BIOLOGIA PLANTARUM 50 (2): 295-299, 2006

BRIEF COMMUNICATION

Optimization of primer screening for evaluation of genetic relationship in rose cultivars

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Abstract

Optimization of primer screening for evaluation of genetic relationship in 34 cultivars of rose through random amplified polymorphic DNA (RAPD) markers was investigated. Four series of decamer primers were used for screening and optimization of RAPD analysis between which A and N series performed good amplification of fragments as compared with other series. The primers OPN-07 and OPN-15 produced maximum number of DNA fragments in *Rosa hybrida* cv. Anuraag. Some primer either did not produce amplification or produced very poor amplification. Further, ten selected primers were used for genetic analysis of 34 rose cultivars. The primer OPN-15 amplified 21 fragments in all cultivars tested. A total of 162 distinct DNA fragments (bands) ranging from 100 to 3400 base pairs were amplified by using 10 selected random primers. The cluster analysis indicated that these rose cultivars formed nine clusters.

Additional key words: polymerase chain reaction, random amplified polymorphic DNA markers, *Rosa hybrida.*

Random amplified polymorphic DNA technique has been widely used in many plant species for cultivar analysis, population studies and genetic linkage mapping (Willams *et al*. 1990, Yu *et al*. 1993, Debener *et al*. 1996, Rout *et al*. 2003). Optimization of the RAPD method depends on selection of primers. Although, the RAPD method uses arbitrary primer sequences, many of these primers must be screened in order to select primers that provide useful amplification product. The amplification and the type of reaction buffer on RAPD profiling of various species have been reported (Levi *et al*. 1993, Samal *et al*. 2003, Das née Pal and Raychaudhuri 2003/4, Betal *et al*. 2004, Grilli Caiola *et al*. 2004). Few reports are available on the use of high G + C percentage in primers used in RAPD analysis in cassava and *Nepenthes* (Wong *et al*. 1997, Lim *et al*. 2000).

The genus *Rosa* is large and complex, comprising of wild and domesticated species, grouped on the basis of morphological characters. Hybridization and allopolyploidization have occurred frequently in this genus and hence the classification and the search for relationships between species and cultivars are complicated (Zhang and Gandelin 2003). There are very few reports on molecular characterization of rose plants (Rajapakse *et al*. 1992, Ben-Meir and Vainstein 1994, Debener *et al*. 1996, Millian *et al*. 1996). In this communication, we report the optimization of primer screening and evaluation of genetic relationship in 34 rose cultivars through RAPD markers.

Fresh leaves of *Rosa hybrida* cvs. Paradise, Lover`s Meeting, Lal Bahadur, Confidence, Sadabahar, Arunima, Montezuma, Mrinalini, Charisma, Don Don, Queen Elizabeth, Jantar Mantar, Love, Indian Princess, Shocking Blue, Super Star, Papa Meilland, Taj Mahal, Raktagandha, Christian Dior, Crimson Glory, First Prize, Anuraag, Italy Famous, Chandrama, Sofia Loren, Banjaran, Tata Centenary, Gold Medal, Dr. John Snow, Landora, My Valentine, Echo and B.P. Pal were collected in an ice box from the greenhouse of the Regional Plant Resource Centre, Bhubaneswar, Orissa, India.

The leaves (2 g) were ground to a fine powder in liquid nitrogen in a mortar using a pestle. The fine powder was resuspended in 10 cm^3 of preheated DNA extraction buffer [1 M boric acid (pH 8.0), 2 mM EDTA, 1.4 M $NaCl_2$, 1.5 % hexadecyltrimethylammonium bromide (CTAB), 0.2 % β-mercaptoethanol (v/v)]. The

Received 21 May 2004, *accepted* 12 January 2005.
Abbreviations: PCR - polymerase chain reaction; RAPD - random amplified polymorphic DNA.
Acknowledgement: The authors wish to acknowledge to Department of Forest and Environment, Government of Orissa for necessary facilities.
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mixtures were subsequently heated at 60 °C for 3 h. This was followed by addition of 10 cm^3 of chloroform and centrifugation at 8 000 *g* for 20 min (*Kubata KR-2000 C*, Tokyo, Japan). After centrifugation, DNA was precipited with 5 M NaCl and absolute ethanol. The crude DNA pellet was resuspened in 1 cm^3 of 10 mM Tris, pH 8.0, and 0.1 mM EDTA (TE) buffer. Subsequently, it was treated with 0.003 cm^3 RNase (10 mg cm^{-3}) and incubated for one hour at 37 °C. DNA purification was made through clean *Genei* kit (*M/S Bangalore Genei*, Bangalore, India). The DNA was washed in 70 % ethanol, air dried and resuspended in 0.05 cm^3 of TE buffer. DNA quantification was performed by UV-Spectrophotometer (*UV/DEC-650, JASCO*, Tokyo, Japan) and visualized under UV light, after electrophoresis on 0.8 % (m/v) agarose gel and compared with λ-DNA digested by Hind-III as marker. The resuspended DNA was then diluted in TE buffer to concentration 5 mg cm^{-3} for use in PCR amplification.

A set of 40 random decamer oligonucleotides (series A, B, D and N) purchased from *Operon Technologies Inc*. (Alameda, USA) was used as primers for the amplification of RAPD fragments. Polymerase chain reactions (PCR) were carried out in a final volume of 0.025 cm^3 containing 20 ng template DNA, 125 μM each deoxinucleotide triphosphate, 15 ng of decanucleotide

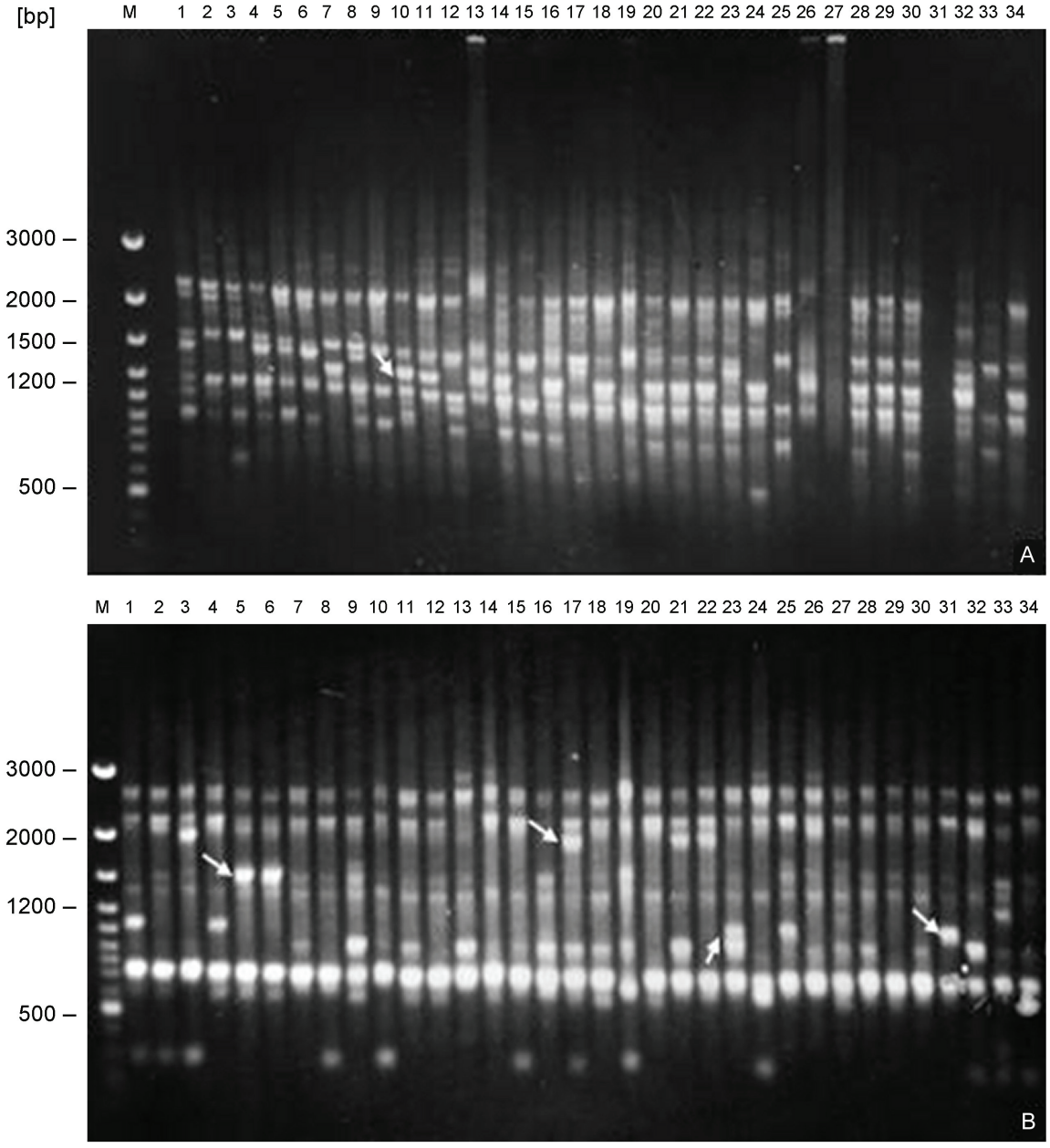

Fig. 1. RAPD patterns of 34 cultivars of *Rosa hybrida* generated by the primer OPN-07 (A) and OPN-15 (B). M - molecular mass ladder; 1 - 34 hybrid culticars.

primers (*M/S Operon Technology Inc.*), 1.5 mM $MgCl_2$, 1× Taq buffer (10 mM Tris-HCl, pH 9.0, 50 mM KCl, 0.01 % (m/v) gelatin) and 0.5 U Taq DNA polymerase (*M/S Bangalore Genei*). DNA amplification was performed in a PTC 100 thermal cycler (*M.J. Research*, Watertown, USA) programmed for 45 cycles: 1st cycle of 3.5 min at 94 °C, 1 min at 37 °C and 2 min at 72 °C; then 44 cycles each of 1 min. at 94 °C, 1 min at 37 °C and 2 min at 72 °C followed by one final extension cycle of 7 min at 72 °C. Amplified products were separated alongside a molecular mass marker (1kb ladder, *MBI Fermentas Inc*., Amherst, USA) by electrophoresis on 1.2 % (m/v) agarose gels run in 1× TAE (Tris acetate EDTA) buffer, stained with ethidium bromide and visualized under UV light. Gel photographs were scanned through *Gel Doc System 2000* (*Biorad*, Hercules, USA). All reactions were repeated at least two times.

Data were recorded as presence (1) or absence (0) of bands from the examination of photographic negatives. Each amplification fragment was named by the source of primer (*Operon, Advanced Biotechnologies Inc.*), the kit letter, the primer number and its approximate size in base pairs. Bands with similar mobility to those detected in the negative control, if any, were not scored. Similarity indexes were estimated using the Dice Coefficient of similarity (Nei and Li 1979). Cluster analysis was carried out on similarity estimates using the unweighted pair-group method of arithmetic average (UPGMA) using *NTSYS-PC* version *1.80* (Rohlf 1995).

The present investigation offers a optimization of primer screening for evaluation of genetic relationship of 34 rose cultivars through RAPD analysis. Cultivar Anuraag was used for screening primers (derived from A, B, D and N series) obtained from different series for amplification by using polymerase chain reactions. The results showed that A and N series primers produced relatively more amplification fragments as compared to B and D series (data not shown). The amplification generated by primers OPB-07, OPB-12 and OPD-11 produced least number of fragments by using the cv. Anuraag. The primers OPN-07 and OPN-15 produced maximum number of DNA fragments; the size of the DNA fragments ranged from 200 to 3000 bp. Primer OPN-07 amplified 12 fragments whereas OPN-15 produced 14 bands. It was also noted that some decamer primers did not show any amplification by using this cultivar (data not shown). The number of fragments varied from one series of primers to other series. The twenty decamer primers produced good amplification of RAPD fragments ranging from 200 to 3200 bp (Table 1). Subsequently, ten decamer primers were selected and used to analyze the genetic relationship among 34 rose cultivars through polymerase chain reaction. The RAPD analysis showed that 162 consistent RAPD markers were produced with ten selected primers involving 34 cultivars, ranging in size from 100 to 3400 bp; of these, 76 bands were monomorphic and rest was polymorphic (Table 2). Thirty four cultivars of *Rosa hybrida* with two selected primers (OPN-05 and OPN-15) showed different amplification pattern (Fig. 1*A*,*B*). Most of the cultivars produced more than 10 amplified DNA fragments by using primer OPN-05 and OPN-15.

Table 1. Primers used for PCR amplification of *Rosa hybrida* cv. Anuraag.

Name of primer	Sequence (5-3)	Number of bands	Size range of bands [bp]
OPA-02	5`-TGCCGAGCTC-3`	08	500 - 3000
OPA-04	5`-AATCGGGCTG-3`	10	650 - 2400
OPA-08	5`-GTGACGTAGG-3`	07	750 - 1850
OPA-15	5`-TTCCGAACCC -3`	11	400 - 1200
OPA-20	5`-GTTGCGATCC -3`	10	600 - 2900
OPB-06	5`-TGCTCTGCCC-3`	08	200 - 2200
OPB-07	5`-GGTGACGCAG-3`	06	550 - 2150
OPB-10	5`-CTGCTGGGAC-3`	09	400 - 1900
OPB-12	5`-CCTTGACGCA-3`	06	500 - 2300
OPD-02	5`-GGACCCAACC-3`	08	350 - 2600
OPD-03	5`-GTCGCCGTCA-3`	08	400 - 2800
OPD-05	5`-TGAGCGGACA-3`	10	550 - 1800
OPD-08	5`-GTGTGCCCCA-3`	07	450 - 1750
OPD-11	5`-AGCGCCATTG-3`	06	600 - 3000
OPN-02	5`-ACCAGGGGCA-3`	10	500 - 2300
OPN-05	5`-ACTGAACGCC-3`	08	300 - 2550
OPN-07	5`-CAGCCCAGAG-3`	12	500 - 3200
OPN-08	5`-ACCTCAGCTC-3`	10	450 - 2700
OPN-10	5`-ACAACTGGCG-3`	08	650 - 2500
OPN-15	5`-CAGCGACTGT-3`	14	200 - 2800

Table 2. Total number of amplified fragments generated by polymerase chain reaction using selected random 10-mer primers with 34 cultivars of rose.

Name of primer	Sequence of the primer (5 - 3)	Number of amplification products	Size range [bp]
OPA-02	5´-TGCCGAGCTG-3´	16	300 - 2800
OPD-02	5´-GGACCCAACC-3´	16	500 - 3000
OPD-03	5´-GTCGCCGTCA-3´	14	300 - 2200
OPD-05	5´-TGAGCGGACA-3´	17	400 - 2400
OPD-08	5´-GTGTGCCCCA-3´	15	200 - 2600
OPD-11	5´-AGCGCCATTG-3´	12	400 - 2900
OPN-02	5´-ACCAGGGGCA-3´	17	100 - 2700
OPN-07	5´-CAGCCCAGAG-3´	18	500 - 3400
OPN-10	5´-ACAACTGGGG-3´	16	200 - 3000
OPN-15	5´-CAGCGACTGT-3´	21	100 - 3000

The present findings showed close genetic variation among the 34 hybrid rose cultivars. The similarity matrix obtained after multivariate analysis using Nei and Li`s coefficient was used to construct a dendrogram with the UPGMA method (Fig. 2). The dendrogram shows nine clusters within these cultivars. There is narrow variation within the hybrid cultivars. Our results showed that the distances among the cultivars were not significantly

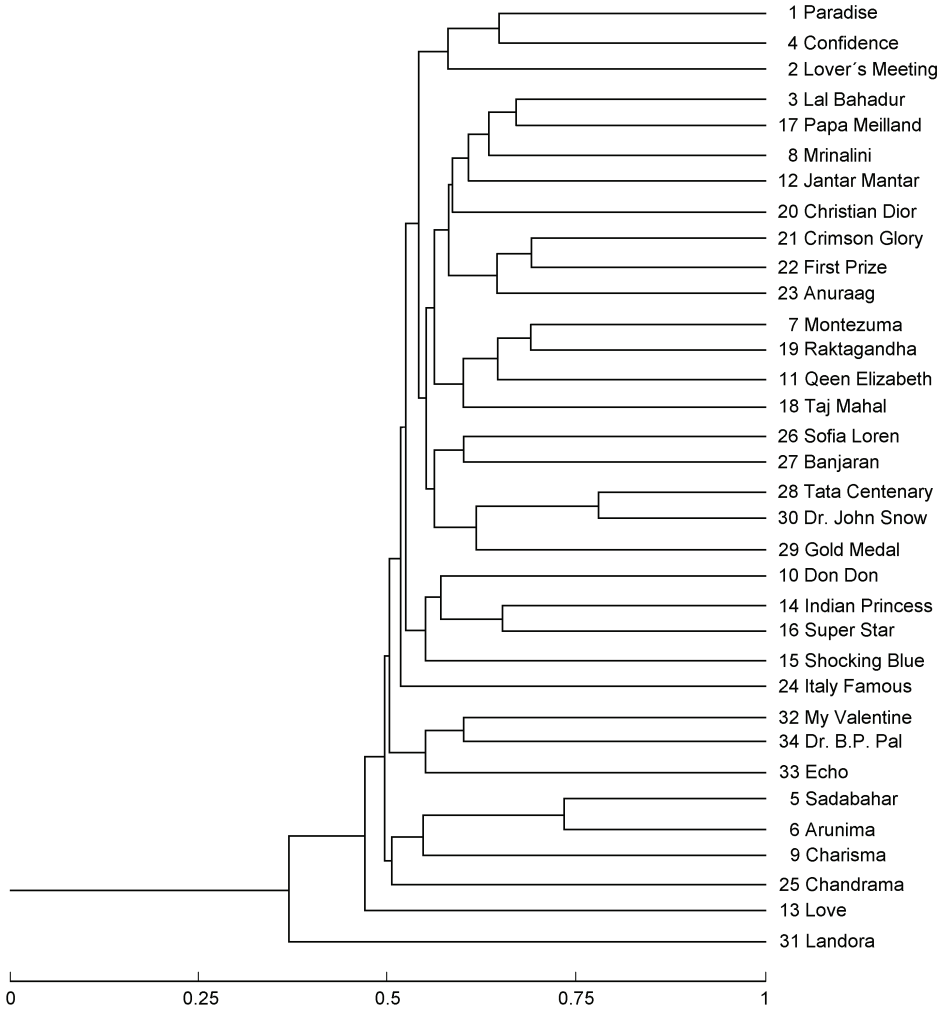

Fig. 2. Dendrogram of cluster analysis of RAPD markers. The scale indicates the fractional similarities among cultivars.

different from those within the cultivars. Among the nine clusters formed, one cluster had eight cultivars (Lal Bhadur, Mrinalini, Jantar Mantar, Papa Meilland, Christian Dior, Crimson Glory, First Prize and Anuraag), three clusters had five cultivars each, one cluster had four cultivars, two clusters had three cultivars each and two clusters had one cultivar each. The genetic distance indicated that the cultivar Tata Centenary has 81 % similarity with Dr. John Snow. Sadabahar has 72 % similarity with Arunima. The cvs. Landora and Love have 37 % and 50 % similarity with the 34 cultivars, respectively. Indian Princess and Super Star have 65 % similarity among themselves. Our findings are consistent with the findings of Vainstein *et al.* (1993) and Ben-Meir and Vainstein (1994). Vainstein *et al*. (1993) reported that the genetic similarities were small within the cultivated rose groups (hybrid tea, floribunda, polyantha and miniature) by using 28 DNA fragments from micro satellite fingerprints. The optimization of primer screening for RAPD analysis has been used for the analysis of diversity and identification of duplicates within the large germplasm collection (Virk *et al.* 1995), identification of varieties/species (Prince *et al.* 1995), phylogenetic relationship (Nair *et al.* 1999) and conservation and management of genetic resources (Bretting and Widrelechner 1995). Thus, this investigation is an understanding of the level and partitioning of genetic variation within the cultivars and would provide an important input into determining appropriate management strategies and also future breeding program in roses.

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