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MOLECULAR CHARACTERISATION OF *CATHARANTHUS ROSEUS* CULTIVARS FROM VARIOUS REGIONS OF RAJASTHAN BASED ON RAPD MARKER

Asheesh Kumar [*1], K. C. Singhal [1], R. A. Sharma [2], Govind K. Vyas [3] and Vinod Kumar [4]

Department of Biotechnology [1], Institute of Advance Science and Technology, NIMS University, Jaipur - 303121, Rajasthan, India.
Department of Botany [2], University of Rajasthan, Jaipur - 302004, Rajasthan, India.
Institute of Applied Science and Biotechnology [3], Chemind Diagnosis and Biosolutions, Jaipur - 302019, Rajasthan, India.
Seed Technology Research [4], Agriculture Research Station, SK Rajasthan Agricultural University, Durgapura, Jaipur - 334006, Rajasthan, India.

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Correspondence to Author:
Asheesh Kumar

Department of Biotechnology, Institute of Advance Science and Technology, NIMS University, Jaipur - 303121, Rajasthan, India.

E-mail: asheesh8282@yahoo.com

ABSTRACT: Genetic relationship between ten wild cultivars of *C. roseus* L. from four different regions of Rajasthan (Jaipur, Bikaner, Kota, and Jhalawar) with the marked difference in climatic condition was investigated using RAPD markers. *Catharanthus roseus* L. is a storehouse of several secondary metabolites such as vindoline, catharanthine, vinblastine, and vincristine, *etc*., thus making it an important source of drugs. A number of varieties of *C. roseus* L. occurs in wild and are cultivated in India. However, an evolutionary study has been limited. Using 24 decamer primers, we obtained a total of 221 amplicons with an average of 9.20 bands per primer. Out of 221 bands, 187 were found to be polymorphic, and the level of polymorphism was 84.61 percent. The average number of polymorphic bands per primer was 7.79. The average genetic similarity coefficient observed was 0.51 ± 0.186. Phylogenetic tree constructed using the neighbor-joining method of cluster analysis separated all the 10 samples of the *C. roseus* into two clusters. RAPD analysis thus was effective in differentiating the various cultivars, and the resulting high percentage of variation seems to have accumulated in the cultivars of different regions as a result of adaptation.

INTRODUCTION: *Catharanthus Roseus* L. (commonly known as periwinkle) is popular for its high medicinal value and as one of the richest source of a variety of secondary metabolites [1]. It is believed to be of, Indian Ocean Island, Madagascar, origin. It has been generally known as *Vinca rosea*, *Ammocallis rosea*, and *Lochnera rosea*.

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It is a perennial plant belonging to the Apocynaceae family. It is found in the areas of India, Australia, Africa and southern Europe [2].

In India, *C. roseus* is found in all parts of India. *C. roseus* has potent secondary metabolism responsible for monoterpenoid glucosides and other terpenoid compounds, steroids, phenolics, flavanoids, anthocyanins, and 130 terpenoid indole alkaloids (TIAs) [3, 4, 5]. It includes some medically important constituents like ajmalicine, Serpentine and Reserpine, which are well known for their hypotensive and antispasmodic properties [6]. *C. roseus* species are particularly important due to their medicinal alkaloids contents such as vindoline, catharanthine, vinblastine. Vincristine

and vinblastine (VBL) are one of the most important alkaloids produced from *C. roseus* due to its antitumor activity and wide pharmaceutical use [7, 4].

Genetic diversity plays an important role in plant conservation and their survival in adverse conditions. It can be measured at any functional level from a blueprint (DNA) to phenotype [2]. DNA-based molecular markers have been proposed as an excellent tool for identifying geographical variation, genetic diversity, phylogenetic relationship, authentication of plant species, pharmacognostic characterization, species characterization and genetic mapping in medicinal plants.

Among the polymerase chain reaction (PCR)-based molecular techniques, random amplified polymorphic DNA (RAPD) is convenient in performance and does not require any information about the DNA sequence to be amplified [8]. Due to its procedural simplicity, the use of RAPD as molecular markers for taxonomic and systematic analysis of plants [9], as well as in plant breeding and the study of genetic relationships have considerably increased [10]. Although several varieties of *C. roseus* is commonly seen in India, there are a few reports on a genetic-based study on the phylogenetic relationship. In our previous work, variations in the alkaloid content of the different cultivars from different parts of Rajasthan with marked differences in environmental conditions, namely Jaipur, Bikaner, Kota, and Jhalawar [11]. The aim of the present study was to find out genetic variations of the same cultivars of *C. roseus* using RAPD (random amplified polymorphic DNA).

MATERIAL AND METHODS:

Sample Collection: Fresh mature leaves of *Catharanthus roseus*, ranging in age from 7 to 8 months were randomly collected from four different locations of Rajasthan **Table 1.** The voucher specimen (RUBL 20862) is deposited at the Herbarium of the Department of Botany, University of Rajasthan Jaipur. Morphological details of the ten cultivars are given in **Table 2**.

TABLE 1: DETAILS OF DIFFERENT LOCATIONS OF RAJASTHAN SELECTED FOR SAMPLE COLLECTION

Location	Latitude	Longitude	Temperature (Degree)	Climate
Jaipur	26° 55' N	75° 52' E	28-34	Semi Arid
Bikaner	28° 01' N	73° 11' E	34-40	Hyper Arid
Kota	25° 10' N	75° 52' E	32-35	Humid
Jhalawar	25° 11' N	75° 51' E	30-34	Humid

TABLE 2: MORPHOLOGICAL DETAILS OF THE 10 PLANT SAMPLES COLLECTED FROM DIFFERENT PARTS OF RAJASTHAN

	Kota			Bikaner			Jaipur		Jhalawar	
Characteristics	K1	K2	K3	B1	B2	B3	J1	J2	JH1	JH2
Plant height	88 cm	70 cm	60 cm	70 cm	45 cm	62 cm	34 cm	60 cm	85 cm	60 cm
Plant branching	Many	Many	Many	Medium	Many	Many	Medium	Medium	Many	Many
Petal color	White	White	White+ pink spot	White+ pink spot	White+ pink spot	White+ pink spot	Red, dark flower	Pink flower	Pink flower	Pink flower
Plant Branching pattern	O S P	O S P	O P	O P	O P	O P	O P	O P	O P	O P
Stem color	Green	Green	Reddish brown	Reddish brown	Reddish brown	Greenish brown	Reddish brown	Reddish brown	Dark reddish	Reddish brown
Leaf size	Large	Large	Large	Large	Medium	Medium	Medium	Medium	Medium	Medium
Leaf serration	Strong	Strong	Weak	Weak	Weak	Weak	Weak	Weak	Weak	Weak
Pod shape	E F	E F	E F	E F	E F	E F	E F	E F	E F	E F
Pod length	4 cm	3.8cm	3.5 cm	3.0 cm	3.1 cm	3.5 cm	3.3 cm	3.8 cm	3.4 cm	3.1 cm
Seed color	Black	Black	Black	Black	Black	Black	Black	Black	Black	Black
1000 seed weight	2.4 g	2.3 g	2.0 g	2.2 g	2.2 g	2.0 g	2.1 gram	2.1 g	2.0 g	2.0 g
3th branch length	36.2 cm	30.5 cm	28.5 cm	24.6 cm	28.8 cm	25.4 cm	22.3 cm	26.7 cm	30.7 cm	26.1 cm
3th internode length	5.4 cm	5.3 cm	5.7 cm	4.1 cm	4.2 cm	4.1 cm	4.2 cm	5.2 cm	3.2 cm	3.8 cm
Total number of leaves	350	245	212	150	210	212	188	230	350	226
Flower number	15	8	10	6	10	7	5	9	12	8

Total number of branch	7	5	6	5	5	4	3	5	6	4
Total pod number	23	21	16	10	12	12	8	13	24	16

DNA Extraction and Purification: Three grams of tender twigs along with leaves were homogenized in liquid Nitrogen (liq. N_2). The homogenized material was handled as per the method described by Doyle and Doyle [12]. RNA was removed by treating the sample with DNase free RNase procured from Bangalore Genei, Banglore. Protein, including RNase, was removed by treating with chloroform: Isoamyl alcohol (24:1). DNA concentration and purity were determined by measuring the absorbance of diluted DNA solution at 260 nm and 280 nm.

The quality of the DNA was determined using agarose gel electrophoresis stained with ethidium bromide [13]. The purity and quantity of the isolated DNA were determined by Nanodrop spectrophotometer (ND1000). The 260/280 ratio was found between 1.8 – 1.99 that indicated the DNA was pure enough for RAPD.

RAPD Analysis: For RAPD analysis, 50 random decamer primers obtained from OPRON TECHNOLOGY, USA were screened, of which 24 were finally used for amplification. The PCR reactions were performed in a 25 µl reaction mixture containing 1X Taq assay buffer, 0.5 units of Taq DNA polymerase, 200 µM of each dNTPs (Bangalore Genei Pvt. Ltd., India), 0.2 µM primers and 50 ng of template DNA. The PCR reactions were carried out in DNA thermal cycler (Model-CGI-96, Corbett Research, Australia). The PCR reactions were repeated twice for each primer to ensure the reproducibility of RAPD results.

The PCR amplification conditions for RAPD consisted of an initial extended step of denaturation at 94 °C for 4 min followed by 44 cycles of denaturation at 94°C for 1 min, primer annealing at 37 °C for 1 min and elongation at 72 °C for 2 min followed by a final step of extension at 72 °C for 4 min.

The PCR reaction products were fractionated on 1.2% agarose gel containing 0.5 µg/µl ethidium bromide. After separation, gels were documented using Biovis Image Plus software (LIAS, Avgene, Taiwan).

Data Analysis: RAPD data were scored for the presence (1), or absence (0) and bands with the same molecular weight and mobility were considered as a single locus. Similarity matrix for RAPD primers was constructed using the Jaccard's similarity coefficient values to find out the genotypic relationship. These data were then subjected to UPGMA (unweighted pair-group method with arithmetic averages) analysis to generate dendrograms using NTSYSpc-version 2.02e [13].

RESULT AND DISCUSSION:

DNA Isolation and RAPD Analysis: A single sharp band is corresponding to λDNA was observed for all the samples of DNA on 0.8 percent agarose gel. The quality of DNA was determined as the ratio $A_{260}/A_{280,}$ which ranged from 1.8 to 2.0, which is indicative of good quality plant DNA, and the ratio was almost consistent. The concentration of DNA preparation varied from 5.68 µg/µl to 0.94 µg/µl.

All the ten varieties of *C. roseus* were examined for random amplified polymorphic DNA (RAPD) genetic marker with 27 decamer primers (OPRON TECHNOLOGY, USA). However, out of these 27 primers, three primers did not amplify (OPC-5, OPG-1, and OPG-7). All the primers showing amplification were repeated at least twice to confirm the reproducibility and polymorphism.

This is because the stoichastic nature of the band, and banding pattern of DNA amplification with RAPD, reproducibility of the banding pattern has been found to change. Finally, only those bands were considered as polymorphic, which did not amplify in certain varieties on repetition. The banding pattern generated by each primer was primer and species dependent and varied from 5-14 at 37 °C annealing temperature. A total of 221 amplicons were obtained with 24 primers with an average of 9.20 bands per primer. Out of 221 bands, 187 were found to be polymorphic, and the level of polymorphism was 84.61 percent **Table 3**. The average number of polymorphic bands per primer was 7.79.

TABLE 3: LIST OF ARBITRARY PRIMERS WITH TOTAL POLYMORPHIC AMPLICONS AND POLYMORPHISM GENERATED FOR 10 *C. ROSEUS* SAMPLES

Primers	*Sequence 5'-3'*	Total no. of bands (a)	Total No. of the polymorphic band (b)	Polymorphism (%) b/a × 100
OPA-17	GACCGCTTGT	14	14	100
OPB-11	GTAGACCCGT	8	7	87.5
OPB-13	TTCCCCCGCT	14	14	100
OPB-15	GGAGGGTGTT	10	9	90
OPB-16	TTTGCCCGGA	10	9	90
OPC-6	GAACGGACTC	6	5	83.33
OPC-7	GTCCCGACGA	13	13	100
OPC-8	TGGACCGGTG	5	4	80
OPC-9	CTCACCGTCC	13	12	92.30
OPC-10	TGTCTGGGTG	5	3	60
OPC-11	AAAGCTGCGG	7	7	100
OPC-13	AAGCCTCGTC	7	6	85.71
OPC-14	TGCGTGCTTG	5	2	40
OPC-16	CACACTCCAG	14	14	100
OPC-17	TTCCCCCCAG	5	4	80
OPC-18	TGAGTGGGTG	3	2	66.66
OPC-19	GTTGCCAGCC	10	9	90
OPC-20	ACTTCGCCAC	12	4	33.33
OPG-2	GGCACTGAGG	12	10	83.33
OPG-3	GAGCCCTCCA	11	8	72.72
OPG-6	GTGCCTAACC	14	14	100
OPG-8	TCACGTCCAC	9	8	88.88
OPG-9	CTGACGTCAC	5	2	40
OPG-10	AGGGCCGTCT	9	7	77.77
		221	187	84.61

The most informative primers were OPB-13, OPC-16, OPC-17and OPG-6 with all polymorphic bands and least informative primer was OPC-18 with 4 polymorphic bands out of total 12 bands, polymorphism being 33 percent **Fig. 1**. None of the primer produced all monomorphic bands.

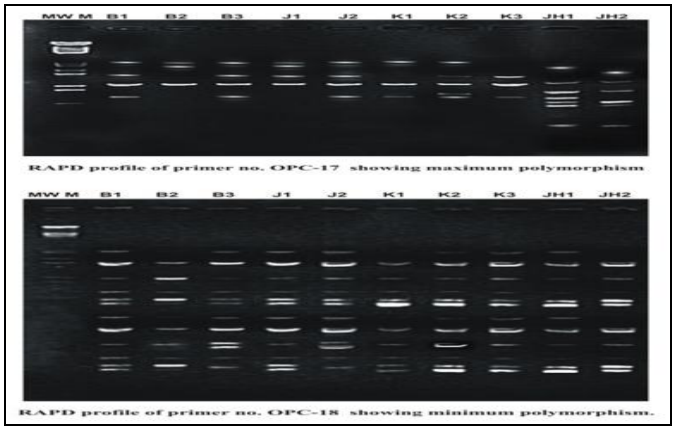

FIG 1: RAPD PROFILE USING OPERON PRIMERS – 17 AND 18

Varietal Identification: To identify different accessions of *C. roseus,* different patterns generated by each primer were considered. The primer generating unique banding patterns for all the varieties are most useful for varietal identification or discrimination of varieties. In the present study, only one primer (OPB-17) produced unique patterns for all the varieties; hence, this primer can be used without the help of other primers to distinguish all the varieties studied.

The discriminatory power of a primer is determined by a number of types defined by the profiles (banding patterns) generated and the relative frequency of these types, *i.e.* the number of genotypes generating that same pattern. These two facets of discrimination are not generally presented as a single numerical value and therefore, cannot be used for a straight forward comparison of different methods, we have used a single numerical index of discrimination (D) based on the probability that two unrelated strains sampled from the test population will be placed into different typing groups. This probability was calculated by Simpson's index of diversity as described by Hunter and Gaston [6].

The discriminatory power ranged from 0.53 to 1.0 for pattern generated by various primers. The discriminatory index of value one was obtained for the primer OPB-13, which produce unique banding

pattern for all the varieties **Table 4**. The discriminatory power of protein profile was less than one [0.93], *i.e.* it failed to generate unique banding for all varieties. However, polypeptide patterns show a few variety of specific bands.

The maximum discriminating power was assessed for OPB-17 (D = 1) followed by OPA-13 (D = 0.95) producing maximum different types of banding patterns. The minimum discriminating power was assessed for OPC-10 (D = 0.53).

TABLE 4: PRIMER DISCRIMINATIVE POWER (D)

Primer	D at 37º
OPA-17	0.95
OPB-11	0.73
OPB-13	1
OPB-15	0.73
OPB-16	0.62
OPC-06	0.86
OPC-07	0.91
OPC-08	0.64
OPC-09	0.91
OPC-10	0.53
OPC-11	0.86
OPC-13	0.88
OPC-14	0.6
OPC-16	0.84
OPC-17	0.91
OPC-18	0.75
OPC-19	0.77
OPC-20	0.82
OPG-02	0.86
OPG-03	0.8
OPG-06	0.82
OPG-08	0.8
OPG-09	0.71
OPG-10	0.73

Genetic Relationship among the Varieties/Lines: The pairwise genetic similarity estimates (Jaccard's coefficient) based on RAPD banding pattern were used for cluster analysis to present genetic relationship in the form of the dendrogram. The similarity coefficient matrix was subjected to algorithm unweighted pair group method for average arithmetic analysis (UPGMA) to generate clusters. The Jaccard's pairwise similarity coefficient value for ten *C. roseus* is presented in **Table 5**.

The range of genetic similarity was found to be between 0.335 (B-1 and B-2, J-1, and K-3) to 0.87 (B-2 and B-3). The average genetic similarity coefficient observed was 0.51 ± 0.186. The genetic similarity was found between group the 80 and 65% and within group 36%.

Dendrogram constructed using the neighbor-joining method of cluster analysis separated all the 10 samples of the *C. roseus* into two clusters **Fig. 2**. Cluster 1, which is separated into 2 sub-clusters, first includes one Bikaner region, and second includes three Kota regions and two Jhalawar regions. Cluster 2 contains Bikaner and Jaipur regions. The cultivars thus show close relation in terms of origin. It can be explained from the observation that geographically isolated individuals tend to accumulate genetic variations during environmental adaptations [14].

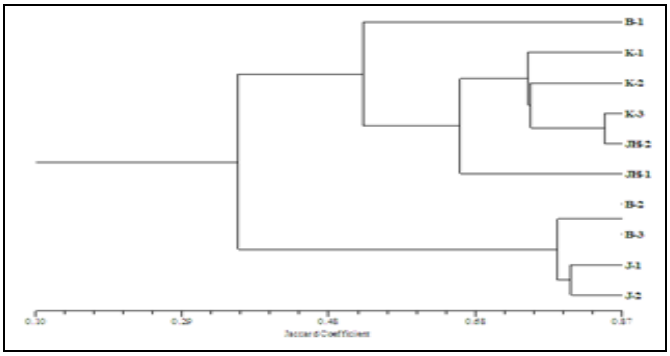

FIG. 2 : DENDROGRAM GENERATED FOR TEN *C. ROSEUS* CULTIVARS USING UPGMA CLUSTER ANALYSIS BASED ON JACKARDS' SIMILARITY COEFFICIENT FOR RAPD DATA

TABLE 5: JACCARD'S SIMILARITY COEFFICIENT FOR TEN VARIETIES OF *C. ROSEUS* BASED ON RAPD PROFILING

Varieties	B-1	B-2	B-3	J-1	J-2	K-1	K-2	K-3	JH-1	JH-2
B-1	1									
B-2	0.33	1								
B-3	0.35	0.87	1							
J-1	0.35	0.8	0.82	1						
J-2	0.37	0.76	0.78	0.81	1					
K-1	0.55	0.34	0.36	0.37	0.39	1				
K-2	0.56	0.35	0.38	0.34	0.36	0.77	1			
K-3	0.54	0.34	0.34	0.33	0.34	0.74	0.78	1		
JH-1	0.49	0.40	0.42	0.41	0.40	0.62	0.64	0.66	1	
JH-2	0.53	0.35	0.36	0.35	0.36	0.75	0.79	0.66	0.7	1

CONCLUSION: RAPD analysis revealed that a high percentage of polymorphism among the ten selected cultivars of *C. roseus*, which indicates that genetic variation, has been preserved over the different regions of Rajasthan.

These results will be useful to establish and maintain a germplasm collection of *C. roseus* and may guide us in designing strategies for breeding programs that maximize the utility of *C. roseus* genetic resources.

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CONFLICT OF INTEREST: Nil

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