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Analysis of genetic relationships of Indian grape genotypes using RAPD markers

A.K. Singh[*] and Rakesh Singh[**]
Division of Fruits and Horticultural Technology, Indian Agricultural Research Institute, New Delhi 110 012

ABSTRACT

The 34 grapevine genotypes grown in India were studied for the genetic relatedness using RAPD, markers. Out of 120 primers tested, 31 were found polymorphic for all the genotypes. The molecular diversity of these genotypes was estimated based on 205 RAPD bands using Jaccard's and Dice's similarity coefficients. The two diversity indices for RAPD marker system depicted a high level of correlation (>99%). The resolving power (Rp) and polymorphism information content (PIC) was found highest for primers OPB-15 (16.7) and OPG-15 (9.17), respectively. The UPGMA dendrogram obtained from the cluster analysis using Jaccard's similarity matrix showed two clusters at 35% genetic similarity. In the dendrogram, all the seedless varieties clustered together and other grouped at different similarity levels that suggest the development of seedless grapevine cultivars from related ancestor(s). The RAPD primers OPG-7, OPB-12, OPB-3 and OPB-4 amplified a unique band for Hybrid 72-51 (Angoor Kalan x Pusa Seedless) whereas, OPW-19 and OPB-4 amplified unique band for Bharat Early and Julesky Muscat, respectively. The Mantel's test between the Dice's and Jaccard's similarity matrices provided r = 0.99518 (t value = 8.45) for RAPD, suggesting very good correlation between the two similarity indices. The analysis with RAPD markers provides a quick, reliable and highly informative system to establish genetic relationship among grape genotypes.

Key words: *Vitis*, RAPD, genetic relationship, diversity, Indian genotypes.

INTRODUCTION

Grapevine (*Vitis vinifera* L.) is the most economically important fruit crop in the world with a long history of domestication being first reported in Occidental Europe in the Tertiary-Quarternary transition (Levadoux, 11). Contrary to other fruit crops grapevine is not only used as a fruit in its multiple uses (fresh fruit, fruit juice, dried fruit etc.), but it is the basis for the production of high-value added products such as wine and spirits. It is a diploid plant and can be easily crossed and selfed. It has a small genome of approximately 500 Mbp, equivalent to four times the genome size of *Arabidopsis*, *i.e.*, 125 Mb (AGI, 1) and has a number of unique features including a novel shoot architecture and non-climacteric fleshy fruit produced from a perennial deciduous woody vine (www.viteacae.org). The fruit also has unique secondary metabolism producing colour pigments, tannins, flavour and aroma compounds. Cultivars are propagated vegetatively and therefore each elite cultivar represents a unique, usually highly heterozygous genotype.

Vitis has vast genetic diversity in the form of species, rootstocks and cultivars. India has emerged as a major producer of grape with the total annual production of about 1.8 million tonnes and has the productivity of about 23.6 tonnes per hectare, which is the highest in the world (NHB, 15). Grapevine varieties have been characterized for identification purposes by ampelographic characters (Viala and Vermorel, 22), isoenzyme (Arulsekar and Parfitt, 2; Benin *et al*., 3) and DNA markers (Gogorcena *et al*., 7; Vidal *et al*., 23; Herrera *et al*., 9). The new techniques for DNA analysis allow obtaining an unlimited number of polymorphic markers for genetic relationship studies. With this objective several DNA studies on grapevine have been carried out by using RFLP (Bourquin *et al*., 4), RAPD (Grando *et al*., 8; Herrera *et al*., 9), AFLP (Sensi *et al*., 19), ISSR (Herrera *et al*., 9) and SSR (Bowers and Meredith, 5).

There are two widely used PCR-based techniques that are both cheap and generate polymorphism of value for genetic distance analysis. In the first, RAPD (Random Amplified Polymorphic DNA), a ten base long decamer of random sequence is used as a primer for PCR. Several studies with RAPD markers on genetic relationship have been reported in grapevine. The present study aimed to study diversity and the genetic relationship among a collection of Indian grape cultivars with RAPD marker systems.

MATERIALS AND METHODS

The 34 grapevine genotypes used in the study were sampled from the Field Genebank of IARI,

*Corresponding author's E-mail : aksingh36@yahoo.com
**National Research Centre on DNA Fingerprinting, National Bureau of Plant Genetic Resources, New Delhi

New Delhi. These genotypes comprised of exotic and indigenous collections from different sources (Table 1) and inter-varietal hybrids developed at IARI. Young leaves were collected and stored at -80°C until total genomic DNA was extracted using a modification of the cetyl-trimethyl ammonium bromide (CTAB) protocol described by Doyle and Doyle (6), as modified by Steenkamp *et al*. (21). Frozen tissue (2-3 g) was grounded with mortar-pestle in liquid nitrogen and homogenized in 15 ml of preheated (65°C) DNA extraction buffer containing 100 mM Tris-HCl (pH 8.0); 1.4 M NaCl; 20 mM EDTA (pH 8.0); CTAB (20% w/v) and mercaptoethanol (0.2% v/v) with addition a 50 mg polyvinyl pyrollidone (PVP).

The mixture was incubated for 45 min. at 65^{0}C with occasional mixing with gentle swirling. DNA was extracted with an equal volume of chloroform: isoamyl alcohol (24:1 v/v) and precipitated with 1 volume of cold isopropanol. The precipitated DNA was centrifuged and washed with 70% ethanol for 5 min., dried at room temperature and re-suspended in 500 µl Tris-EDTA pH 8.0 (1 M Tris and 0.5 M EDTA). The genomic DNA was treated with RNase to avoid RNA contamination. The DNA was further purified using phenol: chloroform: isoamyl alcohol (25:24:1) and re-precipitated with isopropanol. The DNA was re-suspended in 200 µl Tris-EDTA buffer and quantification was carried out by using 'Hoefer's Dyna quant' (Pharamacia Biotech, USA) and diluted in autoclaved double-distilled water to 5 ng/µl.

A total of 120 RAPD primers (A, B, C, D, E, and F series) from Operon Technology (Alameda, California) were employed on three cultivars with distinctive differences in morphology to screen the polymorphism. Based on their ability to generate polymorphic and reproducible profiles, 31 RAPD primers were selected for the subsequent analyses (Table 2). Polymerase chain reaction amplification for RAPD was performed in a 25 µl reaction volume, containing 1× reaction buffer (10 mM TrisHCl pH 8), 1.5 mM $MgCl_2$, 200 µM of each dNTP, 1 u of *Taq*polymerase, 30 ng of primer and approx. 25 ng genomic DNA template. PCR amplification was carried out using thermal cycler (Perkin Elmer 9600), which was programmed for an initial step of 5 min. at 94°C, followed by 40 cycles of 1 min. at 94°C, 1 min. at 37°C, and 2 min. at 72°C. A final extension step at 72°C was programmed for 7 min. and product was stored at 4°C until loading. PCR products were separated on 1.4% agarose gel and then stained with ethidium bromide (1×), products were visualized and photographed with gel documentation system (Flourchem™ 5500, Alpha Innotech, USA).

The band profiles of each gel of RAPD were scored visually. The consensus profiles were recorded on the basis of presence (1) or absence (0) of bands and binary qualitative data matrices was constructed. Data analysis was performed using the NTSYS-pc (Numerical Taxonomic System, Rohlf, 17) version 2.1 programme. Among the various similarity indices those of Jaccard and Dice were chosen as the most appropriate ones for dominant markers, like RAPD, since they do not attribute any genetic meaning to the coincidence of band absence. The similarities are calculated as follows:

Dice $= 2N_{AB} /(2N_{AB} + N_A + N_B)$

Jaccard $= N_{AB} /(N_{AB} + N_A + N_B)$

Where, N_{AB} is the number of bands shared by samples, N_A represents amplified fragments in sample A and N_B represents fragments in sample B.

Correlation between two indices was estimated by means of the Mantel's matrix correspondence test (Mantel, 13) using 1000 random permutations. The rationale of the test is the displacement of the quantity: $Z_{OBS} = \sum_{i<j} X_{ij} Y_{ij}$, where X_{ij} and Y_{ij} are the off-diagonal elements of the matrices X and Y to be compared, with respect to the random variate Z_{rdm} calculated after random permutations of rows or columns in X or Y; lower is the frequency of $Z_{rdm} \geq Z_{OBS,}$ higher is the correlation 'r' between X and Y. The degree of fit can be interpreted as follows: $r \geq 0.9$, very good fit; $0.8 \leq r \geq 0.9$, good fit; $0.7 \leq r \geq 0.8$, poor fit; $r \leq 0.7$, very poor fit. Cluster analysis of the similarity matrices was performed and the result summarized as unweighted pair group method for arithmetic average (UPGMA) using SAHN function.

Resolving power is based on distribution of alleles within the sampled genotypes. For most molecular fingerprinting systems, the division of genotypes into two groups is based on the presence or absence of a band at a particular position. Ideally, each band position would therefore be present in half of the genotypes and absent from the other half. The value of a particular band position can be measured simply then by its similarity to the optimal conditions (50% of genotypes containing the band). This 'band in formativeness' (I_b) can be represented into a 0-1 scale by the formula: $I_b = 1- \{2 \times (0.5-p)\}$, where, p being the proportion of the genotypes containing the band (Prevost and Wilkinson, 15). The I_b value was calculated for all the informative bands for 31 RAPD primers that were scored in the study. If all bands were optimally informative, then the most useful primer would be those that generated the most band positions. Given that bands can be weighted according to their similarity to optimal informativeness, the ability of a primer distinguish between large numbers of genotypes, *i.e.*, Resolving Power of the primer (Rp) could be represented by the sum of these adjusted values. This can be described as: $Rp = \sum$ Ib. The Rp of 31 RAPD primers were determined in this way.

Table 1. List of grapevine genotypes used for RAPD analysis along with their source.

Code	Genotype	Source	Remarks
1	Hybrid 70-56	IARI, New Delhi	Hur x Beauty Seedless
2	Hybrid 72-51	IARI, New Delhi	Angoor Kalan (AK) x Pusa Seedless (PS)
3	New Perlette	Maharashtra	Seedless
4	Hybrid 74-4	IARI, New Delhi	Banqui Abyad (BA) x Perlette (P)
5	Victory	Unknown Source	Seeded
6	Pusa Urvashi	IARI, New Delhi	Towards Seedless
7	Hybrid 76-3	IARI, New Delhi	Madeleine Angevine (MA) x Ruby Red (RR)
8	Madeleine Angevine	Yuogoslavia	Seeded
9	Hur	Hyderabad	Seeded, Reflexed stamen
10	Hybrid 144	Hyderabad	Cheema Sahibi x Catwaba
11	Gold	Unknown Source	Seeded
12	Hybrid 76-1	IARI, New Delhi	Hur x Cardinal
13	Hybrid 71-35	IARI, New Delhi	Banqui-Abyad (BA) x Beauty Seedless (BS)
14	Beauty Seedless	California	Seedless
15	Perlette	California	Seedless
16	Black Prince	Hyderabad	Seeded
17	Anab-e-Shahi	Aurangabad, Hyderabad	Seeded
18	Hybrid 75-9	IARI, New Delhi	Kata Khurgan x Gold
19	Alumwick	Aurangabad, Hyderabad	Seeded
20	Kishmish Beli	Hyderabad	Seedless, Introduction from USSR, Best for raisin making
21	Hybrid 74-6	IARI, New Delhi	Hur x Gold
22	Banqui-Abyad	Aurangabad	Seeded, Reflexed stamen
23	Pusa Seedless	IARI, New Delhi	Seedless
24	Tas-a-Ganesh	Aurangabad	Seedless
25	Black Muscat	Aurangabad	Seeded
26	Fakhri	Hyderabad	Seeded
27	Haitha	Unknown Source	Seeded
28	Centennial Seedless	USA	Seedless
29	Bharat Early	Hyderabad	Seeded
30	Julesky Muscat	Unknown Source	Seeded
31	Cardinal	Italy	Seeded, A hybrid-seeded Tokay x Ribier USA
32	Hybrid 75-32	IARI, New Delhi	Banqui Abyad x Perlette
33	Angoor Kalan	Hyderabad	Seeded
34	Flame Seedless	USA	Seedless

The basic information that determines their application in genetic mapping of both the marker system was calculated for each marker by using the PIC (Lynch and Walsh, 12). PIC expresses the discriminating power of the locus by taking into account not only the number of alleles that are expressed, but also their relative frequencies and frequency of alleles per locus, expressed as: $PIC = 1- \sum p_i^2$, where p_i is the frequency of the ith allele.

RESULTS AND DISCUSSION

Among the primers tested, all the RAPD primers except OPA-20 showed 100% polymorphism. Total number of amplified bands, number of polymorphic bands and the bands shared among the 34 genotypes were recorded. The genotypes specific bands are listed in Table 2. These specific bands will prove to be useful for varietal identification and purification in case of any physical mixture or mis-numeration. Resolving power

Table 2. RAPD primer sequence and their properties.

Primer	5' ---------- 3'	TNB	NPB	P %	Unique genotype(s) identified	PIC	Rp	Size range (bp)
OPX-15	5'-CAGACAAGCC-3'	9	9	100	-	6.159	9.176	400-1800
OPG-2	5'-GGCACTGAGG-3'	6	6	100	-	4.425	5.764	250-2000
OPG-5	5'-CTGACGTCAC-3'	11	11	100	-	9.173	13.704	300-1900
OPW-19	5'-CTGAGACGGA-3'	9	9	100	Bharat Early	6.625	8.058	250-2600
OPA-2	5'-CAAAGCGCTC-3'	8	8	100	-	5.699	7.588	400-3000
OPG-9	5'-TGCCGAGCTG-3'	4	4	100	-	3.384	3.000	250-1200
OPN-19	5'-GTCCGTACTG-3'	2	2	100	-	1.538	1.704	1000-1800
OPW-5	5'-GGCGGATAAG-3'	5	5	100	-	4.683	2.116	250-2200
OPK-6	5'-CACCTTTCCC-3'	4	4	100	-	1.737	5.704	300-2000
OPG-11	5'-TGCCCGTCGT-3'	5	5	100	-	4.278	3.410	350-2500
OPG-7	5'-GAACCTGCGG-3'	9	9	100	Hybrid 72-51	6.667	8.352	600-3000
OPW-8	5'-GACTGCCTCT-3'	3	3	100	-	2.138	2.940	1000-1800
OPB-12	5'-CCTTGACGCA-3'	7	7	100	Hybrid 72-51	5.06	5.764	800-2500
OPP-5	5'-CCCCGGTAAC-3'	5	5	100	-	4.294	3.528	300-2500
OPW-2	5'-ACCCCGCCAA-3'	5	5	100	-	3.669	4.840	600-1500
OPK-7	5'-AGCGAGCAAG-3'	7	7	100	-	4.265	8.156	400-2200
OPA-20	5'-GTTGCGATCC-3'	9	8	88.89	-	5.724	9.646	250-2750
OPA-15	5'-TTCCGAACCC-3'	2	2	100	-	0.823	3.058	300-600
OPG-18	5'-GGCTCATGTG-3'	5	5	100	-	3.106	5.470	500-1500
OPK-8	5'-GAACACTGGG-3'	6	6	100	-	3.303	7.176	250-1700
OPL-14	5'-GTGACAGGCT-3'	5	5	100	-	2.540	6.882	800-2000
OPK-19	5'-CACAGGCGGA-3'	8	8	100	-	6.647	6.058	400-2600
OPF-7	5'-CCGATATCCC-3'	5	5	100	-	3.322	5.352	450-2250
OPY-6	5'-AAGGCTCACC-3'	5	5	100	-	4.138	3.882	600-1000
OPL-17	5'-AGCCTGAGCC-3'	7	7	100	-	6.403	4.000	1000-2500
OPB-3	5'-CATCCCCCTG-3'	3	3	100	Hybrid 72-51	2.469	1.822	750-1600
OPY-15	5'-AGTCGCCCTT-3'	7	7	100	-	5.474	5.940	750-2200
OPY-16	5'-GGGCCAATGT-3'	8	8	100	-	5.531	9.432	850-2250
OPB-15	5'-GGAGGGTGTT-3'	12	12	100	-	5.522	16.70	650-1500
OPA-9	5'-GGGTAACGCC-3'	10	10	100	Julesky Muscat	7.563	7.940	250-1031
OPB-4	5'-GGACTGGAGT-3'	9	9	100	Gold, Hybrid Hybrid 72-51	5.751	9.000	300-2250
Total		200	199	-		196.16	142.6	
Average		6.45	6.42	99.64		6.327	4.600	

(Rp) and Polymorphism information content (PIC) and size range of bands obtained are presented in Table 2. The UPGMA dendrogram obtained from the cluster analysis using Dice's and Jaccard's similarity matrices gave the similar pattern with only the differences in values of similarity coefficients, therefore, the dendrogram based on Jaccard's similarity coefficient values only are presented here.

The 31 RAPD primers amplified a total of 205 markers with an average of 6.61 bands per primers. The band size ranged from 250 to 3000 bp (Table 2). Maximum number of bands was produced by OPB-15 (12 bands), whereas minimum number of bands was produced by OPA-15 and OPN-19 (2 bands). A high level of polymorphism, ranging from 88.8-100%, was detected in all the genotypes examined with the

tested primers with an average of 99.6%. A RAPD amplification profile is shown in Figure 1. The resolving power (Rp) of the 31 RAPD primers ranged from 1.704 for primer OPN-19 to 16.704 for primer OPB-15. Three other primers (OPG-5, OPA-20, and OPX-15) posses the high Rp values of 13.704, 9.646 and 9.176, respectively. The results suggest the primers with high Rp values are able to distinguish among the genotypes studied. Polymorphism information content ranged from 0.823 (OPA-15) to 9.173 (OPG-5) with high values for PIC for OPA-9 (7.563), OPG-7 (6.667) OPK-19 (6.647) and OPL-17 (6.403). The basic difference between the two genetic parameters lies on the fact the former is based on the similarity to the optimal frequency, *i.e.*, 0.50 for each of the two alleles of a locus, whereas, the latter is based on frequency of alleles per locus. The result suggests primer OPG-5 to be the best for DNA fingerprinting and genetic relationship studies in grape. Unique bands amplified with the RAPD primers suggested the genotype Hybrid 72-51 (Angoor Kalan x Pusa Seedless) amplified uniquely with four different primers (OPG-7, OPB-12, OPB-3 and OPB-4). The combination of these four primers would prove to be highly useful for identification and selection of this hybrid. The other genotypes uniquely identified are: Bharat Early (with OPN-19), Julesky Muscat (with OPA-9) and Gold (with OPB-9). The Mantel's test between the Dice's and Jaccard's similarity matrices provided r = 0.99518 (t value = 8.45) for RAPD, suggesting very good correlation between the two similarity indices. The Dice's index (Sorensen, 20) differs from the Jaccard's index (Jaccard, 10) for the higher weight that it gives to the coincidences of the band present with respect to the non-coincidences. That means the two indices are almost equal for very low (< 0.1) or very high (> 0.9) similarity levels, but diverge quite a lot at intermediate values making more significant the comparison of their analysis in that region (Mattioni *et al.*, 14).

The genetic similarity based on Jaccard's similarity index ranged 20.51% (Black Muscat and Hybrid 74-4) to 88.12% (Banqui-Abyad and Beauty Seedless). The UPGMA dendrogram obtained from the cluster analysis using Jaccard's similarity matrix shows two clusters at 35% genetic similarity (Fig. 1). Cluster I has two genotypes Fakhri and Hybrid 75-32 (Banqui-Abyad x Perlette) sharing 40% genetic similarity. Both the genotypes are seeded and susceptible to anthracnose. Fakhri is an old variety of unknown origin cultivated primarily in southern India. The cluster II has the rest 32 genotypes and sub-divided into successive subclusters. The sub-cluster II-a has only two genotypes Black Prince (mid-season) and Bharat Early (a late genotype) with similarity of 61.1%. Black Prince and Bharat Early are similar in some

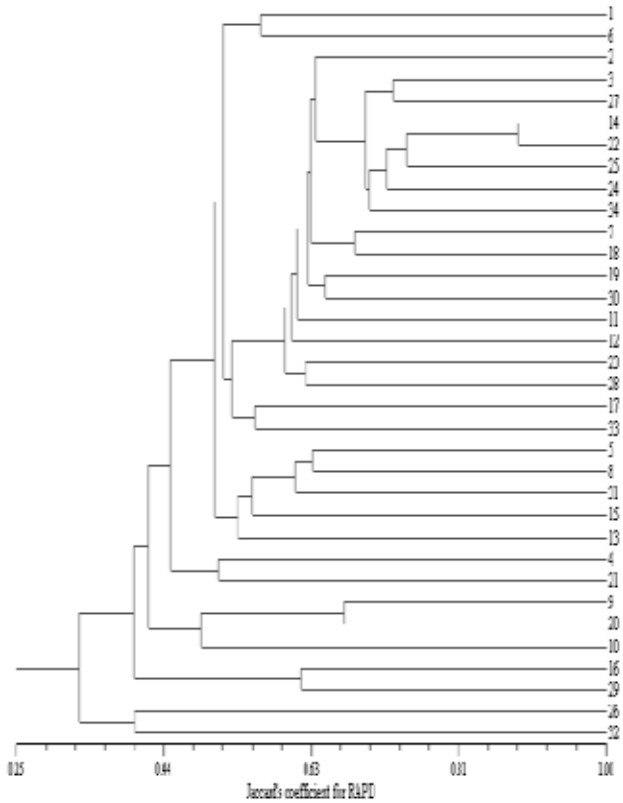

Fig. 1. Dendrogram of 34 *Vitis vinifera* accessions obtained after NTSYS analysis with RAPD markers.

morphological traits (musky flavour, seededness and coloured berries etc.), moreover both these varieties were procured from Hyderabad and possibly the breeder had a specific selection criteria resulting their grouping in same sub-cluster. The sub-cluster II-b has three genotypes, the Hybrid 74-6 (F_1 hybrid between Cheema Sahebi, a seedling selection made in 1927, and Catawba, an introduction form unknown origin), Kishmish Beli (introduced from USSR) and Hur (introduced but the undefined source). The sub-cluster II-c had two hybrids Hybrid 74-4 and Hybrid 74-6. Sub-cluster-II-d have five genotypes, *viz*., Victory, Madeline Angevine (MA), Cardinal, Perlette and Hybrid-71-35. Genotypes MA and Perlette are two earliest maturing varieties in India, whereas Cardinal is late. All three varieties develop flavour to some extent at maturity. Sub-cluster II-d had five genotypes, *viz*., Victory, Madeline Angevine, Cardinal, Perlette and Hybrid 71-35. These all share several attributes that make them suitable for table purpose and grouped here together. The biggest sub-cluster II-e has rest 20 genotypes clustered together at different similarity levels and the grouping of the genotypes in this cluster was random. The overall results for RAPD clustering suggest the

development of grape genotypes from the unknown primitive cultivars.

The results suggest that RAPD marker system is reliable to trace larger portion of genome with less number of primers. Moreover, the present study suggest that RAPD markers are useful to study the genetic relationships between grape genotypes, providing the RAPD markers to be a powerful tool for the generation of potential fingerprinting diagnostic markers for grape cultivars.

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