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Confirming Hybridity in Grape (*Vitis vinifera* L) using Microsatellites

Venkata Rao

Associate Professor of Fruit Science, College of Horticulture, Bengaluru, University of Horticultural Sciences, Bagalkot, Karnataka, India

ABSTRACT

The microsatellites markers used in genetic analysis does not only allow differentiation but also identification and parentage analysis of grapevine cultivars. The main purpose of this study was to confirm the hybridity of these accessions and to construct a molecular database including the parents commonly grown in India. A total of eighteen hybrids and seven parents were analyzed using twelve microsatellite simple sequence repeat (SSR) markers. The hybrid nature of all the progenies used was marked by VVMD-32. In VVMD-32, unique banding pattern was observed in Black Champa (male parent) in Group A. In which six bands were prominent, out of which first and last band were absent in Thompson Seedless (female parent). While in case of primer VVS-29, three prominent bands were observed in male parents. This primer was able to confirm the hybridity of progenies of group A, F, G and H. The primer VVS-2 confirmed the hybridity of progenies of group-B, C and H. In case group B, the 3rd band of male parent was distinct from the female parent. These three primers were thus able to confirm the hybrid nature of all the progenies. The use of twelve polymorphic microsatellite markers to detect the hybrids within Indian grapevine germplasm suggested that this is a reliable, efficient, and effective marker system that can be used for parentage studies in grapevine and subsequently in crop improvement programs.

Key words: hybridity, microsatellites markers, *Vitis vinifera* L.

INTRODUCTION

Grape cultivars (*Vitis vinifera* L) have a long history of domestication. The world's vineyards occupy about 8.7 million hectares. More than 9600 grape cultivars exist around the world (Galet, 2000) and almost 16,000 prime names appear in the *Vitis* International Variety Catalogue (Maul and Eibach, 2003). Some of these are not easily distinguishable by morphology and many cultivars appear to be synonyms, having been distributed around the world and acquiring new names in the process. Moreover, the wide distribution and long history of cultivation have led to the development of numerous cultivars with many synonyms, resulting in complexity among germplasm collections (Galet, 1990). Grape in India are reported to have been introduced in 620 BC. (Olmo, 1976) and commercial cultivation was started in the beginning of the 20th century. Presently, grapes are successfully grown in India over an area of 1,11,000 ha with a production of approximately 1.23 million MT (Anonymous, 2011), primarily for use as fresh fruit.

Grape breeding had mainly relied on selection among naturally occurring spontaneous crosses for ages and to a lesser extent, due to conventional breeding during the last century (Adam-Blondon et al., 2004). The varieties currently available are the results of a selection process by human and ecogeographical conditions (Bisson, 1995). Information on genetic diversity among plant species is important for efficient utilization of genetic resources.The existence of close genetic relationships among cultivars grown in the same region or under similar climatic influence could lead to dilution of genetic resources. Hence, studies on grape have been carried out to characterize the commercially important germplasm available in India. Microsatellite technology has been extensively used in grapevine biology and genetics. The number of microsatellite loci available has greatly increased in the last few years largely through the establishment of the International Vitis Microsatellite Consortium, leading to the discovery of more than 350 new loci. Microsatellite markers, being abundant, multiallelic and highly polymorphic, provide an efficient and accurate means of detecting genetic polymorphism. Most importantly, their codominant nature makes them the markers of choice for population genetic analysis to assess genetic organization in germplasm collections.

Microsatellites have been used to determine parent-progeny relationships in grape (Bowers and Meredith, 1997; Bowers et al., 1999a), to develop a database of DNA profiles for use in cultivar identification (Bowers et al., 1996; Lamboy and Alpha, 1998), and as markers for mapping genetic linkage (Riaz et al., 2004) The present study evaluates the hybrids for their parentage analysis to confirm the hybrid nature using polymorphisms revealed by twelve microsatellite loci.

MATERIALS AND METHODS

Plant Materials

Plant material from 25 grape genotypes was collected from Department of Horticulture, University of Agricultural Sciences, Bangalore and Indian Institute of Horticultural Research, Bangalore. Approximately, 50 g of recently matured leaves (15–20 d old) were collected, washed using distilled water, wiped with 70% (v/v) ethanol, then air dried prior to storage in sealed plastic bags at 4°C.

DNA Isolation DNA was extracted from the stored leaves of grapevine using acetyl trimethyl ammonium bromide (CTAB) method (Simon et al., 2007). 2 g of leaf sample were powdered in liquid nitrogen to extract the DNA. The powder was mixed with 10 ml extraction buffer, preheated to 65°C, containing 100 mM Tris-HCl, pH 8.0, 20 mM EDTA, 1.4 M NaCl, 3% (w/v) CTAB, 2% polyvinylpyrrolidone and 1% (v/v) ¾β-mercaptoethanol, then incubated at 65°C for 90 min. The mixture was cooled to room temperature, 10 ml cold 24:1 (v/v) chloroform:isoamylalcohol was added, and the contents were mixed well. After centrifugation at 6,000 × *g* for 10 min at 4°C, the supernatant was transferred to a fresh tube and the chloroform:isoamylalcohol step was repeated until a clear supernatant was obtained. 5 M NaCl was added to the supernatant [0.5 (v/v)] and mixed gently, followed by addition of 2 volume of cold isopropanol to precipitate the DNA. The mixture was incubated overnight at 4°C, then centrifuged at 10,000 × *g* for 5 min. The resulting pellet was washed with 75% (v/v) ethanol, air-dried, and dissolved in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). 10 µg RNase (bovine pancreatic ribonuclease; Bangalore Genei, Bangalore, India) was added to each sample which was incubated for 30 min at 37°C, mixed with an equal volume of phenol, and centrifuged at 6,000 × *g* for 20 min at room temperature. This step was followed by washing with an equal volume of 1:1 (v/v) phenol:chloroform, then with chloroform alone. The DNA was precipitated overnight at 4°C with 0.5 (v/v) 5 M NaCl and 1 volume of cold isopropanol. The resulting pellet obtained after centrifugation was dissolved in TE buffer, analyzed in an agarose gel and quantified using a spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

Microsatellite Analyses A total of twelve SSR primers characterized in previous studies were used. The primers were VVS1, VVS2, VVS29, VVMD 7, VVMD 14, VVMD 25, VVMD 27, VVMD28, VVMD31, VVMD32, VVMD 36 and VMC7b1 (Bowers et al., 1996 and 1999b).These microsatellites were selected as they were the same core set in the screening programme used to access the target grapevine collections. Primer pairs were synthesized from MWG Biotech, Bangalore, India based on their published gene sequence. PCR was performed in 96-well plates in MJ Research PTC100 thermocyclers (Bio-Rad Laboratories, Bangalore, India).

PCR reactions were carried out in 25 µl reactions containing 50 ng of DNA, 5 pmoles of each primer, 10x of *Taq* polymerase buffer (50 mM KCl, 10 mM Tris-HCl, pH 9.0, 0.05% (v/v) NP40, and 0.05% (v/v) Triton X-100), 1.5 mM of MgCl2, 0.5 mM of dNTPs (Finzymes Pvt. Ltd., India), and 1 U of *Taq* polymerase (Sigma-Aldrich Pvt. Ltd., India). The final volume was adjusted with sterile distilled water. The PCR amplifications were carried out with respect to the protocols for primer sets published in Bowers et al., 1996 and 1999b and Thomas and Scott, 1993. Amplification was confirmed with agarose gels, and alleles were separated by running on 6% polyacrilamide denaturing gels and electrophoresed in 1 × TBE at 55 W for 2 h. The amplified products were visualized with silver staining previously described (Bowers et al., 1996).

Statistical Analysis

Amplified fragments from each SSR primer set were scored manually for their presence (1) or absence (0). The profiles of 25 accessions of grapevine using 12 primer pairs were assembled for statistical analysis. The sizes of the fragments were estimated using 50 bp standard DNA markers (Bangalore Genei Pvt. Ltd., India), coelectrophoresized with the amplified products. A genetic dissimilarity matrix was developed using Euclidean Distances, which estimates all pairwise differences in the amplification products (Sokal and Sneath, 1973). A cluster analysis was based on Ward's method using a minimum variance algorithm (Ward, 1963).

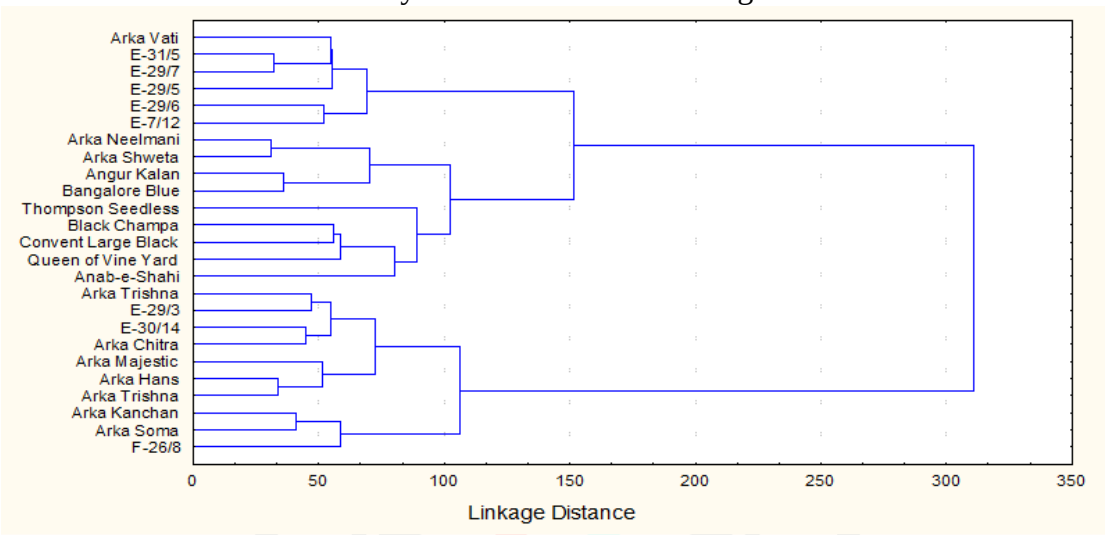
RESULTS AND DISCUSSION

The objective of this study was to confirm the parentage of the hybrids developed. To detect hybridity there must be polymorphism between the parents. The polymorphic bands which are present in male parent should be present in all the hybrids and should not be present in female parent (Magdalita *et al*., 1998).

Analysis of F_1 hybrids and parents

Cluster analysis

A total of 15 primers were used for the analysis out of which 12 primers were polymorphic. Linkage among 18 hybrids and their parents as revealed by a dendorgram is presented in Fig.1. The cluster analysis grouped the parents and hybrids into two major groups. Group I is divided into group I (a), I (b) and I (c). Under group I (a) Arkavati, E-31/5 and E-29/7 come under one cluster to which Arkavati and E-29/5 are related at a linkage distance of 54 and 55, respectively. In the same group E-29/6 and E-7/12 formed a single sub cluster and are closely related at a linkage distance of 51. Groups I (b) comprises of 4 genotypes. Among them Arka Neelamani and Arka Shweta are closely related at linkage distance of 34. Angur Kalan and Bangalore Blue Share much similarly and are related at a linkage distance of 40.



Group I (c) comprised of 5 genotypes, among them Black Champa and Convent Large Black are closely related and formed a single sub cluster at a linkage distance of 58, whereas, Thompson Seedless, Queen of Vine Yard and Anab-e-Shahi separated from this group and formed individual clusters.

Group II comprises of 10 hybrids and is divided into group I (a), I (b) and I (c), under group II (a), Arka Trishna and E-29/3 are closely related at a linkage distance of 45 whereas E-30/14 and Arka Chitra formed a separate sub cluster at a linkage distance of 42. Group II (b) comprises 3 hybrids among them Arka Hans and Arka Trishna are closely related at a linkage distance of 45 whereas E-30/14 and Arka Chitra formed a separate sub cluster at a linkage distance of 42. Group II (b) comprises 3 hybrids among them Arka Hans and Arka Trishna are closely related at a linkage distance of 40 whereas Arka Majestic formed a separate entity and is linked to Arka Hans and Arka Trishna at a linkage distance of 51. Group II (c) also comprises 3 hybrids among them Arka Kanchan and Arka Soma are closely related at a linkage distance of 43. Hybrid E-26/8 formed a separate sub cluster and is related to Arka Kanchan and Arka Soma at a linkage distance of 57. The linkage distance among the hybrids and parents varied from 31 to 104 (Table 1).

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
1	0	46	53	56	48	50	59	63	86	84	86	71	82	81	84	75	69	73	70	82	79	67	57	73	81
2	46	0	31	56	64	68	73	65	88	74	78	79	78	81	74	79	73	69	68	74	81	63	67	65	75
3	53	31	0	47	55	61	74	72	93	81	83	92	73	78	83	90	84	82	79	87	76	68	70	70	72
4	56	56	47	0	44	54	61	63	70	72	66	89	74	85	88	87	91	89	84	90	69	63	81	71	77
5	48	64	55	44	0	32	45	47	72	84	76	77	86	83	80	89	91	87	90	98	71	71	77	79	83
6	50	68	61	54	32	0	53	63	74	76	58	69	70	71	74	87	77	73	84	88	61	81	79	87	93
7	59	73	74	61	45	53	0	52	79	91	89	78	87	90	83	82	86	80	85	87	90	74	84	82	82
8	63	65	72	63	47	63	52	0	69	71	83	74	79	80	59	74	70	70	75	85	86	84	90	90	96
9	86	88	93	70	72	74	79	69	0	62	60	69	74	67	82	89	89	75	92	82	79	83	93	91	95
10	84	74	81	72	84	76	91	71	62	0	42	43	48	57	58	83	71	61	74	66	85	63	85	81	83
11	86	78	83	66	76	58	89	83	60	42	0	47	48	53	64	85	69	63	72	74	77	83	89	77	91
12	71	79	92	89	77	69	78	74	69	43	47	0	49	52	47	60	50	46	59	63	94	82	80	92	94
13	82	78	73	74	86	70	87	79	74	48	48	49	0	45	46	75	55	41	64	64	85	81	85	85	85
14	81	81	78	85	83	71	90	80	67	57	53	52	45	0	57	76	52	56	67	67	98	86	78	90	94
15	84	74	83	88	80	74	83	59	82	58	64	47	46	57	0	65	51	43	66	68	97	85	89	91	93

16	75	79	90	87	89	87	82	74	89	83	85	60	75	76	65	0	44	58	41	59	104	84	76	82	90
17	69	73	84	91	91	77	86	70	89	71	69	50	55	52	51	44	0	34	43	61	102	96	82	96	110
18	73	69	82	89	87	73	80	70	75	61	63	46	41	56	43	58	34	0	49	59	96	86	78	94	94
19	70	68	79	84	90	84	85	75	92	74	72	59	64	67	66	41	43	49	0	50	99	91	67	87	95
20	82	74	87	90	98	88	87	85	82	66	74	63	64	67	68	59	61	59	50	0	103	85	85	93	91
21	79	81	76	69	71	61	90	86	79	85	77	94	85	98	97	104	102	96	99	103	0	68	84	76	92
22	67	63	68	63	71	81	74	84	83	63	83	82	81	86	85	84	96	86	91	85	68	0	64	58	56
23	57	67	70	81	77	79	84	90	93	85	89	80	85	78	89	76	82	78	67	85	84	64	0	70	84
24	73	65	70	71	79	87	82	90	91	81	77	92	85	90	91	82	96	94	87	93	76	58	70	0	58
25	81	75	72	77	83	93	82	96	95	83	91	94	85	94	93	90	110	94	95	91	92	56	84	58	0

Table 1: Distance Matrix Analysis of hybrids and parents

Legend

1	Arkavati	14	Arka Kanchan
2	Arka Neelamani	15	Arka Hans
3	Arka Shweta	16	Arka Trishna
4	E-29/5	17	Arka Soma
5	E-31/5	18	F-26/8
6	E-29/7	19	Thompson Seedless
7	E-29/6	20	Black Champa
8	E-7/12	21	Anab-e-Shahi
9	Arka Krishna	22	Queen of Vine Yard
10	E-29/3	23	Convent Large Black
11	E-30/14	24	Angur Kalan
12	Arka Chitra	25	Bangalore Blue
13	Arka Majestic		

Analysis for confirmation of hybridity

Eighteen hybrids from different parent combinations were tested for their hybridity. Out of 18 hybrids 9 were from cross between Black Champa x Thompson Seedless, two each from cross between Anab-e-Shahi x Queen of Vine Yard, Angur Kalan x Black Champa. One each from a cross between Anab-e-Shahi x Thompson Seedless, Anab-e-Shahi x Convent Large Black, Angur Kalan x Anab-e-Shahi, Bangalore Blue x Anab-e-Shahi and Bangalore Blue x Convent Large Black.

Fifteen primers were used for analysis to confirm the hybridity and the 7 primers giving clear and reproducible bands were chosen for PCR analysis of samples. The banding patterns of the parents and progenies were compared to test the hybridity of the progenies used.

Of the seven primers used individually for amplification of samples, three primers VVMD-32, VVS-2 and VVS-29 gave the amplification patterns which reveal the hybrid nature of the progenies. In case of primer VVMD-32 (Group A), unique banding pattern was observed in Black Champa (male parent) in which 6 bands were prominent, out of which first and last band were absent in Thompson Seedless (female parent). Whereas the first band was present in all the hybrids except 9^{th} and the last band was absent in 8^{th} hybrid. In group B, 5^{th} band was present in male parent and the hybrids but was absent in female parent. In group C and D, the last band was prominent among the male parent and hybrids. In case of groups E and F, 4^{th} and 5^{th} bands were prominent in male parent and hybrids. This primer has not proved the hybridity of progenies of group G and H.

While in case of primer VVS-29, three prominent bands were observed in male parent. This primer was able to confirm the hybridity of the progenies of groups A, F, G and H. In case of group A, the last band was distinct from female parent and is present in all the hybrids except 1^{st}, 4^{th} and 9^{th} hybrids. In case of group F, 1^{st}, 2^{nd} and 3^{rd} bands of male parent were distinct from the female parent and it was present in hybrid. In group G and H, the 1^{st} band of male parent was distinct from female parent and was present in hybrids. The bands generated by primer VVS-2 reveals that the 2^{nd} band of the male parent of group-A was known to be distinct from the female parent. This primer has also proved the hybridity of progenies of group B, C and H. In case of

group B, the 3rd band of male parent was distinct from the female parent and was present in hybrids also. While in group C, the 2nd band of male parent was present in hybrid and was distinct from female parent. In group H, the 4th band of male parent was unique from female parent and was present in hybrid. These three primers were able to confirm the hybridity of all the progenies used in the investigation.

While the remaining primers including above three primers produced banding pattern which were common in both the parents and were present in all the hybrids progenies too. But these bands cannot be taken for analysis as these represent conserved sequences of grape.

Thus, it can be concluded that, all the progenies under investigation were confirmed to be hybrids. Similar kind of observations for hybridity confirmation in grapes were reported by Narayanaswamy *et al* (2009), Sawazaki *et al*. (1996) by using RAPD markers, Sefc *et al*. (1997), Warren and Cristopher (1998) by using SSR markers, Moreno *et al*. (1998) by using ISSR markers.

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