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Microsatellite and RAPD analysis of grape (*Vitis* spp.) accessions and identification of duplicates/misnomers in germplasm collection

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ABSTRACT

Microsatellite and RAPD primers were used to analyze forty four grape accessions from the National Grape Germplasm Repository, Pune, India. Forty four microsatellite primers generated 433 and were more informative than RAPD primers. Thirty three RAPD primers resulted in 420 bands among 44 accessions and generated unique bands for several accessions. The dendrograms of genetic relationship obtained with two classes of markers were comparable. Microsatellite analysis of this set of accessions identified six duplicates and misnomers and genetic identity of duplicates was established. This study will help in better germplasm management and for devising strategies for identifying core selection.

Key words: *Vitis,* molecular markers, microsatellite, RAPD, germplasm.

INTRODUCTION

Grapevine (*Vitis vinifera*) is one of the most remunerative fruit crop in India. Grape in India is grown under subtropical to tropical climates over an area of 65,000 ha mainly in the states of Maharashtra, Karnataka, Andhra Pradesh and Tamil Nadu (NHB, 8). Grape germplasm in India is scattered at various research institutes. The germplasm has been collected from different countries during last few decades. (Chadha and Shikhamany, 5). With passing time several accessions have been lost and there is possibility of mis-nomenclature during material transfer from one place to another. National Research Center for Grapes after its establishment was identified as site for National Grape Repository in India and extensive efforts were made to bring the scattered germplasm at one place.

Genetic analysis techniques based on molecular markers are able to provide objective information on the genetic potential of a species, thus helping the effective characterization and exploitation of germplasm in modern agriculture. Molecular markers such as RFLP (Bourquin *et al.*, 1), RAPD (Vidal *et al.*, 18; Tamhankar *et al.*, 14), microsatellites or SSR (Bowers *et al.,* 2; Thomas and Scott, 16; Upadhyay *et al.*, 17) and AFLP (Cervera *et al.,* 4) are in use for characterization of grape varieties, parentage analysis, identification of clones, studying genetic relationships, genetic maps and marker-assisted selection. Microsatellites and RAPDs are the two very popular and widely used classes of molecular markers. Microsatellites markers have been most widely used for the identification and discrimination of grape cultivars for germplasm management. RAPD though a dominant class of markers are useful for cultivar identification by converting them to cultivar specific SCAR markers. In this study we report the analysis of 44 grape accessions with microsatellite and RAPD primers in an effort to characterize the available germplasm and establish the genetic identity of duplicate/misnomers in the germplasm.

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MATERIALS AND METHODS

Forty-four grape accessions including several *Vitis* species and interspecific hybrids, maintained in germplasm collection at NRC for Grapes, Pune were used for this study. The names of these accessions and their known pedigree are listed in Table 1. Fresh young, tender leaves were used to extract DNA or using DNeasy® Plant Kit (Qiagen, CA, USA).

Forty four microsatellite primer pairs belonging to VVMD (Bowers *et al.*, 2, 3); VVS (Thomas and Scot, 16), VMC (*Vitis* Microsatellite Consortium), VrZAG (Sefc *et al.*, 13*)* and VVI (Merdinoglu *et al.*, 7) sets and included 6 primers recommended as reference primers for characterizing the grape germplasm (This *et al.*, 15). The forward primer was fluorescent labeled with *6 FAM*, *VIC* or *NED.* The PCR amplification reaction mixture (10 µ1) contained 0.66 µM labeled forward primer, 0.66 uM reverse primer, 100 µM of each dNTP, 3.0 mM $MgCl_2$ and 1.0 U *Taq* polymerase (Bangalore Genei Pvt. Ltd., India). The PCR was performed either on a PTC 200 gradient thermal cycler (MJ Research, USA) or GeneAmp PCR system 9700 (Applied Biosystems, USA). The temperature profile consisted of the following steps: 10 min. at 94 °C followed by 35 cycles of 1 min. at 94 °C, 1 min. at 55°C and 1 min. at 72°C and a final extension for 10 min. at 72 °C. PCR

Table 1. Pedigree of grape accessions analysed.

S. No.	Name	Pedigree*
1.	Madhu Angoor	Unknown
2.	Khalili	*V. vinifera*
3.	Carolina Black Rose	Aurelia x Black Rose
4.	Katha Angoora	*V. vinifera*
5.	Sevye Villard 23501	Interspecific hybrid
6.	Sevye Villard 18402	Interspecific hybrid
7.	Sevye Villard 12309	Interspecific hybrid
8.	Sevye Villard 18315	Interspecific hybrid
9.	Sevye Villard 12364	Interspecific hybrid
10.	Sevye Villard 12375	Interspecific hybrid
11.	Seibel 9813	Interspecific hybrid
12.	Seibel 9308	Interspecific hybrid
13.	Cardinal (EC32186),	Flame Tokay × Ribier
14.	Chasselas Tompa (EC32473)	Queen Victoria × Chasselas Jalabert
15.	Trollinger (EC36587)	Queen Victoria
16.	1613C (EC61856)	Solonis × Othello
17.	EC20627	Unknown
18.	Periquita (EC 198244)	*V. vinifera*
19.	Pearl of Csaba	Bronnerstraube × Muscat Ottonel
20.	Suavis (IP365)	Angelo Pirovano × Moscato Rossao Di Malaga
21.	H533	Joannes Sevye 23416 × Traminer Rot
22.	Concord	*V. labrusca*
23.	Champanel	*V. champinii* × Wordon
24.	*V. lanata*	*V. labrusca* var. *lanata*
25.	Catawba	*V. labrusca* × *V. vinifera*
26.	Lake Emerald	Pixiola × Golden Muscat
27.	Friihroter Veltliner	Silvaner × Veltliner rot
28.	Crimpson Seedless	Emperor × C33-199
29.	Gulabi 1	Muscat Hamburg (Sciava Grossa × Muscat of Alexandria)
30.	Gulabi 2	Muscat Hamburg (Sciava Grossa × Muscat of Alexandria)
31.	Charas	*V. vinifera*
32.	Shweta Seedless	Unknown
33.	E2/1	Unknown
34.	Alamwick	Unknown
35.	Bharat Ruba Black	*V. vinifera*
36.	E-32/8	Unknown
37.	E8/5	Unknown
38.	Flame Seedless	(Cardinal × Sultanina) × (Red Malaga × Tifafihi Ahmer) × (Muscat of Alexandria × Sultanina)
39.	Anab-e-Shahi	*V. vinifera*
40.	*V. parviflora*	Syn. *V. flexuosa*
41.	Tas-A-Ganesh	Thompson Seedless
42.	Kali Sahebi	*V. vinifera*
43.	Thompson Seedless	*V. vinifera*
44.	Delight	Koenigin Der Weingaerten × Sultanina

*Source : Vitis International Variety Catalogue (www.vivc.baz.de)

products were diluted 50 times and 1 µl (for *FAM and VIC* labeled) or 2 µl (for *NED* labeled) of diluted mix was added to a mixture of 10 µl HI-DI formamide and 0.10 µl of GeneScan 500 ROX internal size standard. The mix was denatured at 94°C for 5 min. and analyzed on ABI 3130 genetic analyzer using 36 cm capillary filled with POP7 polymer. GeneMapper ver 4.0 was used to determine the peak size using local Southern method and allele call.

Thirty-three RAPD primers (Operon Technology, USA) were used to analyse the grape accessions. The RAPD reaction mixture (25 µl) contained 25 ng DNA, 15 p mole primer, 100 µM of each dNTP, IX PCR buffer, 4.0 mM $MgCl_2$ and 1U of *Taq* polymerase. The PCR programme for RAPD was as follows: 5 min. at 94°C (initial denaturation); 40 cycles of 1 min. at 94°C, 1 min. at 50°C and 2 min. at 72°C, followed by final extension for 10 min. at 72°C. The PCR products were resolved on 1.2% agarose gel using IX TAB (Tris: acetic acid: EDTA) as electrophoresis buffer. A,-DNA double digested with *Eco*Rl and *Hin*dlll and 100 bp ladder were used as size standard. The gels were stained with ethidium bromide and photographed using gel documentation system (Alphainotech, USA). The bands in each gel were scored as 0 or 1 for absence and presence respectively. The gels were scored manually at least thrice.

The allelic data from microsatellite analysis and binary data from RAPD analysis was used to calculate heterozygosity and other parameters using Genealex 6.1 (Peakall and Smouse, 10). The SSR allele data was converted to a binary matrix by assigning 1 or 0 to the presence or absence respectively. The binary matrix from microsatellite and RAPD analysis was used to estimate similarity among accessions using Jaccard's similarity coefficient which takes into account the presence or absence of the bands. Similarity matrix was used for performing cluster analysis using UPGMA and a dendrogram was constructed using NTSYS-PC software package, version 2.10 (Rohlf, 12). Tree file was converted to cophenetic matrix and the robustness of the tree was tested by estimating correlation between cophenetic and similarity matrix. Cophenetic matrix of microsatellite and RAPD data were used to compare the two dendrograms.

RESULTS AND DISCUSSION

The results of microsatellite primers are given in Table 2. Forty-four primers detected a total of 433 alleles with an average of 9.8 alleles per primer. All the alleles were polymorphic. The number of alleles detected by each primer ranged from 4 (VVIM07, VVIQ66, VVIQ57 and VVMD26) to 23 (VVMD14). The average expected heterozygosity was 0.766, indicating that most of these primers are very informative. The primer VVMD 14 was the most informative primer. The information index and heterozygosity value for this primer was 2.855 and 0.927 respectively. VVIQ66 was found to be the least informative primer with an information index of 0.229 and heterozygosity value of 0.086.

Similarity among 44 accessions based on microsatellite data ranged between 0.020 (E2/1 and Suavis) to 0.938 (1613C and Pearl of Csaba). The average similarity based on Jaccard's Coefficient was 0.303. Similarity matrix when subjected to cluster analysis grouped these accessions based on their relatedness. The dendrogram shown in Fig. 1 demonstrates grouping of accessions based on their genetic relationship. Suavis, although belonging to *vinifera* type was found to be the most divergent and separated early. Other accessions clustered in five groups. Generally, accessions belonging to same species or common parents were grouped together. Cophenetic correlation for the tree was 0.95 indicating a very good fit for the tree.

The results of RAPD primers are given in Table 3. Thirty three RAPD primers detected 420 bands with an average of 12.7 bands per primer. Of the 419 bands, 416 (99%) detected polymorphism among accessions. The number of bands detected by individual primer

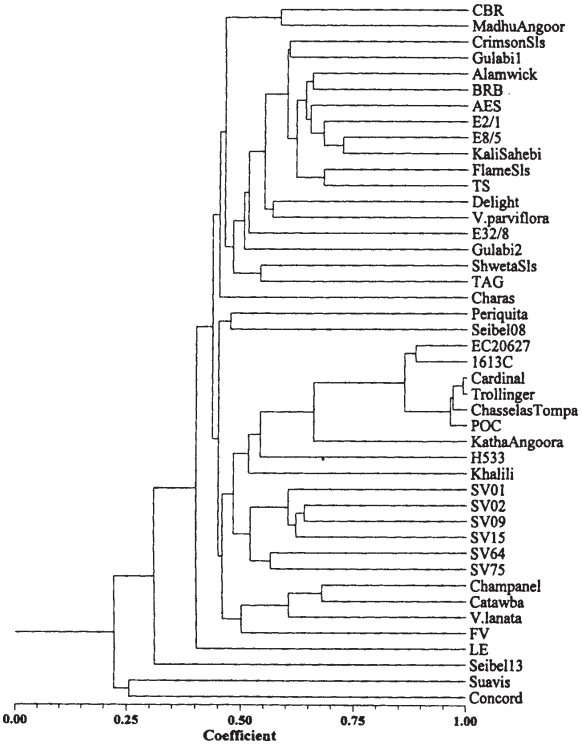

Fig. 1. Dendrogram of genetic relationship among grape accessions based on SSR data.

Table 2. Detailed results of microsatellite primers.

Locus	No. of alleles	No. of effective alleles	Information index	Observed heterozygosity	Expected heterozygosity
VMC4d9.2	8	4	1.60	0.13	0.75
VMC4f8	13	5	1.92	0.72	0.78
VMC7bl	14	7	2.27	0.34	0.86
VMC7c3	8	7	1.95	0.10	0.85
VMC7f2	9	6	1.90	0.83	0.82
VMC7g3	7	3	1.28	0.57	0.61
VMC8a4	9	8	2.07	0.69	0.87
VMC8b5	10	7	2.12	0.59	0.86
VMC8e6	12	8	2.21	0.24	0.87
VMC8g3.2	9	4	1.59	0.76	0.71
VMC8g6	10	5	1.88	0.61	0.79
VrZAG62	10	6	2.00	0.72	0.83
VrZAG79	14	9	2.37	0.65	0.89
VVIB01	8	4	1.536	0.34	0.72
VVIB23	10	6	1.95	0.71	0.82
VVIB66	14	9	2.36	0.48	0.89
VVIB94	8	4	1.67	0.788	0.78
VVIC72	7	5	1.66	0.608	0.78
VVIH01	12	8	2.25	0.49	0.88
VVIH54	7	4	1.61	0.36	0.73
VVII52	9	3	1.54	0.29	0.71
VVIM01	5	2	1.06	0.37	0.55
VVIM07	4	3	1.29	0.63	0.70
VVIN33	14	7	2.24	0.58	0.86
VVIP77	11	5	1.91	0.41	0.81
VVIQ57	4	2	0.83	0.36	0.44
VVIQ66	4	1	0.23	0.09	0.09
VVIT30	6	2	1.15	0.30	0.57
VVIV37	10	6	2.00	0.55	0.84
VVMD14	23	14	2.86	0.58	0.93
VVMD21	13	4	1.87	0.86	0.77
VVMD25	14	8	2.29	0.76	0.87
VVMD26	4	3	1.20	0.67	0.66
VVMD27	13	8	2.22	0.86	0.87
VVMD31	13	6	2.01	0.72	0.82
VVMD32	12	5	1.95	0.18	0.81
VVMD5	7	4	1.54	0.54	0.72
VVMD6	10	7	2.12	0.83	0.86
VVMD7	8	6	1.85	0.47	0.82
VVMD8	11	6	2.05	0.71	0.84
VVS16	11	3	1.52	0.54	0.62
VVS2	16	8	2.34	0.79	0.88
VVS29	5	5	1.59	0.71	0.79
VVS3	7	6	1.80	0.66	0.82

ranged from 8 (OPF12) to 21 (OPH03). The size of amplified fragments varied between 200 bp and 4900 bp with most bands in the range of 300 to 2300 bp. In spite of the large proportion of the polymorphic bands, most of the primers were only moderately informative as indicated by mean heterozygosity values (0.156 to 0.419) and information index (0.270 to 0.607).

The binary data obtained from RAPD was used to calculate similarity among different accessions. Similarity based on Jaccard's coefficient varied between 0.156 (Concord and Flame Seedless) to 0.991 (Cardinal and Trollinger) with an average of 0.446. In cluster analysis, Suavis and Concord formed a group and separated early (Fig. 2). Similarly, Seibel 9813 and Lake Emerald grouped separately. Remaining accessions formed two major groups. First group contained accessions belonging to *vinifera* and was further subdivided into many sub-groups, whereas other group consisted of accessions, which belonged to other *Vitis* spp. or interspecies hybrids. The cophenetic coefficient for this matrix was 0.93 indicating robustness of the tree.

The dendrograms of genetic relationship obtained with microsatellite and RAPD were compared. The correlation between cophenetic matrix of two trees was 0.65 suggesting considerable similarity between the two dendrograms. In both the dendrograms, six accessions belonging to Sevye Villard, which are interspecific hybrids and share their pedigree, were grouped together in both the analysis. Seibel, which is one of the parents of Sevye Villard also clustered closely. All the accessions belonging to *Vitis labrusca* or having it as one of their parents grouped together. *V. lanata* which was earlier grouped under *V. labrusca* and has close similarity was also clustered in the same group. In both the analysis, accessions belonging to *Vitis vinifera* grouped together. One worthy observation was on Tas-A-Ganesh, which is a clone of Thompson Seedless but it could be clearly distinguished and had only 50% similarity with Thompson Seedless.

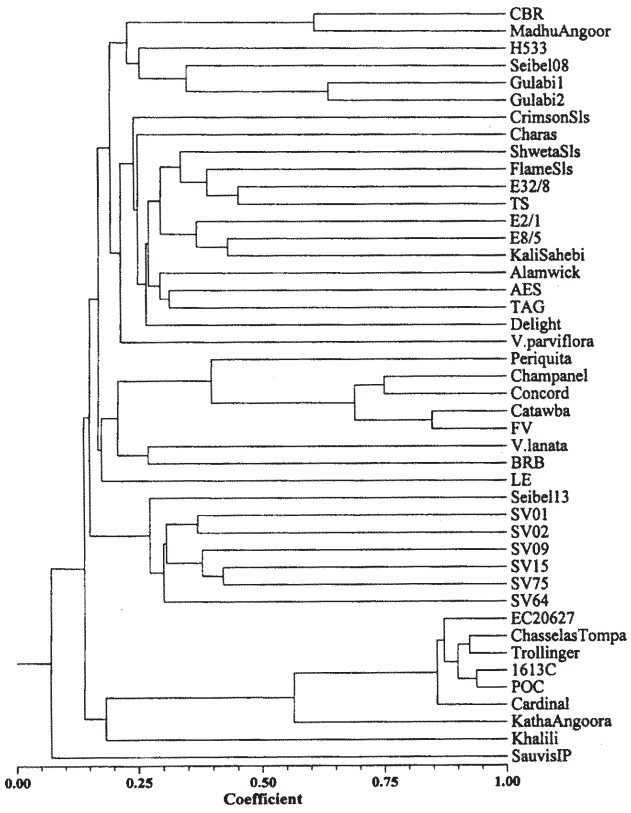

Fig. 2. Dendrogram of genetic relationship among grape accessions based on RAPD data.

Several studies have compared different marker techniques for germplasm analysis. In this analysis while the level of polymorphism obtained with microsatellite and RAPD was comparable, the microsatellite were more informative and exhibited higher information index and heterozygosity values. This was reflected in similarity matrix also. The average similarity among accessions obtained with microsatellite data was much less than that obtained with RAPD data. Powell *et al.* (11) while comparing different marker systems in soybean observed that RAPD produced higher estimates of similarities compared to other markers. Similarly, Garcia *et al.* (6) who while comparing different marker systems in maize found microsatellites to be more informative. The low average similarity in this study could be due to the reason that accessions belonging to different species were also used for this study.

Thirteen bands unique for 11 different accessions were obtained with 9 different primers. Unique bands for Khalili (OPJ01-$_{690}$), Madhu Angoor (OPJOU$_{75}$), Charas (OPA02.$_{890}$), *V. lanata* (OPA04.56o), *V. parviflora* (OPB04.i$_{270}$), Delight (OPB04.$_{440}$), Sevye Villard 12375 (OPF14.$_{200}$), Sevye Villard 12364 (OPH03.i$_{560}$), Concord (OPI20.$_{300}$), Suavis (OPH07_4300 and OPI20.$_{210}$) and Frtihroter Veltliner (OPI04.$_{390}$ and OPI04.$_{300}$) were identified. RAPD, a dominant class of marker though considered to be suitable for genetic diversity analysis, is not preferred for varietal identification. However, when converted to SCAR markers (Paran and Michelmore, 9) to generate screening markers based on simple PCR assays, it could be used for accurate identification. RAPD bands uniquely present in an accession are selected for conversion to SCAR markers. The unique bands identified in this study could be useful for developing SCAR primers for grape variety identification and developing their DNA fingerprint.

In germplasm several accessions are maintained with EC (exotic collection) number and name.

Table 3. Detailed results of RAPD primers.

S. No.	Primer	No. of bands	No. of polymorphic bands	Information index	Mean band heterozygosity	Band heterozygosity (range)
1.	OPA02	12	11	0.453	0.301	0.023-0.499
2.	OPA03	9	9	0.408	0.257	0.045-0.421
3.	OPA04	13	13	0.413	0.268	0.023-0.499
4.	OPA08	12	12	0.520	0.347	0.067-0.499
5.	OPA11	15	15	0.326	0.190	0.067-0.356
6.	OPA13	13	13	0.457	0.298	0.089-0.499
7.	OPB04	10	10	0.493	0.335	0.023-0.499
8.	OPB07	14	14	0.506	0.340	0.045-0.499
9.	OPB12	13	13	0.456	0.298	0.067-0.499
10.	OPC13	13	13	0.439	0.281	0.067-0.486
11.	OPF04	16	16	0.424	0.268	0.067-0.466
12.	OPF09	14	14	0.436	0.283	0.067-0.486
13.	OPF12	8	8	0.529	0.349	0.232-0.479
14.	OFF 14	14	14	0.429	0.272	0.023-0.466
15.	OPG06	16	16	0.545	0.366	0.152-0.496
16.	OPG14	9	9	0.504	0.328	0.193-0.489
17.	OPH03	21	21	0.365	0.227	0.023-0.499
18.	OPH04	14	14	0.452	0.289	0.023-0.486
19.	OPH07	17	17	0.270	0.156	0.023-0.470
20.	OPH19	9	9	0.503	0.341	0.045-0.489
21.	OPI04	16	16	0.384	0.242	0.023-0.427
22.	OPI07	9	9	0.464	0.296	0.131-0.470
23.	OPI10	16	15	0.322	0.190	0.000-0.486
24.	OPI13	11	11	0.420	0.263	0.045-0.447
25.	OPI14	14	12	0.271	0.158	0.000-0.386
26.	OPI20	15	15	0.385	0.246	0.023-0.451
27.	OPJ01	12	12	0.392	0.255	0.023-0.496
28.	OPJ07	9	9	0.438	0.279	0.089-0.451
29.	OPK11	15	15	0.437	0.275	0.089-0.500
30.	OPK12	9	9	0.468	0.300	0.067-0.496
31.	OPK13	12	12	0.372	0.228	0.045-0.499
32.	OPQ04	8	8	0.607	0.419	0.251-0.499
33.	OPK17	12	12	0.463	0.300	0.110-0.496

Accessions maintained as Cardinal (EC32186), Pearl of Csaba, Trollinger (EC36567), Chasselas Tompa (EC32473) and 1613C (EC61857) and EC20627 showed high level of similarity based on morphological and ampelometric analysis and they were suspected to be duplicates or misnomers. Microsatellite and RAPD analyses revealed very high level of similarity among these accessions. The known pedigree of these accessions is very different from each other and such a high level of similarity obtained with microsatellite (0.94) and RAPD (0.95) analyses suggested that these accessions belong to the same genotype. Comparison of microsatellite profile of these accessions at eight loci including six recommended as reference loci for universal grapevine identification (This *et al.,* 15), with that of rootstock 1613C, indicated that all these accessions belong to rootstock 1613C (Table 4).

The analysis revealed wide genetic diversity in the germplasm even though only a set of germplasm was analysed. Six duplicates/misnomers accessions were also identified. This information will be further strengthened by analysis of all the accessions in the germplasm and will be used for better germplasm management and identification of core collection with desirable traits.

Table 4. Comparison of duplicate accessions at 8 microsatellite loci.

Accession	VVS2	VVMD5	VVMD7	VVMD27	VRZAg 62	VrZAG79	VVMD 31	VVMD32
1613C	126:137	239:267	246:254	176:207	188:190	258:267	212:216	252:252
Pearl of Csaba	126:137	239:267	246:254	176:207	188:190	258:267	212:216	252:252
EC20627	126:137	239:267	246:254	176:207	188:190	258:267	212:216	252:252
Cardinal (EC32186)	126:137	239:267	246:254	176:207	188:190	258:267	212:216	252:252
Chasselas Tompa (EC32473)	126:137	239:267	246:254	176:207	188:190	258:267	212:216	252:252
Trollinger (EC36587)	126:137	239:267	246:254	176:207	188:190	258:267	212:216	252:252
1613C (EC61856)	126:137	239:267	246:254	176:207	188:190	258:267	212:216	252:252

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