# Identification of molecular markers for flower characteristics in *Catharanthus roseus* producing anticancer compounds

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**Abstract:** *Catharanthus roseus* or vinca produces over 130 TIAs with pharmaceutical value. Two of them, namely vinblastine (VB) and vincristine (VC), are species-specific and extensively used in anticancer chemotherapeutics. To detect *C. roseus* genotypes abundant in desired TIAs, contrasting genotypes should be analyzed via marker-assisted selection (MAS) to avoid analytical quantitation. A trustable morphological marker, like a flower characteristic, will lessen the selection efforts in improving such a quantitative trait. The present work aims at detecting PCR-based molecular markers, i.e., RAPD, ISSR and AFLP, for eight *C. roseus* cultivars for flower characteristics as possible markers for high levels of VC and VB. The study indicated high levels of polymorphism generated across different type of marker; AFLP was the most powerful, while ISSR was the least. The polymorphism information content (PIC), average of heterozygosity ($H_e$), the effective multiplex ratio (E), and the marker index (MI) revealed that data of ISSR is the least trustable. The overall results separated the cultivars with white versus those with pink colors, on one hand, and versus colored flowers, on the other hand. A number of 79 cultivar-specific markers were detected across type of marker. Other 100 markers for important flower characteristics were also detected. They are white petal, colored petal, pink petal, petal white center and yellow flower eye center. The highest number of flower trait markers was scored during AFLP analysis (65), while the lowest was scored during RAPD analysis (14). Recent efforts indicated that some of these markers can be linked to the levels of the anticancer compounds VC and VB in *C. roseus*.

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## 1. Introduction

Plant species are known to synthesize over 2000 different types of terpenoid indole alkaloids (TIAs) (Barleben *et al.*, 2007, Ziegler & Facchini, 2008, Guirimand *et al.*, 2010). TIAs are naturally synthesized as a defense against pests and diseases (Roepke *et al*., 2010). More than 130 TIAs are produced in the periwinkle, *Catharanthus roseus* or vinca (van der Heijden *et al.*, 2004, Wang *et al*., 2011) mostly act as valuable pharmaceuticals. Two of them, namely vinblastine (VB) and vincristine (VC), are species-specific and extensively used in anticancer chemotherapeutics (van der Heijden *et al*., 2004, Pasquier & Kavallaris, 2008).

Among the various plant breeding procedures available, the selection breeding involves isolation of individual plants bearing superior phenotype from the segregating population (Sharma *et al*., 2012). Hybrids between parents possessing desirable features are used to generate segregating populations. The selection breeding brings together genes/alleles favorable for the improved expression of the traits of interest, from diverse parents, into the selected genotype(s). To detect *C. roseus* genotypes abundant in desired TIAs, contrasting genotypes should be analysed (Guirimand *et al.*, 2011a&b). In marker-assisted selection (MAS) (Dekkers & Hospital, 2002), the DNA markers linked to the desired trait should be identified. A trustable morphological maker, like flower or fruit characteristics, will lessen the selection efforts in improving such a trait. Application of this technology in *C. roseus* will allow tracking of trait of high TIAs, specifically VC and VB, in segregating populations

without having actually to quantitate them analytically.

DNA-based genetic markers are recently integrated into several plant systems and expected to play a very important role in the future of plant breeding and molecular genetics. Polymerase chain reaction (PCR) was invented as a genetic assay based on selective DNA amplification (Saiki *et al*., 1988, Innis *et al.*, 1990). Random amplified polymorphic DNAs (RAPDs) (Williams *et al.*, 1990) are useful PCR-based molecular markers for the assessment of genetic diversity among species (Artyukova *et al.*, 2004, Sureja *et al*., 2006, Guerra *et al*., 2010) including vinca (Gupta *et al.*, 2007, Shaw *et al.*, 2009) because of their simplicity, speed and relatively low cost as compared to other types of molecular markers. Inter simple sequence repeats (ISSRs) were developed to access variation in the numerous microsatellite regions dispersed throughout the genome (Zietkiewicz *et al*., 1994). They are simple and reproducible approach based on the amplification of DNA regions between inversely oriented SSRs or microsatellites (Bussell *et al*., 2005). ISSR primers are designed from SSR motifs and can be undertaken for any plant species containing a sufficient number and distribution of SSR motifs in the genome ((Morgante & Olivieri, 1993, Buhulikar *et al*., 2004). Therefore, ISSRs are widely used in many respects such as the study of genetic diversity in barley (Brantestem *et al.*, 2004) and cultivar identification in tobacco (Denduangboripant *et al.*, 2010) and vinca (Shaw *et al.*, 2009). Amplified fragment length polymorphism (AFLP) utilizes fragments of DNA amplified using primers from restriction digested genomic DNA (Vos *et al.*, 1995). It provides the highest levels of resolution to allow delineation of complex genetic structures, to differentiate individuals in a population and in gene flow experiments, and also to register plant varieties (Powell *et al.*, 1996, Law *et al.*, 1998, Barker *et al.*, 1999, Aparajita & Rout, 2010, Misra *et al.*, 2010).

The present work aims at detecting PCR-based molecular markers, i.e., RAPD, ISSR and AFLP, for eight *C. roseus* for color characteristics as possible molecular markers for high levels of TIA, especially VC and VB.

## 2. Materials and Methods

### *Plant material*

The study involved eight cultivars of *C. roseus*, differing in petal color, flower eye and center colors, and petal shape grown naturally in the Mecca region (Table 1 & Figure 1). Detection of different cultivars was based on the petal color and flower eye and center colors as described by Show *et al.* (2009). Flower petal was separated as colorless (white) (a-c) and colored (d-h). Petals of some cultivars overlap (b, c, d, f and g), while the rest non-overlap (a, e and h). Leaf samples of each genotype were collected from plants in three locations (populations). Ten plants of different genotypes were selected in each location based on morphological homogeneity.

### *Genomic DNA extraction and purification*

Extraction of total DNA was performed using the modified procedure of Gawel and Jarret (1991). The minimum number of plants to be bulked for each genotype to saturate polymorphisms within each cultivar subjected to experimentation (data shown upon request). To remove RNA contamination, RNase A (10 mg/ml, Sigma, USA) was added to the DNA samples and incubated at 37$^{o}$C for 30 min. Estimation of the DNA concentration in different samples was done by measuring optical density at 260 nm according to the equation: DNA concentration (ug/ml) = $OD_{260}$ X 50x dilution factor.

**Table 1:** Names, flower petal colors and flower eye and center colors of the eight cultivars of *Catharanthus roseus* (L.) G. Don. Single red line represents cultivars with white flowers, while double blue line represents cultivars with pink flowers.

| Code | Name of cultivar | Petal color | Eye color |
|---|---|---|---|
| *I. Colorless* | | | |
| a. | Patricia White | White | White eye with yellow center |
| b. | First Kiss Polka Dot | Milky white | Red radiating eye with small red center |
| c. | First Kiss Peach | Whitish pink | Pink radiating eye with yellow center |
| *II. Colored* | | | |
| d. | Experimental Rose Pink | Pale pink | Red radiating eye with red center |
| e. | Experimental Deep Pink | Pink | Dark pink radiating eye with pink center |
| f. | Cooler Orchid | Pinkish red | White radiating eye with yellow center |
| g. | Victory Red | Deep red centre | Dark red eye with small red center |
| h. | Blue Pearl | Purple blue | White large radiating base with yellow center |

### *Random amplified polymorphic DNA (RAPD)*

A set of 20 random 10*mer* primers (Operon Technology, USA) from groups A, B, C, O and Z (Table 2) was used in detecting polymorphism among different genotypes, but 14 only were successful in generating reproducible, polymorphic and reliable amplicons. The amplification reaction was carried out in 25-µl reaction volume containing 1x PCR buffer, 4 mM $MgCl_2$, 0.2 mM dNTPs, 21 pmole primer(s), 2 units Taq DNA polymerase and 25 ng template DNA.

**Table 2:** List of random 10*mer* primers (groups A, B, C, O and Z) and their nucleotide sequences used in the present study. Primers succeeded to recover reproducible, polymorphic and reliable amplicons are written in bold, while the rest was written in italics.

| Primer | Sequence (5'–3') | Primer | Sequence (5'–3') |
|---|---|---|---|
| **OP-A04** | **AATCGGGCTG** | **OP-C07** | **GTCCCGACGA** |
| **OP-A09** | **GGGTAACGCC** | *OP-C11* | *AAAGCTGCGG* |
| *OP-A13* | *CAGCACCCAC* | **OP-C14** | **TGCGTGCTTG** |
| **OP-A17** | **GACCGCTTGT** | **OP-C16** | **CACACTCCAG** |
| **OP-B01** | **GTTTCGCTCC** | *OP-C18* | *TGAGTGGGTG* |
| **OP-B02** | **TGATCCCTGG** | **OP-O01** | **GGCACGTAAG** |
| **OP-B03** | **CATCCCCCTG** | *OP-O02* | *ACACACGCTG* |
| **OP-B04** | **GGACTGGAGT** | **OP-O07** | **CAGCACTGAC** |
| **OP-B05** | **TGCGCCCTTC** | **OP-O09** | **TCCCACGCAA** |
| *OP-B07* | *GGTGACGCAG* | *OP-Z04* | *AGGCTGTGCT* |

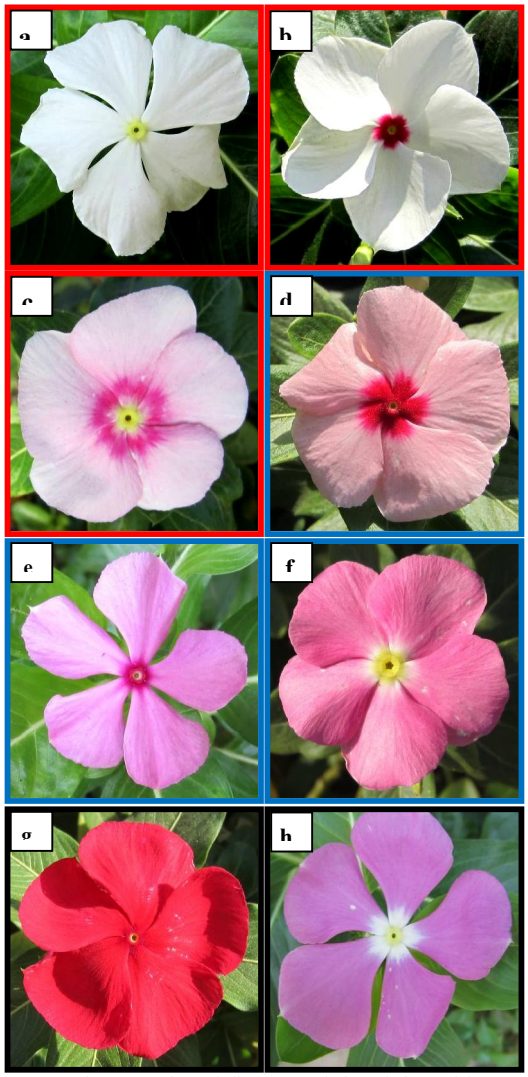


**Figure 1:** Photographs of flowers of the eight cultivars of *Catharanthus roseus* (L.) G. Don. Flower color ranged from colorless or white (a-c, red framed) to pink (d-f, blue framed) and/or colored (d-h). Color of petal eye can be white (a, f and h), pink (c and e) or red (b, d and g). Eye c enter can be yellow (a, c, f and h) or red (b, d and g) or pink (e).

RAPD-PCR amplification was performed in a Perkin Elmer 2400 thermocycler (Germany), programmed to fulfill 40 cycles after an initial denaturation cycle for 4 min at 94°C. Each cycle consisted of a denaturation step at 94°C for 1 min, an annealing step at 37°C for 2 min, and an extension step at 72°C for 2 min, followed by extension cycle for 7 min at 72°C in the final cycle.

***Inter simple sequence repeat (ISSR)***

Seventeen primers for ISSR were successfully used in the present study in generating reproducible and reliable amplicons for different genotypes. Names and sequences of these primers are shown in Table 3. PCR analysis was performed in 25-µl reaction and amplification was programmed to fulfill 40 cycles after an initial denaturation cycle for 4 min at 94°C. Each cycle consisted of a denaturation step at 94°C for 1 min, an annealing step at 40°C for 2 min, and an extension step at 72°C for 2 min, followed by extension cycle for 7 min at 72°C in the final cycle.

***Amplified fragment length polymorphism (AFLP)***

AFLP analysis was performed using the AFLP Analysis System I according to the manufacturer's protocol (Invitrogen, cat. no. 10544-013). Genomic DNA samples were digested with *Eco*RI and *Mse*I restriction enzymes in which *Eco*RI and *Mse*I adapters were ligated to the digested DNA fragments. Pre-amplification was carried out using *Eco*RI primer plus one extension base at the 3' position (A) and *Mse*I primer plus one extension base at the 3' position (C) to amplify fragments that contain complementary sequences. Five combinations of *Eco*RI primers plus three extension bases and *Mse*I primers plus three extension bases were used to selectively amplify the DNA fragments matching the primer-extension sequence, four of them succeeded to recover good quality polymorphic patterns. These four combinations are: M-CCA/E-ACT, M-CAC/E-ACA, M-CAG/E-AAC and M-CTC/E-AAG.

**Table 3:** List of ISSR primers and their nucleotide sequences used in the present study.

| No. | Name | Sequence | No. | Name | Sequence |
|---|---|---|---|---|---|
| 1 | 814 | $(CT)_8TG$ | 10 | HB10 | $(GA)_6CC$ |
| 2 | 844A | $(CT)_8AC$ | 11 | HB11 | $(GT)_6CC$ |
| 3 | 844B | $(CT)_8GC$ | 12 | HB12 | $(CAC)_3GC$ |
| 4 | 17898A | $(CA)_6AC$ | 13 | HB13 | $(GAG)_3GC$ |
| 5 | 17898B | $(CA)_6GT$ | 14 | HB14 | $(CTC)_3GC$ |
| 6 | 17899A | $(CA)_6AG$ | 15 | HB15 | $(GTG)_3GC$ |
| 7 | 17899B | $(CA)_6GG$ | 16 | UCB-820 | $(GT)_8C$ |
| 8 | HB8 | $(GA)_6GG$ | 17 | UCB-827 | $(AC)_8G$ |
| 9 | HB9 | $(GT)_6GG$ | | | |

***Detection of PCR products***

The products of both RAPD and ISSR were detected using electrophoresis on agarose gel (1.2% in 1x TBE buffer), stained with ethidium bromide (0.3 ug/ml), then visually examined with UV transilluminator and photographed using a CCD camera (UVP, UK). AFLP products were detected by capillary electrophoresis and virtual gels were prepared and analyzed. Fragments were separated and sized on an ABI 3500 DNA sequencer (Applied Biosystems, Foster city, California). Using the program Genemapper 4.1 (Applied Biosystems, Foster city, California), a genetic fingerprint was produced for each individual sample by scoring for the presence or absence of a standardized set of markers between 50 and 600 base pairs in size (Rogers, 2008).

***Computer and statistical analysis***

The bands recovered by different techniques were considered reproducible and scorable only after observing and comparing them in three separate amplifications for each primer (data provided upon request). Clear, unambiguous and reproducible bands recovered through different techniques were considered for scoring. Each band was considered a single locus. Data were scored as (1) for the presence and (0) for the absence of a given DNA band. Band size was estimated by comparing with 100-bp ladder (Bioron, Germany) using Gel Works 1D advanced gel documentation system (UVP, UK). The binary data matrices were entered into the TFPGA (Ver. 1.3) and analyzed using qualitative routine to generate similarity coefficient. Dissimilarity coefficients were used to construct a dendrogram using un-weighted pair group method with arithmetic average (UPGMA) and sequential hierarchical and nested clustering (SHAN) routine.

Similarity matrix produced by RAPD, ISSR and AFLP were compared based on the genetic distance of the TFPGA, the normalized Mantel statistic (Mantel, 1967). The PIC (polymorphism information content) was calculated by applying the following formula given by Powell *et al*. (1996) and Smith *et al*. (1997): $PIC = 1 - \sum fi^2$ in which $i = 1 - n$, and $fi$ is the frequency of the $i^{th}$ amplicon. The number of amplicons refers to the number of scored bands. The frequency of an amplicon was obtained by dividing the number of cultivars, where it was found, by the total number of cultivars. The PIC value provides an estimate of the discriminating power of a marker. Marker index (MI) was calculated for each primer or primer combination as the product of PIC and the number of polymorphic bands.

Analysis of molecular variance (AMOVA) is a method of estimating population differentiation directly from molecular data and testing hypotheses about such a differentiation. A variety of molecular marker data (for example, RAPD or AFLP), direct sequence data, or phylogenetic trees may be analyzed using this method (Excoffier *et al*., 1992). AMOVA was performed using GENALEX 6 (genetic analysis in excel, Peakall & Smouse, 2006) in RAPD, ISSR and AFLP to partition the total molecular variance between and within populations.

## 3. Results and Discussion

In this work, the three marker types, namely RAPD, ISSR and AFLP were utilized to analyze eight cultivars of *C. roseus* (see Table 1 & Figure 1). We estimated the optimal number of primers for PAPD and ISSR or primer combinations for AFLP required to discriminate among genomic DNAs of different plant genotypes based on the reproducibility of data and level of polymorphism obtained by each type of molecular analysis (e.g., RAPD, ISSR, AFLP, RFLP, etc.). The argument of the required value of genetic distance to classify correlated plants accessions as distinct cultivars have been raised (Cabrita *et al*., 2001, Papadopoulou *et al*., 2002). In the present study, primers (14 for RAPD, 17 for ISSR and four combinations for AFLP) with informative patterns were selected (samples are shown in Figure 2). Selection of primers was based on the number of amplicons recovered through PCR and the stability (or reproducibility) of the patterns. These primers were used in the characterization of eight genotypes belonging to the *C. roseus* species. Less than 7% intra-plant polymorphism (within) was found across the three types of analyses for the plants of the same genotype (data provided upon request).

**Table 4:** List of cultivar-specific markers of C. *roseus* for different marker types. The table indicates the type and number of markers along with their molecular weights (MW) in bp for different cultivars (a-h). Single red line represents cultivars with white flowers, while double blue line represents cultivars with pink flowers.

| Marker type | Primer | Number (and MW in bp) of cultivar-specific markers | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | a[1] b | c | d | e | f | g | h |
| **RAPD** | A04 | 1 (470) | - | - | - | -- | - | - |
| | A09 | - 1 (240) | - | - | 1 (1440) | - | - | - |
| | A17 | - - | - | - | - | - | - | - |
| | B01 | - 1 (980) | - | - | - | 1 (720) | - | - |
| | B02 | - - | - | 1 (1150) | - | - | 1 (520) | - |
| | B03 | - - | 1 (2080) | - | - | - | - | - |
| | B04 | 2 (880, 1450) | - | -- | | 1 (2220) | -- | |
| | B05 | - - | - | - | - | - | -2 (260, 420) | |
| | C07 | - - | 1 (1480) | - | - | - | - | - |
| | C14 | - 1 (750) | - | - | - | - | - | - |
| | C16 | 1 (780) | - | - | - | - | -- | - |
| | O01 | - - | - | - | - | - | - | - |
| | O07 | - 1 (1720) | - | 1 (660) | - | - | -- | |
| | O09 | - - | - | - | - | - | - | - |
| | **Total** | **4 4** | **2** | **2** | **1** | **2** | **1** | **2 = 18** |
| **ISSR** | 814 | 1 (180) | - | - | - | - | -- | - |
| | 844A | - - | 2 (620, 1780) | - | - | - | - | - |
| | 844B | - 1 (680) | - | - | - | - | - | - |
| | 17898A | - - | - | - | - | - | - | - |
| | 17898B | - - | - | - | - | 3 (440, 480, 980) | - | - |
| | 17899A | - - | - | - | - | 1 (2100) | - | - |
| | 17899B | 1 (420) | - | - | - | - | -- | - |
| | HB8 | - - | 1 (550) | - | - | - | 1 (960)- | |
| | HB9 | 1 (800) | - | - | - | 1 (2050) | -- | - |
| | HB10 | - - | - | 1 (620) | - | - | 1 (440) | - |
| | HB11 | - 1 (250) | - | - | 1 (480) | - | - | - |
| | HB12 | - - | - | - | - | - | -2 (220, 500) | |
| | HB13 | 1 (580) | - | - | - | - | -- | - |
| | HB14 | 1 (1950) | - | - | 2 (450, 1650) | | --- | - |
| | **Total** | **5 2** | **3** | **3** | **2** | **4** | **2** | **2 = 23** |
| **AFLP** | CCA/ACT | 1 (320) | 1 (250) | 1 (370) 400, 440, 520) | 6 (160, 220, 300),-- | | 3 (200, 460, 550) | 1 (280) |
| | CAC/ACA | - 3 (170, 420, 510) | - | 3 (210, 290, 330) | 1 (280) | - | 2 (260, 400) | 1 (360) |
| | CAG/AAC | 2 (240, 380) | -1 (450) | 1 (510) | - | -1 (300) | 3 (170, 190, 290) | |
| | CTC/AAG | 1 (430) | - | -2 (260, 350) | 1 (520)-2 (190, 220) | | 1 (580) | |
| | **Total** | **4 4** | **2** | **12** | **2** | **-** | **8** | **6 = 38** |
| **Total** | **13** | **10** | **7** | **17** | **5** | **6** | **11** | **10= 79** |

[1]See Table 1

**Table 5:** Numbers of cultivar-specific and flower characteristics markers of C. *roseus* across different marker types.

| Marker type | Marker name | marker[a] | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 | **Total** |
| RAPD | A04 | 1 | - | 1 | - | - | - | 2 |
| | A09 | 2 | - | - | - | - | 1 | 3 |
| | A17 | - | 1 | - | - | - | - | 1 |
| | B01 | 2 | - | - | - | 1 | - | 3 |
| | B02 | 2 | 1 | - | - | - | - | 3 |
| | B03 | 1 | - | - | - | - | - | 1 |
| | B04 | 3 | - | - | 1 | - | - | 4 |
| | B05 | 2 | - | - | - | - | - | 2 |
| | C07 | 1 | - | - | 1 | - | - | 2 |
| | C14 | 1 | - | - | - | - | 1 | 2 |
| | C16 | 1 | - | - | - | - | - | 1 |
| | O01 | - | 2 | - | 1 | - | - | 3 |
| | O07 | 2 | - | - | 1 | - | 1 | 4 |
| | O09 | - | - | - | 1 | - | - | 1 |
| | **Total** | **18** | **4** | **1** | **5** | **1** | **3** | **32** |
| ISSR | 814 | 1 | 1 | - | - | - | - | 2 |
| | 844A | 2 | - | 1 | - | - | - | 3 |
| | 844B | 1 | 1 | - | 1 | - | 1 | 4 |
| | 17898A | - | 1 | - | - | - | 1 | 2 |
| | 17898B | 3 | - | - | - | - | - | 3 |
| | 17899A | 1 | - | 1 | 2 | - | - | 4 |
| | 17899B | 1 | - | - | - | 1 | - | 2 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | HB8 | 2 | 1 | - | - | - | - | 3 |
| | HB9 | 2 | - | - | - | 1 | - | 3 |
| | HB10 | 2 | - | - | 2 | - | - | 4 |
| | HB11 | 2 | 1 | - | - | 1 | - | 4 |
| | HB12 | 2 | - | - | - | - | 1 | 3 |
| | HB13 | 1 | - | - | - | - | 1 | 2 |
| | HB14 | 3 | 1 | - | - | - | - | 4 |
| | **Total** | **23** | **6** | **2** | **5** | **3** | **4** | **43** |
| AFLP | CCA/ACT | 13 | 6 | 2 | 4 | 2 | 4 | 31 |
| | CAC/ACA | 10 | 3 | 3 | 1 | 5 | 2 | 24 |
| | CAG/AAC | 8 | 5 | 2 | 1 | 4 | 6 | 26 |
| | CTC/AAG | 7 | 4 | 4 | 5 | 3 | - | 23 |
| | **Total** | **38** | **18** | **11** | **11** | **14** | **12** | **103** |
| **Total** | | **79** | **28** | **14** | **21** | **18** | **19** | **179** |

[a]1: cultivar-specific, 2: white petal, 3: colored petal, 4: pink petal, 5: petal white eye, 6: yellow eye center.

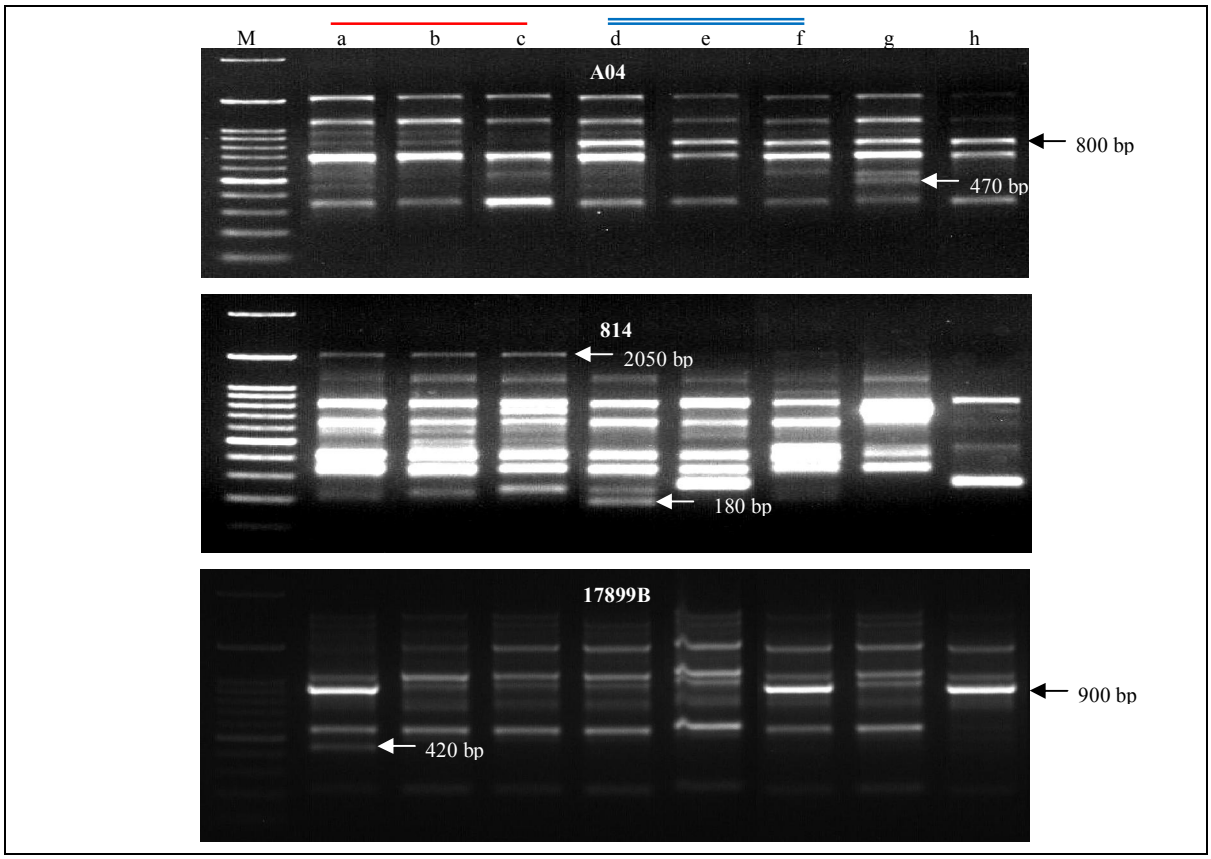


**Figure 2:** Models of different marker profiles including RAPD, ISSR and AFLP of the eight cultivars (a-h, see Table 1). M refers to DNA standard (100-bp ladder, Bioron). RAPD with primer A04 indicates two markers with 470 (cultivar-specific) and 800 bp (for colored petal). ISSR with primer 814 indicates two markers with 180 (cultivar-specific) and 2050 bp (for white petal). ISSR primer 17899B indicates two markers with 420 (cultivar-specific) and 900 bp (petal white eye). AFLP with primer combination M-CCA/E-ACT indicates 31 different markers (see Tables 4 & 5). Single red line represents cultivars with white flowers, while double blue line represents cultivars with pink flowers.

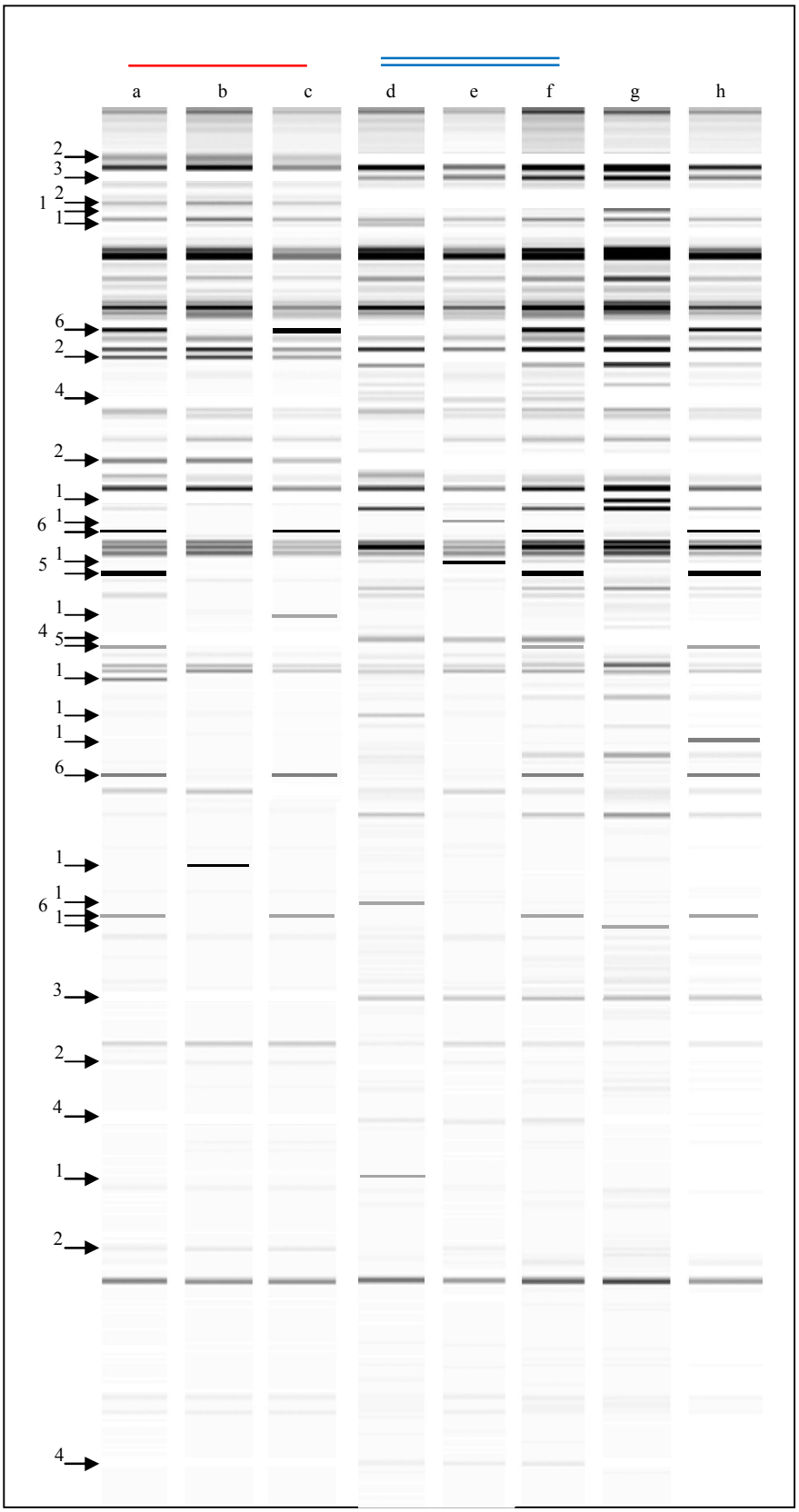


**Figure 2:** continued

**Table 6:** Similarity matrixes based on molecular data for the eight *C. roseus* cultivars (a-h, see Table 1). Single red line represents cultivars with white flower, while double blue line represents cultivars with pink flower.

| Marker type | Cultivar | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | h | g | a | b | c | d | e | f |
| **RAPD** | | | | | | | | |
| h | 1.00 | | | | | | | |
| g | 0.76 | 1.00 | | | | | | |
| a | 0.71 | 0.77 | 1.00 | | | | | |
| b | 0.79 | 0.82 | 0.83 | 1.00 | | | | |
| c | 0.80 | 0.82 | 0.85 | 0.92 | 1.00 | | | |
| d | 0.72 | 0.80 | 0.75 | 0.84 | 0.81 | 1.00 | | |
| e | 0.68 | 0.81 | 0.80 | 0.81 | 0.82 | 0.89 | 1.00 | |
| f | 0.64 | 0.76 | 0.77 | 0.76 | 0.76 | 0.76 | 0.80 | 1.00 |
| **ISSR** | | | | | | | | |
| h | 1.00 | | | | | | | |
| g | 0.87 | 1.00 | | | | | | |
| a | 0.80 | 0.80 | 1.00 | | | | | |
| b | 0.78 | 0.75 | 0.68 | 1.00 | | | | |
| c | 0.74 | 0.74 | 0.79 | 0.69 | 1.00 | | | |
| d | 0.83 | 0.82 | 0.81 | 0.81 | 0.83 | 1.00 | | |
| e | 0.78 | 0.76 | 0.78 | 0.69 | 0.77 | 0.81 | 1.00 | |
| f | 0.79 | 0.79 | 0.79 | 0.77 | 0.81 | 0.85 | 0.82 | 1.00 |
| **AFLP** | | | | | | | | |
| h | 1.00 | | | | | | | |
| g | 0.81 | 1.00 | | | | | | |
| a | 0.76 | 0.74 | 1.00 | | | | | |
| b | 0.76 | 0.80 | 0.80 | 1.00 | | | | |
| c | 0.67 | 0.70 | 0.78 | 0.75 | 1.00 | | | |
| d | 0.79 | 0.75 | 0.77 | 0.76 | 0.75 | 1.00 | | |
| e | 0.82 | 0.72 | 0.71 | 0.73 | 0.68 | 0.81 | 1.00 | |
| f | 0.76 | 0.75 | 0.71 | 0.78 | 0.68 | 0.79 | 0.78 | 1.00 |
| **Overall** | | | | | | | | |
| h | 1.00 | | | | | | | |
| g | 0.81 | 1.00 | | | | | | |
| a | 0.75 | 0.76 | 1.00 | | | | | |
| b | 0.76 | 0.80 | 0.79 | 1.00 | | | | |
| c | 0.71 | 0.73 | 0.79 | 0.78 | 1.00 | | | |
| d | 0.80 | 0.77 | 0.77 | 0.78 | 0.77 | 1.00 | | |
| e | 0.77 | 0.74 | 0.74 | 0.74 | 0.72 | 0.82 | 1.00 | |
| f | 0.75 | 0.76 | 0.73 | 0.78 | 0.72 | 0.79 | 0.79 | 1.00 |

**Figure 3:** Dendrogram based on algorithm of unweighted pair group method with arithmetic averages among cultivars (a-h, see Table 1) within or across type of marker. Single red line represents cultivars with white flowers, while double blue line represents cultivars with pink flowers.

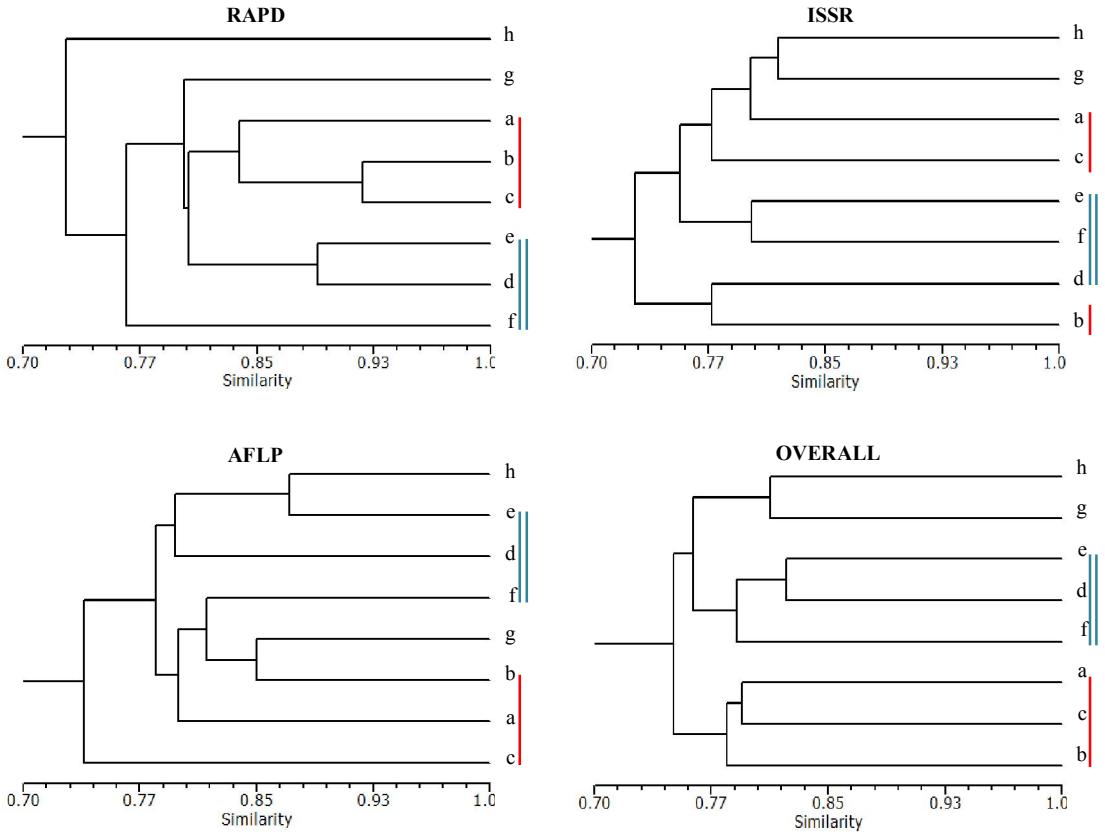


Figure 4. Similarity

**Table 7:** Analysis of molecular variance (AMOVA) of the different *C. roseus* cultivars.

| Source | df[1] | SS[2] | MS[3] | Variance (%) |
|---|---|---|---|---|
| Among Pops | 7 | 1.365 | 0.195 | 1 |
| Within Pops | 704 | 164.225 | 0.233 | 99 |
| Total | 711 | 165.590 | | |

[1]df = Degrees of freedom, [2]SS = Sum of squares, [3]MS = Mean square

**Table 8:** Polymorphism information content (PIC), expected heterozygosity for polymorphic products (He), effective multiplex ratio (E) and the marker index (MI) of each marker type used across *C. roseus* cultivars.

| Marker type | PIC | He | E | MI |
|---|---|---|---|---|
| RAPD | 0.35 | 0.46 | 62 | 28.52 |
| ISSR | 0.37 | 0.49 | 53 | 25.97 |
| AFLP | 0.35 | 0.45 | 236 | 106.2 |

As being dominant markers, pooling (bulk DNA) strategy in ISSR, RAPD and AFLP analyses is thought to be ideal for saturating such an intra-plant polymorphism with no effects on the accuracy of the obtained results. Mengoni *et al*. (2000) indicated that 10% of intra-plant polymorphism, following the procedure of AMOVA (Excoffier *et al*., 1992), is statistically insignificant and acceptable.

***Identification of molecular markers for cultivars and different flower traits***

A high level of polymorphism was generated utilizing the 14 RAPD primers. A total of 672 amplicons, across genotypes and primers, were separated on agarose gel electrophoresis. Of these, 253 bands were polymorphic (38%). The highest number of amplicons was generated for cv. Patricia White (73 amplicons), while cv. Blue Pearl generated the lowest (57 amplicons). The highest number of cultivar-specific markers (18, see Tables 4 & 5), due to the presence of a unique band for a given plant cultivar (positive marker), was scored for cvs. Patricia White and First Kiss Polka Dot (4), while the lowest number was scored for cvs. Experimental Deep Pink and Victory Red (1).

ISSR is a relatively more recent class of molecular markers, which is based on inter tandem repeats of short DNA sequences. Such repeats were proven to be highly polymorphic even among closely-related genotypes due to the lack of functional constraints in these non-functioning DNA regions that was thought to result in the evolutionary changes in their DNA structures. Accordingly, a high level of polymorphism was generated utilizing the 17 ISSR primers. A total of 912 amplicons were

obtained in which 544 of them were polymorphic (60%) and the rest were monomorphic (40%). The highest number of amplicons was generated for cv. Patricia White (85 amplicons), while cv. Blue Pearl generated the lowest (49 amplicons). The highest number of cultivar-specific markers (see Tables 4 & 5) was scored for cv. Patricia White (5), while the lowest number was scored for cvs. First Kiss Polka dot, Experimental Deep Pink, Victory Red and Blue Pearl (2).

Four combinations were used in the AFLP analysis and revealed a total of 1533 amplicons, 981 of them were polymorphic (64%) among the different genotypes. The highest number of amplicons was generated for cv. Patricia White (221 amplicons), while cv. Blue Pearl generated the lowest (176 amplicons). The highest number of cultivar-specific markers (see Tables 4 & 5) was scored for cvs. Experimental Rose Pink (12) and Victory Red (8), while none was scored for Cooler Orchid. In conclusion, the four primer combinations of AFLP used in the present study allowed for the highest rate of distinction, as compared to RAPDs and ISSRs.

The number of cultivar-specific markers scored across cultivars and type of marker was as high as 79 in which 38 of them were generated during AFLP analysis, while 23 for ISSR and 18 for RAPD analyses (Table 5). The highest number of cultivar-specific markers across type of marker was scored for cv. Experimental Rose Pink (17), while the lowest was scored for cv. Experimental Deep Pink (5) (Table 4).

Across the different flower characteristics, a number of 100 markers were generated across type of marker (Table 5). The highest number of markers was scored for white petal trait (28), while the lowest was scored for colored petal trait. The highest number of markers was scored during AFLP analysis (65), while the lowest was scored during RAPD analysis (14).

***Genetic relationships and cluster analysis***

The genetic similarities among the eight cultivars of *C. roseus* species, based on Nei's method (Nei's, 1978), within and across markers are shown in Table 6 and Figure 3. The results of similarity indices and dendrograms within RAPD, ISSR and AFLP data indicated accumulative information towards the complete separation of the cultivars with white flowers (a-c) versus those with pink flowers (d-f). The resulted dendrogram of RAPD data was the closest to that resulted across type of marker. It was obvious that ISSR and AFLP data has diluted these relationships as generated dendrograms have placed the two categories of cultivars with white and pink flowers in three subgroups instead of two. ISSR dendrogram has the advantage of separating the red and blue colored cultivars in a subgroup (Figure 3). The results of genetic relationship between cultivars with white (a-c) and colored (d-h) flowers indicated complete separation across type of marker (Table 6 & Figure 3). The most closely related cultivars were First Kiss Polka Dot (b)/First Kiss Peach (c) for RAPD analysis (similarity index of 0.92), while Victory Red (g)/Blue Pearl (h) for ISSR analysis (similarity index of 0.87), Experimental Deep Pink (e)/Blue Pearl (h) for AFLP analysis (similarity index of 0.82), and Experimental Rose (d)/Experimental Deep Pink (e) across type of marker (similarity index of 0.82). On the other hand, the most genetically distant cultivars were Cooler Orchid (f)/Blue Pearl (h) for RAPD analysis (similarity index of 0.64), while unexpectedly Patricia White (a)/first Kiss Polka Dot (b) for ISSR analysis (similarity index of 0.68), expectedly First Kiss Peach (c)/Blue Pearl (h) for AFLP analysis and across type of marker (similarity index of 0.67 and 0.71, respectively). These results indicate the inadequacy of utilizing ISSR in genetic relationship among *C. roseus* cultivars as it indicated high genetic distance between cultivars with similar flower color (Patricia White (a)/first Kiss Polka Dot (b), Figure 3).

The partitioning of variation within and across *C. roseus* cultivars was studied with the analysis of the Dice's distance matrix by the analysis of molecular variance (AMOVA) approach. A hierarchical analysis of genetic diversity using a two-way nested AMOVA was performed. Results from AMOVA within and among population are shown in Table 7. Data indicated that 99% of the genetic variation is attributed to differences among populations, while only 1% of the genetic variation is attributed to differences within populations. The values of MS indicated the high level of polymorphism among genotypes and the low level of experimental error. This reflects the homogeneity in leaf samples collected for the study as a perfect representative of the target genotypes. The polymorphism information content (PIC), average of heterozygosity ($H_e$), the effective multiplex ratio (E), and the marker index (MI) were computed for each assay based on experimental data (Table 8). ISSR revealed the highest PCI and $H_e$ (0.37 and 0.49, respectively) as compared to AFLP (0.35 and 0.45, respectively), then RAPD (0.35 and 0.46, respectively). The obtained results agreed with these of Powell *et al*. (1996). Muzher (2005) found that $H_e$ of RAPD was more than AFLP. However, the results of ISSR data analysis indicated the inadequacy in utilizing this type of marker in detecting genetic relatedness among *C. roseus* cultivars. With regard to the E value and MI, ISSR indicated the lowest records (53 and 25.97, respectively), while AFLP (236 and 106.2, respectively) and RAPD (62 and 28.52, respectively) indicated higher values. In general, the results of ISSR can be considered less reliable than AFLP or RAPD. Reliability of ISSR can be improved if more primers were used in characterizing cultivars.

It could be concluded that markers differ in their ability to differentiate individuals, in the mechanism of detecting polymorphism, genome

coverage, and the ease of application. They can be complementary to each other, as it is the case in the present study, depending on technical availability. Some of these markers can be linked to flower characteristics in *C. roseus* as well as to the levels of the anticancer compounds VC and VB. There are recent efforts towards breeding genotypes with high TIAs levels via MAS (Dekkers & Hospital, 2002, Lorz & Wenzel, 2005, Sharma *et al.*, 2012) that can be duplicated. The versatility and continuous flowering habit of *C. roseus* have enhanced breeding efforts to expand the availability of flower colors to detect economic traits (van der Heijden *et al*., 2004). Although a little is known about the effects of breeding for flower color on the levels of MIAs, there are few articles indicating that colored flower is linked to the high level of MIAs (Sharma *et al.*, 2012). For example, the accession 'Pink Delhi' has pink-colored flower petals, less salt and drought tolerance and high levels of MIAs, while accession gsr8 has white-colored flower petals, more salt and drought tolerance and low levels of MIAs. These observations were recorded in many recent works (ex., Gupta *et al*., 2007, Chaudhary *et al*., 2011, Sharma *et al.*, 2012).

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