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GENETIC POLYMORPHISM BY RAPD AND ANTHOCYANIN CONTENT IN THE MORPHOLOGICALLY VARIABLE SELECTED CULTIVARS OF *BEGONIA*: SOME OBSERVATIONS

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ABSTRACT

Begonia species forms a highly variable and complex group and are distinguished from each other solely on the size and shape of leaves with reference to recent taxonomic revision. They share similar floral morphology with caudex or swollen stem base. Considerable variation among populations was seen with respect to the size, margin and lobbing of leaves. An attempt was made to compare DNA polymorphism, anthocyanin content and morphological traits among five cultivars of three *Begonia* species. The cultivars of *B. rex* and *B. heracleifolia* showed more or less similar morphometric characters but differ significantly from *B. malabarica*. *Begonia rex* 'baby rainbow' and 'black beauty' showed remarkable anthocyanin content (69.6 and 70.6 mg/g FW respectively) compared to others. Genetic diversity among the cultivars of the species was attempted by DNA finger printing using 15 primers. Optimal quantity of DNA was extracted from the cultivars of young leaves by CTAB method. The quality of DNA was checked by Agarose gel electrophoresis. The isolated DNA was quantified at 260 nm and 280 nm and was subjected to PCR amplification. 106 unambiguous, readable and reproducible RAPD bands were produced using the selected primers. The number of bands obtained with the sizes ranging from 250 to 1500 bp. All the bands were polymorphic i.e., 100 %. Similarly, the cultivars can be identified by unique bands with specific primers. A dendrogram was also constructed using the percentage of genetic distance grouped them in to different clades. Further, the present study also shows correlation between DNA polymorphism and anthocyanin content among the cultivars.

Keywords: *Begonia*, Genetic diversity, DNA, PCR, RAPD, Total anthocyanin, Cultivars.

INTRODUCTION

Begonia is one of the ten largest plant genera found throughout the tropics - wet tropics and is represented by over 1600 species. *Begonia* is a perennial flowering plant belongs to Begoniaceae. The large size of the genus and its variation makes it as the ideal sample for speciation studies[1]. The highly diverse and hyper genus is distinguished from each other on the basis of morphological parameters. They share similar floral morphology with swollen or caudex stem base. At the generic level *Begonias* are easily distinguished by asymmetry of leaf form, succulent petioles, unisexual flowers that are borne within the same inflorescence and winged capsules. Divergent natural selection has promoted speciation in a wide range of taxa. DNA, RNA and protein markers are powerful means to determine genetic similarity and variations among cultivars and accessions used in horticultural/agricultural breeding programmes. Many taxonomic studies have been carried to analyze genetic diversity and phylogenetic kinships among species. Some of these studies used biochemical methods like isoenzyme, while others used molecular approaches to evaluate genetic diversity in crop species. Generally, isoenzymes analysis yield monomorphic patterns. Random amplified polymorphic DNA (RAPD) is a versatile, easy method used for molecular or genetic studies and has been applied for the less-known species. This marker system has the ability to amplify DNA from dispersed polymorphic loci and has its power to detect small genetic differences. To the best of knowledge, such molecular marker is only an indirect DNA sequence analysis technique. To infer a more accurate conclusion on genetic relationships among species, it is necessary to combine with direct DNA sequence analysis techniques. Significant demand was noticed related with the natural pigments such as chlorophylls, carotenoids, anthocyanins and betalains colorants among the consumers as ingredients in food. Anthocyanins are water soluble pigments that occur naturally. In plants, they give protection against the harmful UV irradiations, attractants for seed dispersal and pollination and also provide antimicrobial and antiviral activities. Anthocyanins have been used as part of the human diet throughout the history; however, they have gained renewed attention due to their positive health benefits. Naturally, anthocyanin is dominating over the flowers but *Begonia* is an exception where the leaves are brightly colored with this unique pigment. Anthocyanin content usually varies with the genotypes. Thus, in this scenario the present study aims to evaluate the morphometric, genetic polymorphism among 5 selected cultivars belonging to three species and also their anthocyanin content.

MATERIALS AND METHODS

Plant material

For the whole attempted work, the fresh healthy *Begonia* plants belonging to two species such as *Begonia heracleifolia* Cham. and Schltdl. and *Begonia malabarica* Lam. and three cultivars of *Begonia rex* (*Begonia rex* 'baby rainbow' L.H. Bailey, *Begonia rex* 'black beauty' and *Begonia rex* 'Sir Percy')[2,3] were collected from the department garden and the voucher specimen was deposited in the herbarium of University College, Trivandrum, Kerala, India (Specimen No. P. C. 100006, 100007, 100008,100009, 100010 respectively). Leaf sample at specific growth stage was selected for the morphological, analytical and molecular analysis.

Morphology variation studies

Morphological studies were carried out by analyzing the height of the plant, leaf size, floral variations, epidermal hairs and the stomatal types among the five genotypes. Micrometric techniques were used to calculate the length of the epidermal hairs.

Molecular analysis
Random Amplification of Polymorphic DNA (RAPD)

Genomic DNA was extracted and purified by CTAB method of Saghai-Maroof *et al*.[4] with some modifications. The purity of DNA aliquot was checked at 260 nm. Twenty Oligo nucleotide 10 mer primers of Operon (OPD series, Operon Technologies, USA) were used for the random amplification of the genomic DNA. DNA amplification during PCR reaction was standardized. Agarose gel was casted in a Genei mini model horizontal gel apparatus by melting agarose in 1X TAE buffer, pH 8.0 (100 mM Tris-acetate and 10 mM EDTA). Green view dye (0.5 mg/ml) was added to the buffer after sufficient cooling, which fluorescents DNA[5]. The polymorphic DNA bands that showed consistency in repeated experiments were screened according to their presence ('1') or absence ('0') in each of the genotypes. Percentage of genetic distance between the genotypes was estimated by the pair wise comparison method of Nie and Li[6]. After calculation of all pair wise similarities between varieties, the relationships among them were expressed by performing cluster analysis using the software GENSTAT. It was then graphically represented as a dendrogram[7].

Estimation of anthocyanin content

1 g leaf sample homogenized in 3 ml methanol with 1 % HCl and vortexed for 30 seconds and kept in water bath at 60^{o}C for 20 minutes. The samples were vortexed twice during incubation. Then the sample was centrifuged at 10000 rpm for 10 minutes. The supernatant was transferred to a 10 ml volumetric flask. The residue was again mixed with 3 ml of methanol. The supernatant was again centrifuged and combined with the previous supernatant and made up to 10 ml. The final extract solution was kept at 0^{o}C for further analysis. 1 ml of extract was taken and transferred to 10 ml volumetric flask for preparing two dilutions of sample, one adjusted with KCl buffer, pH 1.0 and the other with sodium acetate buffer pH 4.5. These dilutions were equilibrated for 15 minutes. The absorbance of each dilution was read at 510 and 700 nm against blank distilled water[8].

RESULTS AND DISCUSSION

The remarkable metabolic capacity of accumulating the anthocyanin intensively in the leaves has made this plant, *Begonia* unique.

Morphological

Leaf size was analyzed in terms of width and length in each cultivar. *Begonia rex* 'Sir Percy' cultivar possess longer leaves whereas, the *Begonia rex* 'baby rainbow' possess broader leaves than other cultivars. The heights of the plant were more or less similar. Floral variation is observed mainly in the length of the corolla tube and color of the petals. The epidermal hair also showed changes in the number of cells in it. Dense or numerous epidermal hairs are observed in all the cultivars except *Begonia rex* 'baby rainbow' (Figure 1 a-d). Epidermal cells can either appear flat or peaky. Some epidermal cells develop into trichomes that vary to a great extent in type, size and the density. The presence of trichomes on the leaf surface alters the amount of light absorbed by the leaf, resulting in reduced leaf temperature and potentially reducing the transpiration rate. *Begonia rex* 'Sir Percy' possessed multi cellular colored trichomes and occurred only on the lower surface of the leaves. Contradictorily all other genotypes possessed colorless multi cellular trichomes (Table 1). Stomata are hypostomatous and anisocytic. Both solitary and clustered stomata are found on the *Begonia* leaves.

Molecular analysis

DNA marker techniques offer powerful tool for the characterization of genetic variability, genotypic identification, genetic analysis and selection and breeding programmes in plants. Important advantages of molecular markers include lack of sensitivity to changes in environmental conditions, as well as a nearly unlimited potential number of bands and speed of the marker assays compared with usual field tests. In many species, random amplification of polymorphic DNA has proven useful for revealing polymorphism among genotypes[9]. They can provide additional information such as the amount of genetic divergence between cultivars and other genotypes and the amount of genetic variability between seedling replicates of genotypes. So the present study has been done to reveal the genetic polymorphism of *Begonia* plants with different leaf color using RAPD markers. The RAPD data was subjected to cluster analysis for detecting the genetic polymorphism of the cultivars and their phylogenetic status was also detected from the dendrogram. The genomic DNA was isolated and purified from the leaf samples collected from *Begonia* plants cultivars. Each cultivar was properly identified from the gardens of registered nurseries. For the study the plants were collected from five different cultivars. The DNA polymorphism at the level of genetic variability in *Begonia* genotypes was analyzed by RAPD method. Of the 15 RAPD primers P1 to P15 screened, 14 produced distinct reproducible polymorphic bands within the five genotypes of *Begonia* (Table 2). Reproducibility of the amplification pattern was checked by repeating the reactions in five members of each genotype. Even though diagnostic bands were observed, most of them were faint or not repeatedly formed in all the representative individuals of the five genotypes. Thus, a large number of potentially genotype specific bands were eliminated from consideration. Figure 2-6 represents the amplification pattern obtained with the 15 primers for the five genotypes. The RAPD profile shows a total number of 106 bands with appropriate band size ranged from 250 to 1500 bp. PCR reactions were optimized at annealing temperature 36ºC for 40 cycles. All the 15 primers in the five genotypes were considered as a single reaction. Genetic polymorphic study had been carried out in pumpkin (*Cucurbita maxima*) with 11 primers generated 43 bands[10]. Ahmad *et al*.[11] evaluated genetic diversity in five *Pisum* cultivars using four random primers. The dendrogram based on 16 bands which showed cultivar KPMR 922 as the most divergent one. RAPD analysis of 17 cultivars of Sugarcane using 40 primers generated 325 bands with 134 polymorphic bands. The genetic similarity between the cultivars varied from 0.77 to 0.99[12]. Similarly, RAPD appeared to be an effective tool in identifying 43 accessions of *Phoenix dactylifera* L. Morphologically all the accessions showed similarities but at molecular level each genotype were distinguishable by their banding patterns[13]. Varietal variations among the seven cultivars of *Capsicum annuum* were observed by Cheema and Pant[14] using RAPD markers. The varieties showed remarkable variations with respect to molecular, morphological and phytoconstituents. The dendrogram of the 7 straw berry cultivars (*Fragaria × ananassa* Duchesne) grouped them into three groups based on similarity coefficient while ISSR markers generated two groups with no direct relationship among them[15]. Further, RAPD data was found to be more reliable in identifying the relationship between the 16 cultivars of *Rubus* with *Rubus allegheniensis* having a genetic contribution of 15.9 %[16]. The similarities and difference among the forty cultivars of *Olea europaea* generated 34 reproducible bands which were useful in discriminating closely related cultivars[17]. Duhan *et al*.[18] analyzed genetic stability using ISSR and RAPD markers in Banana Cv. Grand Naine. Twenty six RAPD and twenty ISSR markers were used. RAPD primers produced 87 distinct and scorable bands, with an average of 3.34 bands per primer and the amplification products range was from 100-1200 bps. The number of scorable bands for RAPD primer varied from 2 to 5 with an average of 3.34 bands per primer. ISSR primers produced 71 distinct and scorable bands in the range of 100-1000 bps and the number of scorable bands for each primer varied from 2 to 6 with an average of 3.55 bands per primer. Based on the screening of the RAPD profile of the five genotypes of *Begonia*, it is possible to categorize the primers into three groups. Table 3 demonstrates the list of primers identified from the 15 ones for

detecting the five genotypes. The primers OPD1, OPD3, OPD4, OPD7 (AACCGACGGG, TGCCCTGCCT, CCAGACCCTG, GGCGGACTGT) showed amplifications in all the five genotypes so that the band profile can be used for identifying them together (Figures 2-4); it is obvious from the figure that the primer OPD1 expressed specificity in the amplification pattern in sample 1 and sample 2 the genotypes with specific individual bands in each. OPD4 expressed specificity in the amplification pattern in samples 1 and 5. OPD5 expressed specificity in the amplification pattern in samples 2, 3 and 4. Figures 2 and 3 demonstrates the RAPD profile of genomic DNA amplified by the primers OPD1, OPD4 and OPD5 with the sequence AACCGACGGG, CCAGACCCTG and AAGCTCCCCG respectively. It could be visible from the figure that the primers OPD 6, OPD 7 and OPD 9 showed their ability to detect the genotypes sample 1, sample 4; sample 1, sample 3 and sample 2, 3, 4 respectively. Meanwhile, the primers OPD8, OPD 10 and OPD 15 (GTCACTCCCC,GGACCCAACC, TTGGCACGGG) expressed its odd nature with unique bands exclusively for the samples 3, 4 and 1 respectively (Figure 4-6). A total of 106 polymorphic bands were obtained. Thus the RAPD data of the primers indicates the discriminatory power in amplification and it can be successfully applied to reveal the genetic diversity between genotypes. Molecular variability among the 54 Gladiolus cultivars using 25 arbitrary primers revealed 93.78 % polymorphism[19]. Eight decamer primers were used to assess the level of genetic diversity among the 24 cultivars of *Anthurium andraeanum* with an average genetic similarity of 91.34 %. The higher percentage of genetic similarity indicated the low genetic diversity among the cultivars[20]. Genetic diversity among the selected five genotypes of apocynaceae from different agro-ecological regions and along with their *in vitro* grown callus was analyzed. *In vitro* and *in vivo* samples showed genetic variation with each other which may be developed during the course of ecological adaptations[21]. The RAPD data of the five genotypes of *Begonia* was extended further for statistical analysis in order to measure the genetic distances among them. Table 2 display the number of bands scored by the primers P1 to P15 for each genotype. The bands were scored according to their presence ('1') or absence ('0') and were arranged as per the molecular size. A pair wise genetic distance among the five genotypes was analyzed by statistical method of Nie and Li[6]. It is evident from the percentage of genetic distance that genetic dissimilarity was found between the plants of the five cultivars. RAPD data was further extended for statistical analysis in order to measure the genetic distances among the genotypes. From the genetic distance calculated from the RAPD data, a dendrogram was prepared by GENSTAT cluster analysis software (Figure 7). The cluster tree grouped the genotypes basically into three clusters. The first cluster contains two varieties S2 and S1 which are distant apart from each other by a measure of 0.683 only. S3 and S4 formed another cluster, each pair of which is distant by a measure of 0.683. S5 is found to be positioned apart from both these clusters and it is estimated that S5 is distant with other varieties. Thus, the dendrogram prepared from the RAPD data reflects a unique grouping of genotype. Thus, the RAPD profile of *Begonia* provides sufficient insight to categorize the *Begonia* genotypes based on its genetic relatedness.

Anthocyanin content

As the last phase of the study anthocyanin was quantified according to the method of Sutharut and Sudarat[8]. *Begonia rex* 'baby rainbow' and *Begonia rex* 'black beauty' possessed highest anthocyanin content compared to other cultivars (Table 4).

Table 1: Morphological Parameters in various *Begonia* Genotypes

Variety	Hairs present /absent in leaf	Hairs present /absent in Petiole	Hairs present /absent in rhizome
Begonia rex 'baby rainbow'(S4)	absent	absent	absent
Begonia heracleifolia (Star leaf begonia) (S1)	Hairy (multi cellular), colorless	Hairy (multi cellular) long, dense, not colored	hairy
Begonia rex 'Sir Percy'(S3)	Hairy (multi cellular), colored, seen only at the lower surface of leaf	Hairy (multi cellular) short, not dense seen near the starting of lamina, colored	absent
Begonia malabarica (S2)	Hairy (multi cellular), colorless	Hairy (multi cellular) short, not dense, not colored	absent
Begonia rex 'black beauty'(S5)	Hairy (multi cellular), colorless	Hairy (multi cellular) long, dense, not colored	hairy

Table 2: Polymorphic Bands Scored By the 15 OPD Primers in *Begonia* Genotypes

Primers	*Begonia heracleifolia* (Star leaf begonia) (S1)	*Begonia malabarica* (S2)	*Begonia rex* 'Sir Percy'(S3)	*Begonia rex* 'baby rainbow'(S4)	*Begonia rex*'black beauty'(S5)
1	1500, 1200, 1100, 1000	800	900	900	900
2	-	-	-	900	900
3	900	900	950	950	950
4	900	950	950	950	1300
5	-	300	200	400	
6	800	-	-	1300,1200,1100, 1000	-
7	300,200,150	300,200	350,250	350	350
8	-	750	950,650	750	-
9	950	1000,950,750, 700	950,750,740	1000,960,760	-
10	760	800	700,760	900,800,700	-
11	700	1000,900,700	1000,900,700	1000,900,700	-
12	1000,950,900	1000,950,1100	950,900	1100,950	-
13	-	-	-	-	-
14	1200,1000	1200,1000,900	1200,1100,900	1200,1100,1000, 900	-
15	1300,1100,980, 900	1100,980,900	1100,980,950,80 0	1100,980,950, 800	-
Total polymorphic bands = 106					

Table 3: Specific Primers with Unique Bands among *Begonia* Genotypes

Genotypes	Primers with unique bands
S1 (*Begonia rex*'black beauty')	OPD1 -1500, 1200, 1100, 1000 bps OPD4 -900 bp OPD6 -800 bp OPD7 -150 bp OPD15 -1300 bp
S2 (*Begonia malabarica*)	OPD 1 -800 bp OPD5 -300 bp OPD9 -700 bp
S3 (*Begonia rex*'baby rainbow')	OPD5 -200 bp OPD7 -250 bp OPD8 -950,650 bps OPD9 -740 bp
S4 (*Begonia rex*'Sir Percy')	OPD5 -400 bp OPD6 -1300,1200,1100,1000 bps OPD9 -960,760 bps OPD10 -900 bp
S5 (*Begonia heracleifolia* 'Star leaf begonia')	OPD4 -1300 bp
Total number of unique bands = 25	

Table 4: Athocyanin Content among *Begonia* Genotypes

Sl. No.	Varieties	Monomeric anthocyanin pigment (mg/ml)
1	*Begonia rex* 'baby rainbow'	69.634
2	*Begonia heracleifolia* (Star leaf begonia)	42.749
3	*Begonia rex*'Sir Percy'	21.374
3	*Begonia malabarica*	22.877
5	*Begonia rex* 'black beauty'	92.67

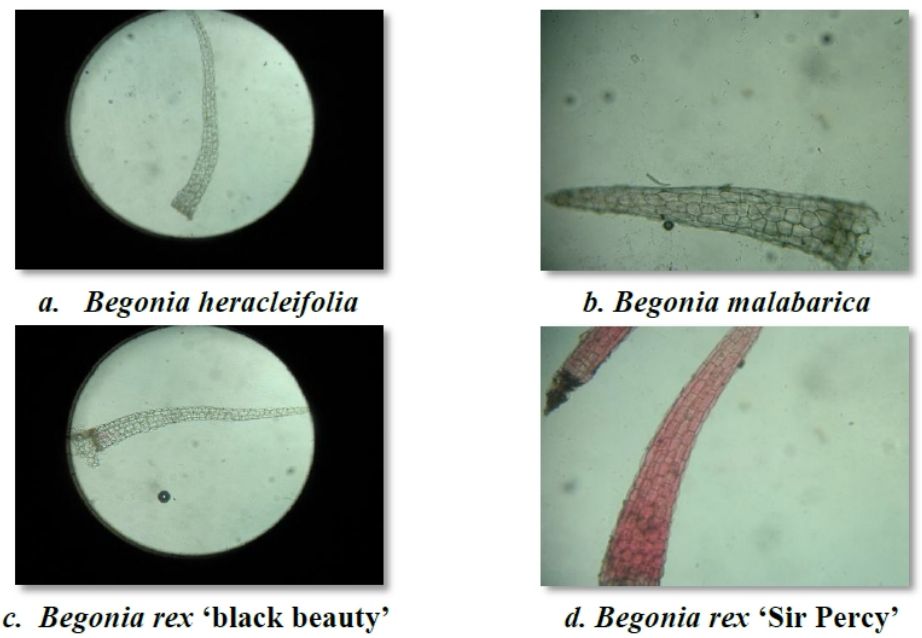

Figure 1 (a-d): Trichome variations among the *Begonia* genotypes

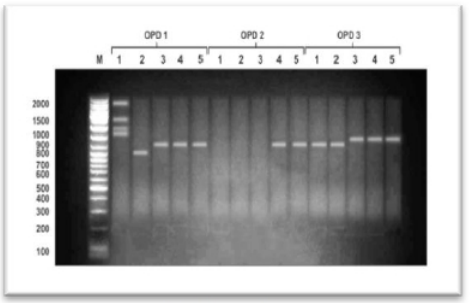

Figure 2

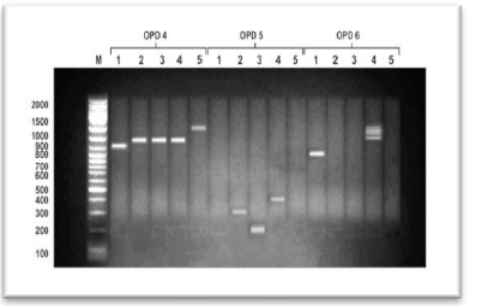

Figure 3

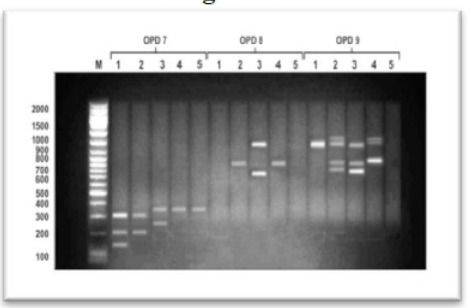

Figure 4

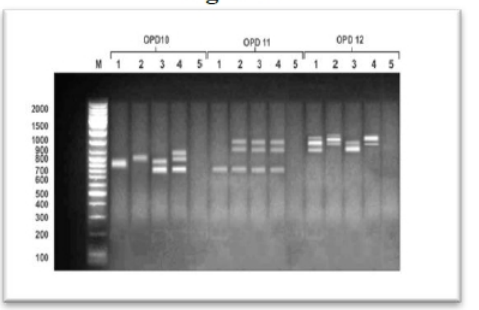

Figure 5

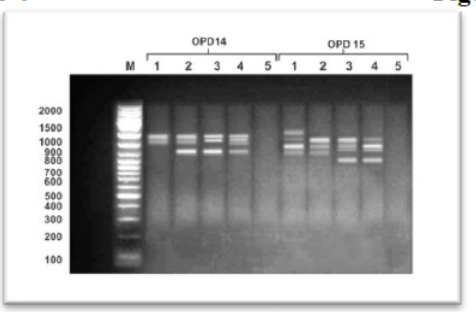

Figure 6

Figure 2-6: RAPD banding profile among various *Begonia* genotypes using 15 primers

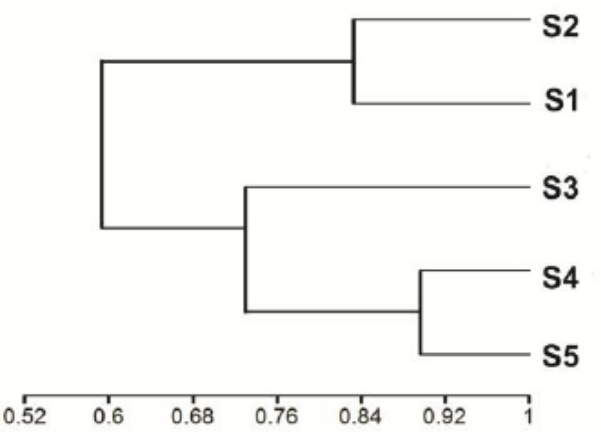

Figure 7: UPGMA dendrogram of *Begonia* genotypes showing the taxonomic relationships

Begonia heracleifolia (Star leaf begonia) (S1), *Begonia malabarica* (S2), *Begonia rex* 'Sir Percy' (S3), *Begonia rex* 'baby rainbow' (S4), *Begonia rex* 'black beauty'(S5)

CONCLUSION

The present study was undertaken as a beginning step for understanding the genetic variability present in different cultivars of *Begonia* using molecular marker. RAPD was selected as the technique because of its simplicity and the moderate cost with which the study could be done. The selected five cultivars showed 100 % polymorphism using 15 random decamer primers. A total of 15 random primers were screened and gave 106 unambiguous scorable polymorphic bands. Most of the selected primers could give high level of polymorphic bands and monomorphic bands were absent. The dendrogram generated clustered the five cultivars into two distinct clusters. Of all the cultivars, two species of *Begonia* comprised the first cluster and the rest of the three cultivars forming the second cluster. Morphologically the cultivars showed variation among each other. As the last phase of the study anthocyanin content also showed variation with the *Begonia rex* 'baby rainbow' and *Begonia rex* 'black beauty' possessing highest anthocyanin content which is morphologically distinguishable. Thus, the present study has demonstrated that RAPD technique in useful in providing an analysis of genetic diversity present in *Begonia*. A further evaluation using more varieties and primers can provide information to the development of strategies for hybrid breeding and determining homogeneity of inbred lines.

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