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Genetic variability studies on *Mussaenda* species variation among five *Mussaenda* species detected by random amplified polymorphic DNA

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ABSTRACT

DNA was isolated from fresh leaves of five *Mussaenda* cultivars to study the genomic relation among them. Genomic relationship was evaluated using 21 random polymorphic DNA markers. Among the 21-decamer primers only 7 primers amplified 12 monomorphic loci. The primer OPM01 has maximum resolution power, while the primer OPQ04 has maximum RAPD primer index. Dendrogram based on the basis SHAN clustering showed two clearcut groups containing *M. leuteola* in an isolated clade. The two varieties of *M. erythrophylla* are coming under same group indicating their close association at the molecular level. Among the 193 amplified loci 181 loci were polymorphic DNA and 65 bands are exclusive amplified only in particular individual. But there were no marker band for the species *M. erythrophylla* 'Rosea'. One interesting outcome of the investigation is the closeness of *M. philipicaca* 'Aurorea' with *M. erythrophylla* 'Gining Imelda'. It had similarity value 84% but the two varieties of *M. erythrophylla* were related at 56% similarity level.

Key words: Mussaenda, RAPD, Molecular characterization, Genomic relations.

INTRODUCTION

Mussaenda L. is a very popular garden plant cultivated in tropical and sub tropical parts of the globe for ornamental purposes. It is an patterned shrub, which can be grown both indoors and outdoors There are around 40 species/cultivars found all over the world. Most of the members of the genus are attractive due to their impressive brilliant sepals. The most widely cultivated species are *M. erythrophylla* cv. Gining Imelda, *M. phillipica* cv. Aurorea, *M. erythrophylla* cv. Rosea, *M. luteola* and other species (http://toptropicals.com/cgi-bin/garden_catalog/cat.cgi?selectengine=catalog&first=1&number=10&lang=en&sale=1&screen_width=1280&search_op=AND&find=Mussaenda). Most of the species differ from each other only by their coloured sepals. Based on the style and stamen lengths, the cultivars and species were classified as either pin or thrum type. The pin cultivars are 'Dona Eva', 'Dona Esperanza', 'Dona Hilaria','Maria Makiling', and 'Queen Sirikit'. The thrum cultivars are 'Mutya','Diwata', 'Dona Luz', 'Baby Aurora' (M. Flava), and 'Dona Trining' (*M. erythrophylla*). Although its floral characters are not significantly different from those of the pin cultivars, 'Lakambini' is the only cultivar whose stamen and style are almost of the same length, a case of an intermediate type in *Mussaenda* reported for the first time. At the early stage of the plant it is very difficult to identify the species/cultivar. As the species/cultivars are not significantly differing from each other morphologically, the essentiality of speciation based on authentic marker system, which is not influenced by environmental factors, is felt. The molecular markers, which are the most reliable and reproducible, can be utilized for studying genetic variations in this genus. Lots of work has been done on the propagation of the plants (Cramer and Bridgen, 6). However, no work on the molecular characterization of the taxa *Mussaenda* has yet been published.

Among the different molecular markers, random amplified polymorphic DNA (RAPD), simple sequence repeats (SSR), inter-simple sequence repeats (ISSR), anchored simple sequence repeats (ASSR), restriction fragment length polymorphism (RFLP) and amplified fragment length polymorphism (AFLP) have extensively been used to quantify accurately the inter and intra-specific variability and inter-generic variability in different plant groups and crop varieties (Karp *et al.*, 5). Among these molecular markers RAPD is the most simple and cost effective in discriminating the species and utilized for different aspects of research in plant sciences (Burdakci, 1). In the present report we report for the first time the application of RAPD markers in studying genetic variation in *Mussaenda* spp.

MATERIALS AND METHODS

Five members of the genus *Mussaenda* L. were

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taken for the present investigation. The members were *M. erythrophylla* cv. Gining Imelda*, M. phillipica* cv. Aurorea*, M. erythrophylla* cv. Rosea, *M. luteola* and *M. mermelada*.

DNA was isolated from young and freshly collected leaves using the CTAB method as described by Saghai-Maroof *et al*. (9). RNA were removed by giving RNaseA treatment (@ 60 μg for 1 ml of crude DNA solution (at 37°C) followed by two washes of phenol: chloroform: iso-amyl-alchohol (25:24:1) and subsequently two washes with chloroform: iso-amyl-alchol (24:1). After centrifugation, the upper aqueous phase was separated, 1/10 volume 3M-sodium acetate (pH 4.8) was added and DNA was precipitated with 2.5 volume of pre-chilled absolute ethanol. DNA was dried and dissolved in $T_{10}E_1$ buffer (Tris-HCl 10 mM, EDTA 1 mM pH 8). Quantification was made by running the dissolved DNA in 0.8% agarose gel alongside uncut λ DNA of known concentration. The DNA was diluted to 25 ng per μl for RAPD analysis.

For RAPD analysis, PCR amplification of 25 ng of genomic DNA was carried out using standard 21 decamer oligonucleotide primers, i.e. OPM01, OPM02, OPM04,OPM06, OPM0-9,OPN04, OPN05, OPN12, OPP01, OPP02, OPP03, OPP05, OPQ01,OPQ04, OPQ05, OPQ19, OPQ20, OPS07, OPT04, OPT07 and OPT08 (Operon Technologies, Alameda, CA. USA). The RAPD analysis was performed as per the standard methods of Williams *et al.* (12). Each amplification reaction mixture of 25 μl contained 20 ng of template DNA, 2.5 μl of 10X assay buffer (100 mM Tris-HCl pH 8.3, 0.5 M KCl and 0.01% gelatin), 1.5 mM $MgCl_2$, 200 μm each of dNTPs, 20 ng of primer and 0.5U *Taq* DNA polymerase (Bangalore Genei Pvt. Ltd., Bangalore). The amplification was carried out in a thermal cycler (Gene Amp 9600, Perkin Elmer, USA). The first cycle consisted of denaturation of template DNA at 94°C for 5 min., primer annealing at 37°C for 1 min. and primer extension at 72°C for 2 min. In the subsequent 42 cycles the period of denaturation was reduced to 1 min., while the primer annealing and primer extension time was maintained same as in the first cycle. The last cycle consisted of only primer extension at 72°C for 7 min. PCR products were separated on a 1.5% agarose gel containing ethidium bromide solution (@ 0.5 μg/ml of gel solution). The size of the amplicons was determined using size standards (100 bp ladder plus, MBI Fermentas, Lithuania). DNA fragments were visualized under UV light and photographed.

The presence/ absence of bands in RAPD analysis was recorded in binary (0,1) form. All the bands (polymorphic and monomorphic) were taken into account for calculation of similarity with a view to avoid over/under estimation of the distance (Gherardi *et al*., 2). Jaccard's coefficient of similarity (Jaccard, 4) was measured and a dendrogram based on similarity coefficients generated by un-weighted pair group method using arithmetic averages (UPGMA) (Sneath and Sokal, 11) and SHAN clustering. All the analyses were done by using the computer package NTSYS-PC (Rohlf, 8). Resolving power of the RAPD was calculated as per Prevost and Wilkinson (7). Resolving power is: $Rp = \Sigma IB$ (IB (Band informativeness) = 1-[2 × (0.5-P)], P is the proportion of the 5 species containing the band.

The RPI was calculated from the polymorphic index. A polymorphic index (PIC) was calculated as $PIC = 1-\Sigma P^2_i$, P_i is the band frequency of the *i*th allele (Smith *et al.* 1997) (10). In the case of RAPDs and ISSRs, the PIC was considered to be $1-p^2-q^2$, where p is band frequency and q is no band frequency (Ghislain *et al*., 3). PIC value was then used to calculate the RAPD primer index (RPI). RPI is the sum of the PIC of all the markers amplified by the same primer.

RESULTS AND DISCUSSION

All the species studied produced a total of 193 distinct bands among which 12 bands are monomorphic and the rest were 182 polymorphic. Among the 21 primers used OPQ01 resulted in the amplification of 15 loci while OPT04 showed amplification of 5 bands only (Table 1, 2). The resolving power is the maximum for OPA02 (14.4) and minimum for the primer OPT 04 (6), but the RAPD Primer index shows its maximum value for the primer OPQ04, (4.96) and the least value for primer OPQM02 is 1.28. The species *M. luteola* showed maximum no of amplification (100) but the species *Mussaenda erythrophylla* (*Gining*

Table 1. Details of the *Mussaenda* species studied.

Name of the taxon	No. of bands amplified	Exclusive band (s)
Mussaenda erythrophylla (Gining Imelda)	81	1
Mussaenda erythrophylla (Rosea)	94	0
Mussaenda mermelada	100	1
Mussaenda philipica (Aurorea)	98	6
Mussaenda luteola	106	57

Table 2. Details of RAPD analysis of *Mussaenda* spp.

Primer	Nucleotide sequences	Total bands	Polymorphic bands	Monomorphic bands	Resolving power	RAPD primer Index
OPM01	5'GTTGGTGGCT3'	11	7	4	14.40	2.88
OPM02	5'ACAACGCCTC3'	7	5	2	10.00	1.28
OPM04	5'GGCGGTTGTC3'	9	8	1	10.00	2.88
OPM06	5'CTGGGCAACT3'	10	10	0	10.80	3.36
OPM09	5'GTCTTGCGGA3'	10	10	0	11.20	4.16
OPN04	5'GACCGACCCA3'	14	14	0	13.20	4.80
OPN05	5'ACTGAACGCC3'	9	9	0	8.40	3.36
OPN12	5'CACAGACACC3'	8	7	1	7.20	2.56
OPP01	5'GTAGCACTCC3'	8	8	0	6.80	2.88
OPP02	5'TCGGCACGCA3'	6	6	0	5.60	2.72
OPP03	5'CTGATACGCC3'	8	8	0	8.40	3.04
OPP05	5'CCCCGGTAAC3'	8	8	0	7.20	3.20
OPQ01	5'GGGACGATGG3'	15	15	0	12.40	4.80
OPQ04	5'AGTGCGCTGA3'	13	13	0	11.60	4.96
OPQ05	5'CCGCGTCTTG3'	10	10	0	7.20	3.84
OPQ19	5'CCCCCTATCA3'	7	7	0	5.20	2.24
OPQ20	5'TCGCCCAGTC3'	7	7	0	6.40	2.56
OPS07	5'TCCGATGCTG3'	7	6	1	8.00	2.72
OPT04	5'CACAGAGGGA3'	5	5	0	6.00	1.92
OPT07	5'GGCAGGCTGT3'	11	10	1	10.00	2.40
OPT08	5'AACGGCGACA3'	10	8	2	11.60	4.00
Total		186	181	12		

Imelda) resulted only the amplification of 81 bands. Highest number of exclusive bands was detected in the species *M. leuteola* (57) (Table 1). The Jaccard's similarity index showed that the two species of *Mussaenda erythrophylla* are closely related having 84% similarity. But the species *M. leuteola* and *Mussaenda erythrophylla* (Gining Imelda) are widely apart and are related only at 14.6% similarity. The dendrogram obtained by SHAN clustering divided the species into two clusters. In the first cluster there are two sub-clusters each group containing two taxa respectively (Fig. 2). The two varieties of *M. erythrophylla* are grouped together in the first group. In the second group the two species placed. As expected from the morphological characters the species *M. leuteolai* is associated with the other species present in an isolated cluster. *M. phillipica* Aurorea and *Mussaenda erythrophylla* are related at 84% of similarity. But the species *Mussaenda leuteola* and *M. phillipica* 'Aurorae' are related at 24% similarity level (Fig. 2).

Two cultivars of *M. erythrophylla* were placed in the same clade. Our finding is exactly what was expected from the morphological observation. *M. leuteola* is quite different from the other four members of the species studied from its morphological features. We also got the same trend using the molecular technique. This indicates that the species *M. leuteola* is fairly distinct from the other species at the molecular level. Our result is further supported by the data from Jaccard's similarity (Table 3). The variety of *M. erythrophylla* (Rosea) have maximum similarity (84%) with *M. philipica* (Aurorea) and the three members of *M. erythrophylla* have a common node at 65% similarity. This indicates that the three varieties of *M. erythrophylla* have a common ancestor and *M. philipica* (Aurorea) the intermediate or hybrid of the other two cultivars of *M. erythrophylla*.

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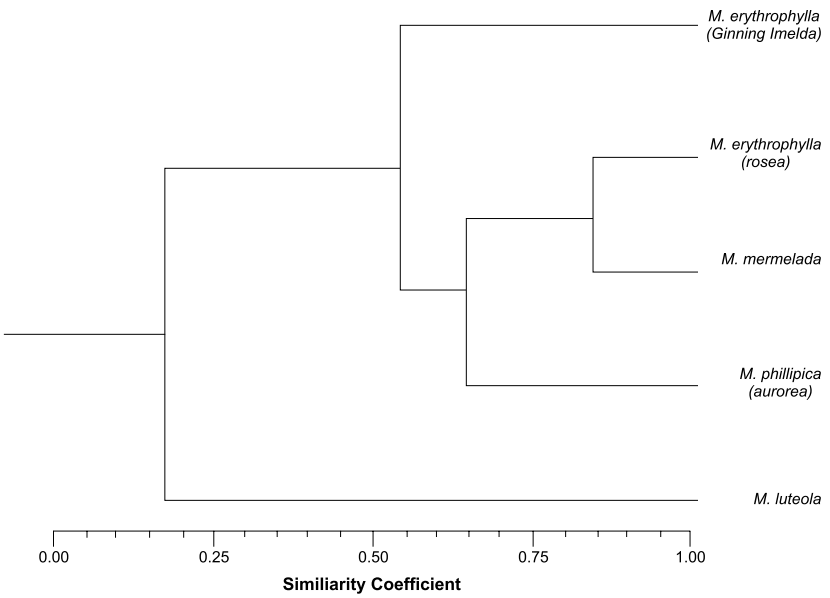

Fig. 2. Dendrogram based on SHAN clustering of the *Mussaeda* species studied.

Table 3. Genetic similarity among 5 *Mussaenda* species using 21 RAPD primers.

Name of the taxon	*M. erythrophylla* (Gining Imelda)	*M. erythrophylla* (Rosea)	*M. mermelada*	*M. philipica* (Aurorea)	*M. luteola*
Mussaenda erythrophylla (Gining Imelda)	1.000				
M. erythrophylla (Rosea)	0.604	1.000			
M. mermelada	0.561	**0.843**	1.000		
M. philipica (Aurorea)	0.466	0.658	0.636	1.000	
M. luteola	**0.146**	0.186	0.177	0.214	1.000

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REFERENCES

1. Burdakci, F. 2001. Random Amplified Polymorphic DNA (RAPD) markers. *Turkish J. Biol.* **25**: 185-96.

2. Gherardi, M., Mangin, B., Goffinet, B., Bonnet, D. and Huguet, T. 1998. A method to measure genetic distance between allogamous populations of alfalfa (*Medicago sativa*) using RAPD molecular markers. *Theor. Appl. Genet.* **96**: 406-12.

3. Ghislain, M., Zhang, D., Fazardo, D., Huamann, Z. and Hismans, R.H. 1999 Marker-assisted sampling of the cultivated Andean Potato *Solanumphu fureja* collection using RAPD markers. *Genet. Resour. Crop. Evol.* **46**: 547-55.

4. Jaccard P. 1908. Nouvelles rescherches sur la distribution florale. *Bull. Soc. Vaud. Sci. Nat.* **44**: 223- 70.

5. Karp, A., Isaac, P. and Ingram, D.S. 1998. *Molecular Tools for Screening Biodiversity: Plants and Animals*. Kluwer Academic Publishers,3300 Dordrecht, the Netherlands, 498 p.

6. Cramer, C.S. and Bridgen, M.P. 1997. Micropropagation of Mussaenda cultivars **In**: *Biotechnology in Agriculture and Forestry 40*, High-tech and micropropagation VI (Y.P.S. Bajaj Ed.). Springer-Verlag, USA, 397 p.

7. Prevost, A. and Wilkinson, M.J. 1999. A new system of comparing PCR primers applied to ISSR fingerprinting of potato cultivars. *Theor. Appl. Genet.* **98**: 661-68.

Fig. 1. Different species/cultivars of *Mussaenda* used for the present study [a) = *M. erythrophylla* Gining Imelda, b) = *M. phillipica* cv. Aurorea, c) = *M. erythrophylla* cv. Rosea, d) = *Mussaenda mermelada,* and e) = *M. luteola*].

8. Rohlf, F.J. 1997. NTSYS-pc : numerical taxonomy and multivariate analyses system verson 1.8. applied biostatistics, New York.

9. Saghai-Maroof, M.A., Soliman, K.M., Jerenson, R.A. and Allard, R.W. 1984. Ribosomal DNA spacer length polymorphism in barley: Mendelian inheritance, chromosomal location and population dynamics. *Proc. Nat. Acad. Sci.* (USA), **81**: 8014-18.

10. Smith, J.S.C., Chin, E.C.L., Shu, H., Smith, O.S., Wall, S.J., Senior, M.L., Mitchell, S.E., Kresovich, S. and Ziegle, J. 1997. An evaluation of the utility of SSR loci as molecular markers in maizes (*Zea mays* L.) : Comparison of data from RFLPs and pedigree. *Theor. Appl. Genet.* **95**: 163-73.

11. Sneath P.H.A. and Sokal, R. 1973. *Numerical Taxonomy*. Freeman, San Francisco, California.

12. Williams, J.G.K., Kubelik, A.R., Livak, K.J., Rafalski, J.A. and Tingey, S.V. 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acid Res.* **18**: 6531-35.

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