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Genetic diversity assessment in nine cultivars of *Catharanthus roseus* from Central Gujarat (India) through RAPD, ISSR and SSR markers

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ABSTRACT:

The genetic relationship study was carried out among nine different cultivars of *Catharanthus roseus* using RAPD, ISSR and SSR markers. In RAPD analysis, out of twenty primers, six primers amplified 592 bands out of which 466 were polymorphic while rest was monomorphic. This gave high (78.71%) polymorphism among nine cultivars. In ISSR analysis, 78.94% polymorphism was observed, while in SSR analysis, 76.62% polymorphism was observed. The dendogram based on all three markers separated the cultivars in two major groups. 'Blue pearl', 'cooler red', and 'pacifica apricot'were in the same group, while rest was in other group. Amongst all 'Pacifica liac' and 'Albus with red eye' were found to be closely related. Our results showed that all RAPD, ISSR and SSR markers are sensitive and effective tool for genomic analysis in *Catharanthus roseus.* This study provides key platform for further crop improvement and cross breeding.

Keywords:
Genetic diversity, RAPD, ISSR, SSR, *Catharanthus roseus.*

Abbreviations:
Random amplified polymorphic DNA (RAPD), Inter simple sequence repeats (ISSR), Simple sequence repeats (SSR), basepair (bp).

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INTRODUCTION

Catharanthus roseus (L.) G. Don (family Apocynaceae) is a beautiful ornamental plant with enormous medicinal values. In India, it's commonly known as "periwinkle", "sada bahar", "sadaphul", or "barmasi". *Catharanthus roseus* (*Vinca rosea*) is native to Madagascar and spread throughout the tropics and subtropics (RK Shaw *et al.*, 2010).

The plant is known to have more than 70 different alkaloids like ajmalicine, vincrystine, vinblastine etc. These alkaloids contribute to anticancer and anti diabetic activity of plant. *Catharanthus roseus* has been used traditionally for its calming effect and its ability to reduce the blood pressure (Flora of China: *Catharanthus roseus*).

Assessment of genetic diversity in crop species is an important component of crop improvement programs. Accurate analysis of the genetic diversity can be useful in plant breeding. The detailed understanding of relationship between inbred lines and pure lines can be useful in planning crosses (Hallauer and Miranda, 1988). Analysis of genetic diversity in germplasm collection can facilitate reliable classification of accessions and identification of subspecies which can be further useful for plant breeding.

Various marker systems have been used for genetic studies and characterization analysis. These include morphological, cytological, biochemical and DNA marker systems. DNA markers are considered the best tools for determining genetic relationships/diversity, as they are unlimited in number, show high polymorphism and are independent of environmental interaction i.e., highly heritable (Singh *et al.*, 2004).

There are various types of DNA markers like Restriction Fragment Length Polymorphism (RFLP, Sambrook *et al.*, 1989), Variable Number of Tandem Repeats (VNTRs, Nakamura *et al.*, 1987), Simple Sequence Repeats (SSRs, Jacob *et al.*, 1991), Inter Simple Sequence Repeats (ISSR, Zietkiewicz *et al.,* 1994*)* and Random Amplified Polymorphic DNA (RAPD, Williams *et al.*, 1990).

Amongst all these techniques, RAPD technique has gained importance due to its simplicity, efficiency, relative ease to perform and non-requirement of DNA sequence information (Karp *et al.*, 1997; Khanuja *et al.*, 1998). The technique has been very useful in studies of genetic diversity (Orozco-Castillo *et al.*, 1994; Chalmers *et al.*, 1994), phylogeny and systematic (Millan *et al.*, 1996; Sun *et al.*, 1998), genetic linkage mapping (Cheung *et al.*, 1997) and gene tagging (Tiwari *et al.*, 1998). SSR and ISSR markers are also being widely used to study genetic diversity in number of plant species.

The current investigation was carried out with the aim to study the genetic diversity among *C. roseus* cultivars using RAPD, ISSR and SSR markers.

MATERIAL AND METHODS

Plant material

Nine different cultivars of *Catharanthus roseus* were collected for this study. All samples were collected from nearby area of Anand, Gujarat, India (**Table-1**). Fresh and young leaves were collected to isolate the DNA.

Genomic DNA isolation

The genomic DNA was isolated from young leaves by standard CTAB (Cetyl trimethyl ammonium bromide) method (Doyle and Doyle, 1990) with slight modifications. Slight higher

Table-1: Name, Petal color and Images of nine cultivars of *Catharanthus roseus* (L.) G. Don

No.	Cultivar name	Colour of petal	Image
1	Patricia white	Milky white	
2	Grape cooler	Light pink	
3	Cooler red	Cherry red	
4	Peppermint cooler	White with red center	
5	Pacifica apricot	Whitish pink	
6	Cooler orchid	Dark pink with white eye	
7	Pacifica liac	Dark pink	
8	Albus with red eye	Milky white with radiating red eye	
9	Blue pearl	Purple	

concentration of detergent was used. RNA was removed by giving RNaseA treatment which was given after incubation at 65^0C in boiling water bath. The isolated DNA was dissolved in 25µl of $T_{10}E_1$ buffer (Tris 10mM and EDTA 1mM, pH 8.0). The DNA was checked through agarose gel electrophoresis and the quantity and purity was checked by Nanodrop-1000 (Thermo Fisher Scientific, USA).

Random Amplified Polymorphic DNA (RAPD) analysis

The RAPD analysis was carried out using the method of Williams *et al.,* (1990). Polymerase chain reactions were performed in 25µl system containing 2.5µl of 10 X assay buffer (10mM Tris-Cl; pH 9.0, 1.5mM $MgCl_2$, 50mM KCl and 0.01% gelatin), 2.5mM of each dNTPs (dATP, dTTP, dCTP and dGTP) (Bangalore Genei Pvt. Ltd, Bangalore, India), 1.5µM of primer (Sigma Aldrich, Bangalore), 0.2 unit of Taq DNA polymerase (Bangalore Genei Pvt. Ltd, Bangalore, India) and 25ng of template DNA. The reaction was carried out using thermo cycler (Corbett research gradient automatic, UK). Reaction was carried put in three steps.

The first step of initial denaturation was performed at 94^0C for 5 min. Following the initial denaturation step, PCR was carried out for 45 cycles. Each cycles consisted of a denaturation step of 1 min at 92^0C, annealing step of 1 min at 37^0C and an extension step of 2 min at 72^0C. The last cycle was followed by final extension step of 7 min at 72^0C. The holding temperature was 4^0C.

Inter Simple Sequence Repeat (ISSR) analysis

In ISSR analysis, the polymerase chain reactions were performed in 25µl system containing 2.5µl of 10 X assay buffer (10mM Tris-Cl; pH 9.0, 1.5mM $MgCl_2$, 50mM KCl and 0.01% gelatin), 2.5mM of each dNTPs (dATP, dTTP, dCTP and dGTP) (Bangalore Genei Pvt. Ltd, Bangalore, India), 1.5µM of primer (Sigma Aldrich, Bangalore), 0.2 unit of Taq DNA polymerase (Bangalore Genei Pvt. Ltd, Bangalore, India) and 25ng of template DNA. The reaction was carried out using thermo cycler (Corbett research gradient automatic, UK). Reaction was carried out in three steps. The first step of initial denaturation was performed at 94^0C for 5 min. Following the initial denaturation step, PCR was carried out for 40 cycles. Each cycles consisted of a denaturation step of 1 min at 94^0C, annealing step of 1 min at various annealing temperatures (Table-3) and an extension step of 2 min at 72^0C. The last cycle was followed by final extension step of 7 min at 72^0C. The holding temperature was 4^0C.

Simple Sequence Repeats (SSR)

In SSR analysis, the polymerase chain reactions were performed in 25µl system containing 2.5µl of 10 X assay buffer (10mM Tris-Cl; pH 9.0, 1.5mM $MgCl_2$, 50mM KCl and 0.01% gelatin), 2.5mM of each dNTPs (dATP, dTTP, dCTP and dGTP) (Bangalore Genei Pvt. Ltd, Bangalore, India), 1.5µM of primer (Sigma Aldrich, Bangalore), 0.2 unit of Taq DNA polymerase (Bangalore Genei Pvt. Ltd, Bangalore, India) and 25ng of template DNA. The reaction was carried out using thermo cycler (Corbett research gradient automatic, UK). Reaction was carried out in three steps. The first step of initial denaturation was performed at 94^0C for 5 min. Following the initial denaturation step, PCR was carried out for 40 cycles. Each cycles consisted of a denaturation step of 1 min at 94^0C, annealing step of 1 min at various annealing temperatures (Table-4) and an extension step of 2 min at 72^0C. The last cycle was followed by final extension step of 7 min at 72^0C. The holding temperature was 4^0C.

Agarose gel electrophoresis

The amplified products were checked on gel electrophoresis (1.5% agarose gel for RAPD and 2% for ISSR and SSR). The electrophoresis was performed for four hours at a constant voltage of 100 volts. The bands were visualized under U.V. light and the photographs were taken by gel documentation system (Alpha Innotech, Alpha Imager EP, USA). 100bp ladder was used to determine the size of the amplicons.

Data scoring and statistical analysis

The band was scored '1' for its presence and '0' for its absence. This data were used to construct the binary matrix. The Jaccard's coefficient (Jaccard, 1908) was used to construct the similarity matrix among the nine cultivars of *Catharanthus roseus*. The phylogram was constructed using the unweighted pair group method using arithmetic means (UPGMA) (Sneath and Sokal, 1973) and SAHN clustering. The entire analysis was performed using the NTSYS-pc (Numerical taxonomy system, applied biostatistics, Inc., New York, USA, software version 2.02e) (Rohlf, 1997). The polymorphism information content (PIC) value was calculated as $PIC=1-\sum P^2_i$; P_i is the band frequency of the i^{th} allele (Smith *et al.,* 1997).

Groupings of cultivars were also evaluated by principle coordinate analysis (PCA) as reported by Thomas *et al.* (2006). PCA was performed by extracting Eigen value and Eigen vectors from a correlation matrix which was generated using a standardized data matrix 2-D and 3-D plots were constructed to evaluate the groupings of *C. roseus* cultivars.

RESULT AND DISCUSSION

In RAPD analysis, total twenty RAPD primers were used to check the genetic variation in nine different cultivars of *Catharanthus roseus.* Out of these twenty, six primers gave satisfactory and reproducible bands. The banding pattern of the RAPD analysis for two primers has been shown in **Fig-1**. The details of the banding pattern are shown in **Table-2**. All these six primers gave amplifications in all nine cultivars. Total 592 bands were observed. From these total bands, 466 were polymorphic while, 126 bands were monomorphic. This resulted in total polymorphism of 78.71%. OPA-03 gave maximum number (146) of bands, while minimum number of bands (62) was observed in OPAF-15 primer. The amplicons were observed ranging from 190-2650bp. The largest amplicon (2650bp) was amplified by OPA-03 primer, while the shortest amplicon (190bp) was amplified by OPN-15 and OPC-12 primer. Similarity matrix was plotted using the Jaccard's coefficient. According to the matrix, the similarity index was observed ranging from 0.198–0.731 with the mean similarity index of 0.56 indicating reasonable variability as obtained by RAPD markers (data not shown). The maximum PIC value (0.955) was observed in OPC 12 primer, while the minimum PIC value (0.900) was observed in OPAF 05. The average PIC value observed from all six RAPD primers was 0.93.

In our study, RAPD markers were successfully used to differentiate all nine cultivars of *Catharanthus roseus* from each other. Thus, on the basis of RAPD, the findings of this study are similar to the observations of Rajaseger *et al.*, (1990).

In ISSR analysis, totally six ISSR primers were used. Out of these six, three primers gave satisfactory and reproducible bands. The banding pattern of one ISSR primer is shown in **Fig-2** and the details are given in **Table-3.** All these three primers gave amplifications in all nine cultivars. These ISSR primers produced total 342 scorable bands. From these total bands, 270 bands were polymorphic while, 72 bands were monomorphic. This gave a total polymorphism of 78.94%. The maximum number of bands (142) was observed in (AGG)6 primer, while minimum number of bands (91) was observed in (GA)9T primer. The amplicons were observed ranging from 190-1635bp. Both the largest and the smallest amplicons were amplified by (GACA)4 primer. The similarity

Table-2: Detailing of RAPD primers and analysis of amplified bands in nine *C. roseus* cultivars.

Primer	Nucleotide sequence	Range of amplicons (inbp)	Total bands	Total polymorphic bands	Total monomorphic bands	PIC
OPA 03	AGTCAGCCAC	230-2650	146	128	18	0.954
OPC 12	TGTCATCCCC	190-2040	136	109	27	0.955
OPD 20	AACCCGGTCA	400-1730	63	45	18	0.922
OPN 15	CAGCGACTGT	190-1740	117	108	09	0.954
OPAF 05	CCCGATCAGA	320-1550	68	23	45	0.900
OPAF 15	CACGAACCTC	270-1940	62	53	09	0.906
Total		**190-2650**	**592**	**466**	**126**	**Avg=0.93**

Table-3: Detailing of ISSR primers and analysis of amplified bands in nine *C. roseus* cultivars.

Primer	Annealing temperature (^{0}C)	Range of amplicons (in bp)	Total bands	Total polymorphic bands	Total monomorphic bands	PIC
(GACA)4	49	190-1635	109	82	27	0.94
(GA)9T	53.1	162-1375	91	91	00	0.94
(AGG)6	45	263-1425	142	97	45	0.94
Total		**190-1635**	**342**	**270**	**72**	**Avg=0.94**

matrix constructed using Jaccard's coefficient showed that the similarity index ranged from 0.257-0.648. The mean similarity index of 0.76 was observed indicating good variability (data not shown). All the ISSR primers showed same PIC value (0.94).

In SSR study, five primers were analyzed. Out of these five primers, only two primers gave satisfactory and reproducible results. The banding pattern of one SSR primer is shown in **Fig-2 (B)** and the detailing of this banding pattern is given in **Table-4.** Both these primers gave amplifications in all *C. roseus* cultivars. Total 231 scorable bands were produced by both SSR primers. Out of these,

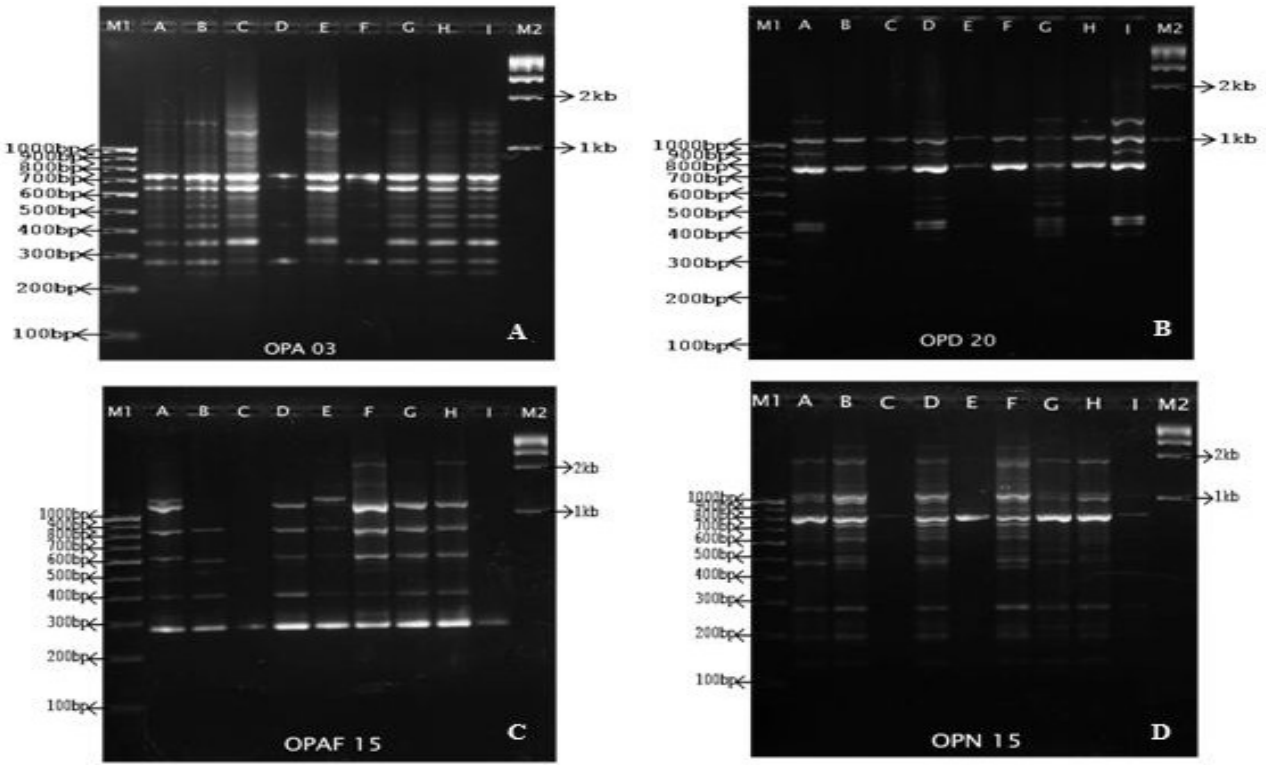

Fig-1: RAPD banding pattern in nine *C. roseus* cultivars. (M1= 100bp ladder, M2=1000bp ladder, Lane A-I are different cultivars as shown in table 1). Fig 1A amplification with OPA 03 primer, Fig 1B amplification with OPD 20 primer, Fig 1C amplification with OPAF 15 primer and Fig 1D amplification with OPN 15 RAPD primer.

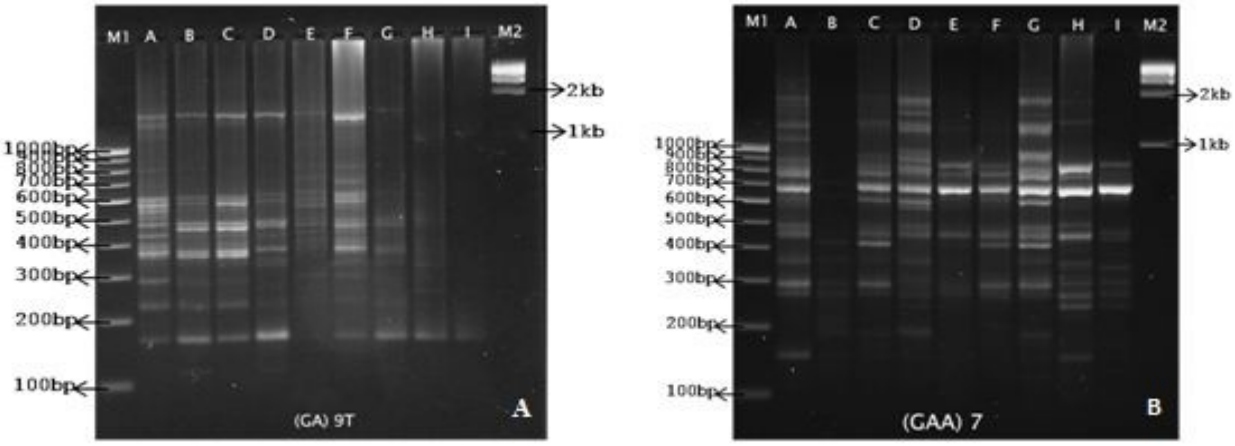

Fig-2: Banding pattern in nine *C. roseus* cultivars using ISSR and SSR primers (M1= 100bp ladder, M2=1000bp ladder, Lane A = patricia white, B= cooler orchid, C= Pacifica liac, D= Albus with red eye, E= blue pearl, F= Grape cooler, G= cooler red, H= first kiss podka dot, I= pacifica apricot). Fig 1A amplification with (GA)9T ISSR primer and Fig 1B amplification with (GAA)7 SSR primer.

177 bands were polymorphic, while 54 bands were monomorphic. This resulted in total polymorphism of 76.62%. Primer (GAA)7 produced more number of bands (142) than (AAGC)3 primer (89). The amplicons were observed ranging from 145-1839bp. The largest amplicon (1839bp) was amplified by (AAGC)3 primer, while the smallest amplicon (145bp) was amplified by (GAA)7 primer. Similarity matrix was plotted using the Jaccard's coefficient. According to the matrix, the similarity index was observed ranging from 0.261–0.736 with the mean similarity index of 0.68 indicating reasonable variability as obtained by SSR markers (data not shown). Primer (GAA)7 showed higher PIC value (0.96) than (AAGC)3 primer (0.91).

The combined RAPD, ISSR and SSR analysis detected high degree of genetic variations. The highest similarity index (0.632) was observed between 'Pacifica liac' and 'Albus with red eye'. The least similarity index (0.287) was observed between 'Cooler orchid' and 'Blue peal'. The mean similarity was observed reasonably high (0.68). This indicates high genetic variation among nine *C. roseus* cultivars. Similar variations were observed in dendogram constructed using UPGMA method (**Fig-3**) as well as in 2-D and 3-D plots (**Fig-4(A) and 4(B**)). In dendogram, two clear clusters were observed (A & B). Cultivars 'patricia white', 'grape cooler', 'pacifica liac', 'albus with red eye', 'First kiss podka dot' and 'cooler orchid' and were in cluster A , while 'blue pearl', 'cooler red', and 'pacifica apricot' were in the cluster B. Cluster A was divided into two sub clusters A1 and A2. Sub cluster A1 was further divided into A1a and A1b. Cultivars 'patricia white' and 'grape cooler' were in A1a, while 'pacifica liac' and 'albus with red eye' were in A1b. 'pacifica liac' and 'albus with red eye' were found to be most similar with 100% similarity. 'First kiss podka dot' and 'cooler orchid' were in the sub cluster A2. Cluster B was further divided into two sub clusters B1 and B2. 'cooler red', and 'pacifica apricot' were in the sub cluster B1, while 'Blue pearl' was in sub cluster B2. The principal coordination analysis (PCA) supported the similar groupings (Fig-4).

Good correlation (r value) was observed among all three markers. The average polymorphism using all three markers was observed 78.09% which indicates high degree of polymorphism among all nine cultivars.

The dendogram constructed using all three RAPD, ISSR and SSR markers successfully differentiated all nine cultivars of *Catharanthus roseus.* These results demonstrate the excellent power of all these three markers in studying the closely related taxa. This supports the findings of Raina *et al.,* (2001). They studied that RAPD and ISSR fingerprints as useful genetic markers for analysis of genetic diversity, varietal identification, and phylogenetic relationships in peanut (*Arachis hypogaea*) cultivars and wild species.

Lalhruaitluanga and Prasad (2009) studied comparative results of RAPD and ISSR markers for genetic diversity assessment in *Melocanna baccifera* (Roxb.) growing in Mizoram State of

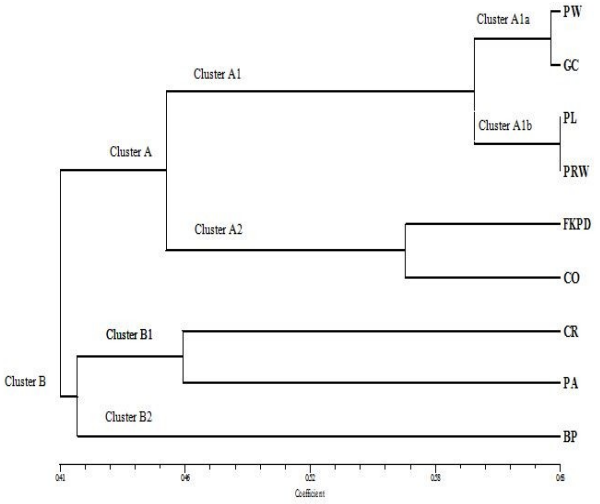

Fig-3: Dendogram based on UPGMA method showing genetic relationship among nine *C. roseus* cultivars as revealed by RAPD, ISSR and SSR markers, PW=patricia white, GC= grape cooler, CR= cooler red, FKPD= first kiss podka dot, PA= pacifica apricot, CO= cooler orchid, PL= pacifica liac, PRW= albus with red eye, BP= blue pearl.

Table-4: Detailing of SSR primers and analysis of amplified bands in nine *C. roseus* cultivars.

Primer	Annealing temperature (^{0}C)	Range of amplicons (in bp)	Total bands	Total polymorphic bands	Total monomorphic bands	PIC
(GAA)7	54	145-1793	142	133	09	0.96
(AAGC)3	60	452-1839	89	44	45	0.91
Total		**145-1839**	**231**	**177**	**54**	**Avg=0.93**

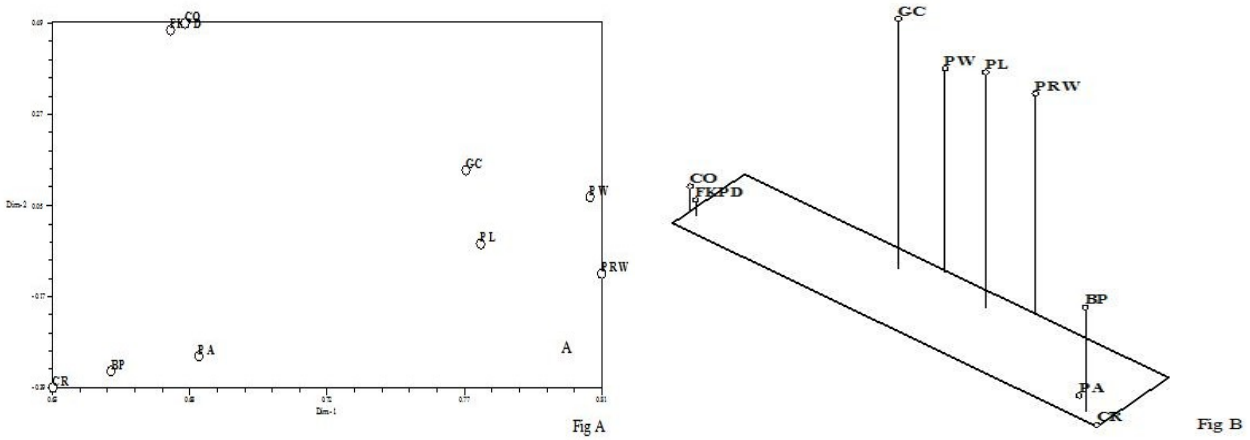

Fig-4: Phylogenetic relationship among nine *Catharanthus roseus* cultivars revealed by RAPD, ISSR and SSR primers. (A) 2-D plot (B): 3-D plot. PW=patricia white, GC= grape cooler, CR= cooler red, FKPD= first kiss podka dot, PA= pacifica apricot, CO= cooler orchid, PL= pacifica liac, PRW= albus with red eye, BP= blue pearl.

India. They found that higher polymorphism (98.02%) was obtained in RAPD markers than ISSR markers (84.1%). However, in our present investigation it was found that slightly higher polymorphism (78.94%) was observed in ISSR markers compared to RAPD markers (78.71%) and SSR markers (76.62%). Similar results were observed by Parsons *et al.*, (1997) who studied the genetic diversity relationship in rice using different marker systems. They observed 56% polymorphism in ISSR markers while, 50% polymorphism was observed in RAPD markers.

Moreover, Lal *et al*., (2010) also reported that higher polymorphism (95%) was obtained in ISSR markers than RAPD (87%) and SSR (93%) markers. They checked the efficiency of all these three PCR based markers in *Cicer arietinum* L. and *Cajanus cajan* L. Millspaugh and found that higher PIC value was observed in ISSR markers (0.70) than in RAPD (0.49) and SSR (0.61) markers. In same manner, in our investigation also, higher PIC value (0.94) was observed in ISSR markers than in RAPD (0.93) and SSR (0.93) markers.

Ajibade *et al.,* (2000) and Galvan *et al*., (2003) concluded that ISSR would be a better tool than RAPD for phylogenetic studies. Nagaoka and Ogihara (1997) have also reported that the ISSR primers produced several times more information than RAPD markers in wheat. Our study also shows that ISSR marker is better tool than RAPD markers for phylogenetic study.

RK Shaw *et al.* (2008) studied the genetic variation in cultivars of *C. roseus* using RAPD and ISSR markers. They observed genetic variability using 18 RAPD and ISSR markers. They concluded that both markers are equally potential to differentiate the closely related cultivars of *C. roseus.* In our investigation also, the high discriminating power of both RAPD and ISSR markers among *C. roseus* cultivars was observed.

To best of our knowledge, no earlier reports are available regarding the genetic diversity analysis in *C. roseus* using all these three markers. The polymorphism data generated can be used for plant breeding, crop improvement programs and also might be helpful in future strategies for

Table-5: Similarity matrix based on Jaccard's coefficient.

	PW	GC	CR	FKPD	PA	CO	PL	PRW	BP
PW	1								
GC	0.628	1							
CR	0.443	0.391	1						
FKPD	0.312	0.447	0.306	1					
PA	0.440	0.408	0.462	0.358	1				
CO	0.471	0.479	0.329	0.562	0.385	1			
PL	0.575	0.568	0.407	0.426	0.453	0.434	1		
PRW	0.631	0.600	0.449	0.407	0.509	0.461	0.632	1	
BP	0.461	0.388	0.370	0.370	0.440	0.287	0.447	0.493	1

evolution of desired genotypes and further development of new *C. roseus* cultivars.

CONCLUSION

All three markers (RAPD, ISSR and SSR) proved potential tools for analyzing genetic variation among nine closely related cultivars. So, all the three markers can be used to design a strategy to maintain or enhance the genetic diversity of future varieties. The polymorphism data generated can be used for plant breeding, crop improvement programs and also might be helpful in future strategies for evaluation of desired genotypes and further development of new *C. roseus* cultivars.

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