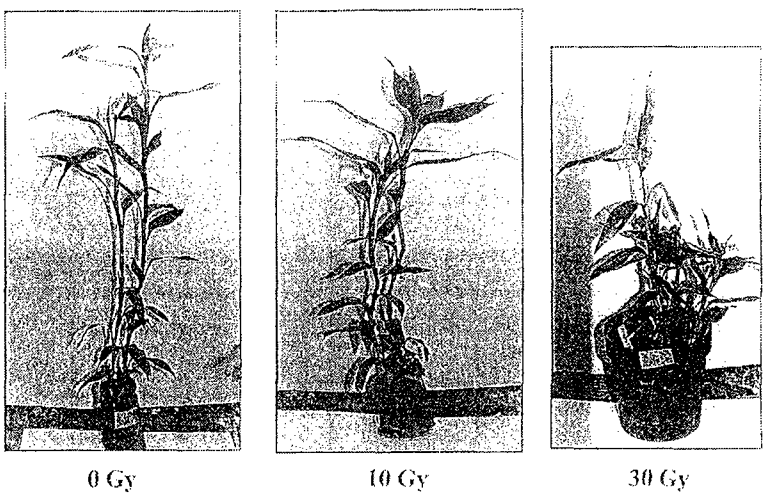


Figure 3. *Dracaena sp*

Morphological characteristics of gamma radiation induced variants of *Murraya* (M_1V_6) and *Dracaena* (M_1V_3) are shown in Table 1. At later stages of *Murraya* (M_1V_8) and *Dracaena* (M_1V_5), dwarfism continued to persist at doses ranging from 10 and 30 Gy cobalt-60 gamma radiation (Table 3). At these developmental stages, gamma radiation-induced variants seem to have reached stable mutation for both foliage plants.

Results of quantitative measurements of microfuge-extracted genomic DNA concentration of variants *Murraya* and *Dracaena* based on absorbance ratio of A_{266}nm/A_{280}nm are shown in Tables 2 and 4. An absorbance ratio of 1.7 – 2.1 indicates high DNA content. With the exception of 0 Gy control *Dracaena*, all extracted DNA samples (using both Eppendorf microfuge in Table 2 and Beckman ultracentrifuge in Table 4) have relatively high DNA content based on their A_{260}/ A_{280} ratio. Extraction of DNA from 0 Gy control *Dracaena* was problematic because the DNA extracts contain more fiberous materials than extracts from irradiated plants. This accounts for lower DNA yield in 0 Gy control *Dracaena* plant extracts using either Eppendorf microfuge or Beckman ultracentrifuge. Extraction of genomic DNA

using ultracentrifuge resulted in cleaner preparation with chlorophyll materials almost completely eliminated at the first centrifugation step. Concentration of DNA from both *Murraya* and *Dracaena* extracts measured using UV spectrophotometry showed very low or negative UV absorbance at 230 nm indicating lesser protein contamination.

Table 1. Morphological Characteristics of ^{60}Co γ-Irradiated Foliage Plants (*Murraya exotica* and *Dracaena sanderiana*) at Doses 10 and 30 Gy

	Murraya exotica (M_1V_6 stage)			*Dracaena sanderiana* (M_1V_3 stage)		
	Dose (Gy)			Dose (Gy)		
Morphological Characteristic*	0	10	30	0	10	30
Height (cm)	normal (35-40)	dwarf (10-12)	dwarf (15-18)	normal (30-33)	normal (21-24)	dwarf (15-18)
Color	green (++++)	green (++)	green (++)	green (+++)	yellow green (++)	yellow green (+)
Texture	rough	smooth	smooth	rough	smooth	smooth
Shape	oblong	ovate	ovate	teardrop	teardrop (thinner)	teardrop (thinnest)
Thickness	+++	++	++	+++	++	++
Firmness	+++	++	++	++++	+	+
Radiance	+	+++	+++	+++	++	++

*All except height are characteristics of the leaves.

Table 2. DNA Concentration of ^{60}Co γ-Irradiated Foliage Plants (*Murraya exotica* and *Dracaena sanderiana*) at Doses 10 and 30 Gy[1]

Ornamental	Dose (Gy)	OD_{260}/OD_{280} Ratio	DNA Concentration (μg/ml)	Total Genomic DNA Isolated (μg)
Murraya exotica (M_1V_6 stage)	0	2.10	2417.5	120.88
	10	1.89	371.2	18.56
	30	1.92	470.0	23.50
Dracaena sanderiana (M_1V_3 stage)	0	1.38	27.5	1.38
	10	1.90	332.5	16.62
	30	1.82	432.5	21.62

[1]extracted using Eppendorf Centrifuge 5415C

Extracted genomic DNA of *Murraya* and *Dracaena* foliage plants were amplified using PCR. With AFLP primer pair using three selective nucleotide combination primer, DNA fingerprint patterns in 2% agarose gel show distinct differences between irradiated and unirradiated control foliage plants (Fig.4). At M_1V_6 stage, dwarfism was observed in 10 and 30 Gy irradiated *Murraya*. This phenotypic variation is demonstrated in agarose gel pattern of AFLP-PCR amplified DNA fragments using primer pair of E-AAG/M-CAA. DNA fingerprint patterns of *Murraya* (10 and 30 Gy) irradiated foliage show

polymorphic bands. In the case of *Dracaena*, (M_1V_3) dwarfism was distinctly associated only with 30 Gy irradiated plants, but not with plant exposed to 10 Gy cobalt-60 gamma radiation. Corresponding polymorphic bands are observed in agarose gel pattern of 30 Gy irradiated dwarfed-*Dracaena* plant. Polymorphic bands are not visible in 10 Gy irradiated *Dracaena* which appears to have similar fingerprint pattern with the unirradiated plant observed in Fig. 4.

Table 3. Morphological Characteristics of ^{60}Co γ-Irradiated Foliage Plants (*Murraya exotica* and *Dracaena sanderiana*) at Doses 10, 20 and 30 Gy

	Murraya exotica (M_1V_8 stage)				*Dracaena sanderiana* (M_1V_5 stage)		
	Dose (Gy)				Dose (Gy)		
Morphological Characteristic*	0	10	20	30	0	10	20
Height (cm)	normal (57-60)	dwarf (23-27)	dwarf (18-20)	dwarf (10-13)	normal (36-60)	stunted (22-47)	stunted (20-44)
Color	green (++++)	green (++)	green (++)	green (++)	green (+++)	green (+++)	y.green to green with ctr stripe
Texture	rough	smooth	smooth	smooth	rough	smooth	corrugated
Shape	oblong	ovate	ovate	ovate & heart-shaped	teardrop	teardrop (thinner)	teardrop (thicker)
Thickness	+++	++	++	++	+++	++	+++
Firmness	+++	++	++	++	++++	++	+++
Radiance	+	+++	+++	+++	+++	++	++
Length (mm)	30-40	20-35	25-37	15-20	220-230	145-240	110-145
Width (mm)	19-21	11-16	16-21	10-13	34-36	35-38	36-40

*All except height are characteristics of the leaves.

Table 4. DNA Concentration of ^{60}Co γ-Irradiated Foliage Plants (*Murraya exotica* and *Dracaena sanderiana*) at Doses 10, 20 and 30 Gy[2]

Ornamental	Dose (Gy)	OD_{260}/OD_{280} Ratio	DNA Concentration (µg/ml)	Total Genomic DNA Isolated (µg)	Sample Weight (g)	Genomic DNA per g Sample (µg/g)
Murraya exotica (M_1V_8 stage)	0	1.86	1522.5	304.5	9.24	32.95
	10	1.93	2722.5	544.5	5.09	107.03
	20	1.95	1352.5	270.5	6.12	44.17
	30	1.83	1657.5	331.5	5.57	59.51
Dracaena sanderiana (M_1V_5 stage)	0	1.52	96.88	19.38	8.61	2.25
	10	1.85	221.88	44.38	8.37	5.30
	20	1.69	248.75	49.75	8.63	5.76
	30	1.79	271.25	54.25	7.51	7.22

[2]extracted using Beckman LS-80 Ultracentrifuge (SW28 Rotor)

AFLP-PCR amplified fragments from genomic DNA extracts of both *Murraya* and *Dracaena* (M_1V_8 and M_1V_5, respectively) were electrophoresed in sequencing gel using ^{32}P labeled EcoR I primer. These were run at the laboratory where Ms. Coloma is undertaking her On-the-Job Training Program in Maryland, USA. Eight different selective primer pairs with 3 selective nucleotides were used to screen for polymorphism in *Murraya* (M_1V_8). A specific primer pair resulted in DNA fingerprint showing polymorphic

bands (arrows) of variant *Murraya* (Fig. 5). Under AFLP Analysis System I protocol, each selective primer pair results in specific DNA fingerprints. More screening trials are set for both *Murraya* and *Dracaena* once the sequencing gel apparatus requested from IAEA is available for our use.

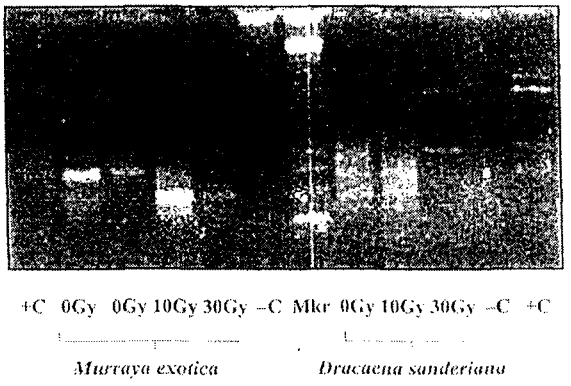


Figure 4. Selective primer pair: E-AAG/M-CAA (AFLP Analysis System I) 2% Agarose gel stained with ethidium bromide

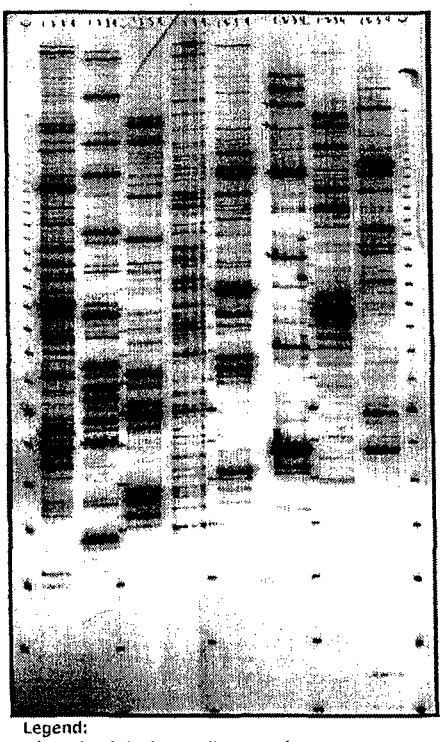
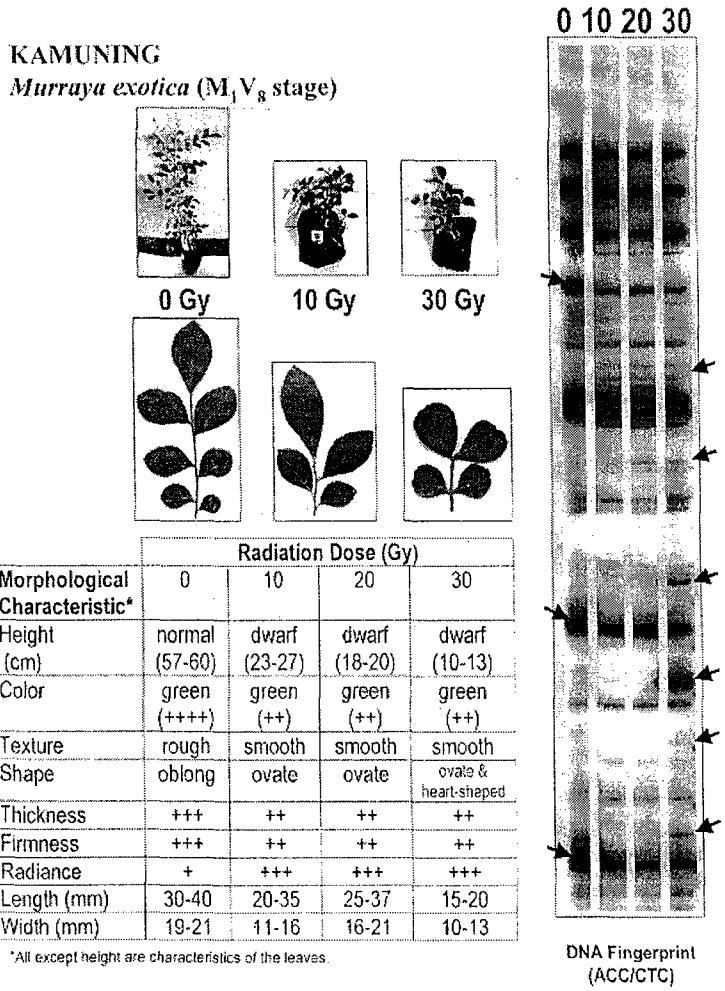


Figure 5. AFLP-PCR Profile of ^{60}Co γ-Irradiated Murraya exotica (M_1V_8 Stage) at Doses 10, 20 and 30 Gy using a Sequencing Gel

Visualization of amplified fragments was achieved in agarose electrophoretic gel and denaturing acrylarnide sequencing gel electrophoresis which are graphically documented as Polaroid hard copies. Between 50 – 100 DNA fragments are generated from PCR-AFLP. Separation of DNA fragments require large sequencing gel in order to look at polymorphic bands. Polymorphic bands in agarose gels stained with ethidium bromide were documented using a Polaroid camera.

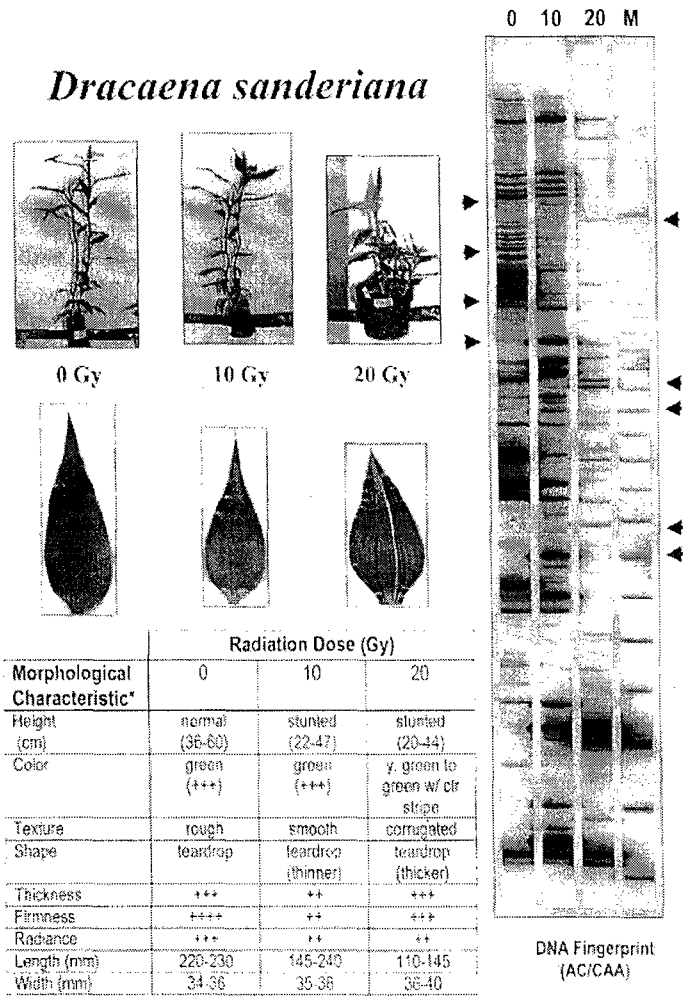


Morphological Characteristic*	Radiation Dose (Gy) 0	10	20	30
Height (cm)	normal (57-60)	dwarf (23-27)	dwarf (18-20)	dwarf (10-13)
Color	green (++++)	green (++)	green (++)	green (++)
Texture	rough	smooth	smooth	smooth
Shape	oblong	ovate	ovate	ovate & heart-shaped
Thickness	+++	++	++	++
Firmness	+++	++	++	++
Radiance	+	+++	+++	+++
Length (mm)	30-40	20-35	25-37	15-20
Width (mm)	19-21	11-16	16-21	10-13

*All except height are characteristics of the leaves.

Figure 6. Database

Results are recorded for database acquisition and encoded for gamma irradiated *Murraya exotica* and *Dracaena sanderiana* foliage plants. Data on polymorphism observed both phenotypically and molecularly were used to generate the database for gamma radiation- induced variants in *Murraya exotica* and *Dracaena sanderiana*. Database includes actual photographs of foliage plants at specifc stage, morphological descriptions and DNA gel fingerprints showing polymorphism. A prototype of the database for both cobalt-60 gamma irradiated variant of *Murraya exotica* and *Dracaena sanderiana* at M_1V_0 and M_1V_3, respectively, are illustrated in Fig. 6 and 7.



Morphological Characteristic*	Radiation Dose (Gy) 0	10	20
Height (cm)	normal (36-60)	stunted (22-47)	stunted (20-44)
Color	green (+++)	green (+++)	y. green to green w/ dk stripe
Texture	rough	smooth	corrugated
Shape	teardrop	teardrop (thinner)	teardrop (thicker)
Thickness	+++	++	+++
Firmness	++++	++	+++
Radiance	+++	++	++
Length (mm)	220-230	145-240	110-145
Width (mm)	34-36	35-36	36-40

Figure 7. Database

REFERENCES

Ausubel, F. M. et al (eds). 1989. Short Protocols in Molecular Biology. John Wiley & Sons, N. Y. pp. 60–61

GIBCO-BRL Instruction Manual. AFLP Analysis System I. Life Technologies USA. pp. 1–18.

Lin, J. J., Kuo, J. 1995. AFLP: A novel PCR-based assay for plant and bacterial DNA fingerprinting. *Focus*. 17, 66–70.

Liscum, M. and Oeller, P. 1998. AFLP: not only fingerinting, but for positional cloning. *http://www.carnegiedpb.stanford.edu/methods/aflp.html*, pp 1–11.

Nucleic Acid Purification Guide. 1996. Purification of genomic DNA from plant tissue. USA. pp. 161–162.

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