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Molecular Characterization and Genetic Relationships of Some Stress Tolerant Grape Rootstock Genotypes as Revealed by ISSR and SSR Markers

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Key words: Diversity analysis, Grape rootstocks, Scion varieties, Genetic relatedness, ISSR, SSR markers

Abstract

Grapevine rootstocks are a complex group of plants; most of them are interspecific hybrids evolved using parent genotypes having inherent tolerance to biotic and abiotic stresses. Fifteen species and interspecific hybrids of grape rootstocks along with three *Vitis vinifera* cultivars from IARI germplasm unit were analyzed with ten ISSR and seven SSR primers combinations. These ISSR produced 69 scorable bands with band size ranged from 200 to 1500 bp, PIC from 0.66 to 0.86 and primer heterozygosity ranged from 0.71 to 0.88. While, SSR primers detected a total of 24 alleles across 15 genotypes with PIC from 0.43 to 0.78 and primer heterozygosity from 0.49 to 0.81.The genetic similarity among the 15 grape genotypes using ISSR and SSR ranged from 0.27 to 1.00 in ISSR and 0.05 to 1.00 in SSR analysis. The genetic similarity matrices using ISSR analysis ranged from 27.0 to 85.0% and SSR 5.0 to 83.0%, respectively. The *Vitis* species Dogridge and Salt Creek had the highest similarity coefficient of 85% with ISSR analysis, while the interspecific hybrids 1103 Paulsen and 110 Richtier had the maximum similarity coefficient of 83% as identified by SSR analysis. The two marker systems formed two main clusters which were almost similar to that of PCA values. The principal coordinate analysis further helped in depicting the variability among species and hybrids of grape genotypes in three dimensional modes. In case of ISSRs, the first three coordinates accounted 51.92% for the existing variability, while with SSRs the variability accounted is 59.69%. The results revealed that ISSR and SSR markers could be exploited for genetic diversity analysis among the highly heterozygous grape rootstock species and interspecific hybrids.

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Introduction

Grape cultivation is one of the most remunerative farming enterprises in India. Grape is grown under a variety of soil and climatic conditions in three distinct agro-climatic zones, namely sub-tropical, hot tropical and mild-tropical climatic regions. Traditionally, grape is propagated as self-rooted plants using hard-wood cuttings. Although grapes are cultivated on own-rooted vineyards, rootstocks play an important role to combat adverse effects of climate change. Salinity is considered as one of the most important abiotic problems facing vine growers. The adverse effects of salinity either of soil or water on growth were confirmed in different grapevine cultivars. Rootstock possesses inherent genetic differences for salt stress tolerance. It is always better to exploit the inherent tolerance to salinity through genetic diversity analysis. Rootstocks Salt Creek and 1103 Paulsen were of the most salt resistant rootstocks as they tolerate up to 0.8 to 1.5% NaCl (Salem et al. 2011).

Conventionally, genetic diversity is estimated on the basis of morphological and phenotypic characters, biochemical markers which devoid of resolving power and only can cover little portion of the genome. However, on the other hand, molecular markers provide powerful tools to reveal polymorphism at the DNA sequence level and are robust to detect genetic variability as there are not influenced by the environment or the developmental stage of a plant, making them ideal for genetic relationships studies (Akhare et al. 2008).

The superiority of molecular markers over ampelography for the characterization of grape cultivars is well established. In grape, molecular markers like RFLP (Bourquin et al. 1993), RAPD (Mohamed and Salah El 2011, Saleh et al. 2015), SSR (Mohamed and Salah El 2011, Rao et al. 2014) and AFLP (Martinez et al. 2003), ISSR (Seyedimorad et al. 2012, Choudhary et al. 2014) are widely used for characterization of clones, parentage analysis, establishing the genetic relationship, molecular mapping etc.

Commercial grape varieties are extensively analyzed with molecular markers, however limited reports are available on molecular characterization of stress tolerant grape rootstocks. The identification of grape rootstock through marker technology was first attempted by Lin and Walker (1998), Sefe et al. (1998), Fatahi et al. (2003) and Gizella et al. (2011). Therefore, the present study was aimed at using two marker systems to assess the level of genetic diversity among the stress tolerant rootstocks in grape.

Materials and Methods

The experiments were conducted at the Division of Fruits and Horticultural Technology, ICAR-Indian Agricultural Research Institute (IARI), New Delhi along with the facilities provided by the Central Tissue Culture Laboratory (NRC

on Plant Biotechnology), IARI and Division of Genomic Resources, ICAR-National Bureau of Plant Genetic Resources, New Delhi. Fifteen grape genotypes including species and interspecific hybrids having diverse genetic backgrounds were collected from different sources were used for the present study. The details of the rootstocks used are Dogridge (*Vitis champinii*), St. George (*Vitis rupestris*), Salt Creek (*Vitis champinii*), *Vitis parviflora*, 110R (*Vitis berlandieri* × *Vitis rupestris*, De Grassette (*Vitis champinii*), 99R (*Vitis berlandieri* × *Vitis rupestris*), Male hybrid [Banqui Abyad × Victory (74-9)], 1103 P (*Vitis berlandieri* × *Vitis rupestris*), H-144 (Cheema Sahebi (*V. vinifera*) × Catawba (*V. labrusca*), 1616C (*Vitisriparia* × *Vitissolonis*), H-516 (*V. labrusca* x *V. vinifera*), 1613C (*Vitis solonis* x *V. labrusca* var. Othello), Perlette (*Vitis vinifera*) and 140 Ruggeri (*V. berlandieri* × *V. rupestris*).

The young leaves of the genotypes were collected for DNA extraction. Genomic DNA was extracted from leaves using acetyl trimethyl ammonium bromide (CTAB) method (Simon et al. 2007). Two grams 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 200 µl β-mercaptoethanol, then incubated at 65°C for 60 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 10,000rpm for 20 min. at 4°C, the supernatant was transferred to a fresh tube and the isopropanol was added until a clear supernatant was obtained. 3 M sodium acetate was added to the supernatant [0.5 (v/v)] and mixed gently to precipitate the DNA. The mixture was incubated overnight at 4°C, and then centrifuged at 10,000 rpm for 20 min. The resulting pellet was washed with 70% (v/v) ethanol, air-dried, and dissolved in TE buffer (10 mM tris-HCl, 1 mM EDTA, pH 8.0). 5 µg RNase (bovine pancreatic ribonuclease; Bangalore Genei, Bengaluru, India) was added to each sample which was incubated for 30 min at 37°C, and analyzed in an agarose gel and quantified using a spectrophotometer (Nano Drop Technologies, Wilmington, DE, USA).

A total of 20 primers each marker (ISSR and SSRs) were used for initial screening. Out of which ten primers each were further screened for polymorphism. PCR was performed in 96-well plates (G-Storm, India). PCR reactions were carried out in 20 µl reaction mixture containing 3 µl of DNA, 2 µl of primer, 10x of *Taq* polymerase buffer with 20 mM $MgCl_2$, 1.5 µl of $MgCl_2$, 1.5 µl of dNTPs and 0.25 µl of *Taq* polymerase for ISSR, whereas 3 µl of DNA, 1 µl of each primer, 10x of *Taq* polymerase buffer with 20mM $MgCl_2$, 1.5 µl of $MgCl_2$, 1.5 µl of dNTPs and 0.3 µl of *Taq* polymerase. The final volume was adjusted with sterile distilled water. The PCR amplifications were performed by

using following thermal profile for each markers, *viz.*, ISSR: 94^{0}C for 5 min (1 cycle); 94^{0}C for 1 min, 40^{0}C for 1 min, 72^{0}C for 2 min (40 cycles); final extension at 72^{0}C for 7 min (1 cycle) and cooling of samples at 4^{o}C, while for SSR the annealing temperature ranged in between 50.9 and 56.7^{0}C, respectively. Amplification was confirmed and alleles were separated by running on 1.5% agrarose gel and electrophoresed in 1.0X TAE at 120 volts for 2 hrs for ISSRs, whereas 4% metaphoreagrose gel and electrophoresed in 0.5 × TBE at 100 volts for 4 hrs for SSRs. The amplified products were visualized on gel documentation system.

The documented marker profiles were carefully examined for banding pattern, polymorphism and number of bands. In both marker systems, scorable bands were recorded as present (1) or absent (0) and based on band data, the similarity matrix was calculated using Jaccard's coefficient. Cluster analysis was carried out using the SHAN module in NTSYS pc 2.1 software (Rohlf 2000). It was further used for construction of dendrogram using unweighted pair group method of arithmetic averages (UPGMA) using Jaccard's coefficient, and three-dimensional (3D) principal coordinate analyses (PCA) carried out through GenAlE x 6.5 software.

Results and Discussion

The preliminary screening was done with 20 five ISSR primers, out of which only ten that produced polymorphic amplification patterns were finally selected for molecular profiling of the grape rootstock genotypes that were known to have wide range of salinity tolerance. In the present study, the detials of total number of bands, number of polymorphic bands, polymorphic information content (PIC) and primer heterozygosity are summarized in Table 1. A total of 69 bands were obtained from selected primers, out of which 61 were polymorphic. Over all the number of bands ranged from a minimum of 4 (UBC 860 and 862) to maximum of 11 (UBC 824). The size of the amplification products ranged from 200 (UBC 859) to 1,500 bp (UBC 809 & 861). The number of polymorphic bands varied from 4(UBC-860 and 862) to 8(UBC-824 and 868) with the average number of polymorphic bands per primer as 6.1. The ten informative ISSR primers produced PIC value which ranged between 0.66 (UBC 862) to 0.86 (UBC 824) with an average of 0.78 and with primer heterozygosity ranging from 0.71(UBC 862) to 0.88 (UBC 824). Similar findings were reported by Choudhary et al. (2014), who found that the average number of bands and average number polymorphic bands/primer detected using seven ISSR primers in four Iranian grape cultivars were 12.8 and 8, respectively. This suggests that there was a wide variation in alleles produced per primer, which may be due to difference among

the genotypes. These differences are tracked as molecular markers to identify desired genes and the resulting traits. It has been reported that the ability to resolve genetic variation may be more directly related to the number of polymorphisms detected by the marker system (Sivaprakash et al. 2004).

For SSR markers, the DNA of 15 grape genotypes (species and interspecific hybrids) was selected to maximize the allelic diversity. Total 24 alleles were identified for the seven analysed loci (VVMD5, VVMD6, VVMD7, VVMD21, VVMD24, VVMD25, VVMD27) with an average of 3.42 alleles per locus (Table 2). Maximum number of alleles were registered with VVDM 5 prime (6 alleles), while minimum was recorded with primer VVDM2.5. The allele heterozygosity for the SSR primers ranged from 0.43 (VVMD 25) to 0.78 (VVMD5). The primer heterozygosity ranged from 0.49 (VVMD 25) to 0.82 (VVMD 5). SSR profile generated for grape genotypes by primer VVMD27 presented in Fig 2. Similar findings were reported by Sefc et al. (1998) who analyzed and discriminated 19 grape rootstock genotypes using microsatellite markers; while Fatahi et al. (2003) for four rootstocks included in Iranian grapevine collections. Several workers also reported diversity and varietal identification in grape rootstocks using SSR markers (Lin and Walker 1998, Dzhambazova et al. 2007, Anuradha et al. 2007, Gizella et al. 2011). Among various marker systems, the most preferential and reliable marker for genotype identification in grape was SSR due to its high level of polymorphism, abundance in the genome, co-dominant nature and Mendelian manner of inheritance. The application of micro satellite markers for genetic characterization of grapevine rootstocks is expected to authentic the genetic purity and uniformity of quality planting material.

The binary data were used to estimate the genetic similarity among the 15 grape genotypes (Tables 3 and 4). It was observed that the genetic similarity ranged from 0.27 to 1.00 in ISSR and 0.05 to 1.00 in SSR analyses. The genetic similarity matrices using ISSR analysis ranged from 27.0 to 85.0%. The closest relationship was observed between Dogridge and Salt Creek, *i.e.,* the two species were 85% similar and Male hybrid and H-144 (71%). On the other hand, the lowest similarity coefficient was recorded between Salt Creek and H-144 (28%).While, the SSRs data used to estimate the genetic similarity among the 15 genotypes ranged from the lowest (5.0%) to the highest as 83.0%. According to the SSR analysis, the highest similarity coefficient value was obtained with the interspecific hybrid 1103Paulsen and 110 Richter, i.e., the two interspecific hybrids were 83% similar as the both shared the common parentage, while the lowest similarity coefficient was observed between Male hybrid and 99 Richter (0.05%).

Table 1. ISSR primers used for finger printing of the grape rootstock and scion genotypes.

Primer name	Primer sequence (5′-3′)	Total No. of bands	Band size (bp)	No. of polymorphic bands	Polymorphic information content (PIC)	Primer heterozygosity
UBC 807	AGA GAG AGA GAG AGA GT	6	300 - 850	5	0.7095	0.7387
UBC 809	AGA GAG AGA GAG AGA GG	9	300 - 1500	7	0.8038	0.8262
UBC 824	TCT CTC TCT CTC TCT CG	11	300 - 2000	8	0.8684	0.8806
UBC 858	TGT GTG TGT GTG TGT GRT	6	300 - 1000	6	0.7835	0.8112
UBC 859	TGT GTG TGT GTG TGT GRC	8	200 - 850	7	0.8508	0.8333
UBC 860	TGT GTG TGT GTGTGT GRA	4	380 - 1000	4	0.6904	0.7387
UBC 861	ACC ACCACC ACCACC ACC	6	300 - 1500	6	0.7526	0.7854
UBC 862	AGC AGCAGCAGC AGCAGC	4	300 - 1150	4	0.6637	0.7178
UBC 868	GAA GAAGAA GAAGAAGAA	9	300 - 1000	8	0.8527	0.8675
UBC 873	GAC AGA CAG ACA GAC A	6	300 - 1100	6	0.7814	0.8080

Table 2. SSR primers used for finger printing of the grape rootstock and scion genotypes.

Primer name	Primer sequence (5′-3′) Forward & Reverse	No. of alleles	Polymorphic information content (PIC)	Primer heterozygosity
VVMD5	CTAGAGCTACGCCAATCCAA TATACCAAAAATCATATTCCTAAA	6	0.787	0.815
VVMD6	ATCTCTAACCCTAAAACCAT CTGTGCTAAGACGAAGAAGA	5	0.751	0.785
VVMD7	AGAGTTGCGGAGAACAGGAT CGAACCTTCACACGCTTGAT	3	0.591	0.665
VVMD21	GGTTGTCTATGGAGTTGATGTTGC GCTTCAGTAAAAAGGGATTGCG	3	0.456	0.551
VVMD24	GTGGATGATGGAGTAGTCACGC GATTTTAGGTTCATGTTGGTGAAGG	3	0.590	0.664
VVMD25	TTCCGTTAAAGCAAAAGAAAAAGG TTGGATTTGAAATTTATTGAGGGG	1	0.438	0.494
VVMD27	GTACCAGATCTGAATACATCCGTAAGT ACGGGTATAGAGCAAACGGTGT	3	0.535	0.611

Table 3. Jaccard's similarity coefficient values based on 10 ISSR primers sequence data in 15 grape species and hybrids.

Genotype	Dogridge	Salt Creek	110R	99R	1103P	1616C	1613C	140Ru	St. George	De Grassette	*V. parviflora*	Male hybrid	H-144	H-516	Perlette
Dogridge	1.00														
Salt Creek	0.85	1.00													
110R	0.68	0.68	1.00												
99R	0.47	0.50	0.43	1.00											
1103P	0.62	0.63	0.66	0.37	1.00										
1616C	0.48	0.45	0.57	0.37	0.63	1.00									
1613C	0.36	0.33	0.47	0.42	0.45	0.61	1.00								
140Ru	0.37	0.34	0.43	0.38	0.35	0.53	0.64	1.00							
St. George	0.38	0.35	0.50	0.39	0.44	0.55	0.57	0.55	1.00						
DeGrassette	0.46	0.42	0.56	0.40	0.54	0.51	0.47	0.43	0.50	1.00					
V. parviflora	0.42	0.35	0.51	0.60	0.36	0.44	0.49	0.44	0.59	0.45	1.00				
Male hybrid	0.31	0.33	0.45	0.49	0.42	0.47	0.49	0.37	0.49	0.61	0.57	1.00			
H-144	0.28	0.27	0.39	0.37	0.42	0.39	0.41	0.34	0.49	0.54	0.44	0.71	1.00		
H-516	0.41	0.43	0.50	0.42	0.38	0.36	0.40	0.46	0.42	0.53	0.35	0.49	0.52	1.00	
Perlette	0.31	0.30	0.32	0.32	0.31	0.32	0.37	0.33	0.33	0.55	0.29	0.40	0.43	0.52	1.00

Table 4. Jaccard's similarity coefficient values based on 7 SSR primers sequence data in 15 grape species and hybrids.

Genotype	Dogridge	Salt Creek	110R	99R	1103P	1616C	1613C	140Ru	St. George	De Grassette	*V. parviflora*	Male hybrid	H-144	H-516	Perlette
Dogridge	1.00														
Salt Creek	0.71	1.00													
110R	0.50	0.57	1.00												
99R	0.37	0.54	0.64	1.00											
1103P	0.37	0.57	0.83	0.70	1.00										
1616C	0.33	0.61	0.50	0.42	0.40	1.00									
1613C	0.14	0.13	0.31	0.08	0.22	0.33	1.00								
140Ru	0.43	0.43	0.66	0.60	0.54	0.46	0.36	1.00							
St. George	0.37	0.46	0.63	0.50	0.64	0.50	0.18	0.33	1.00						
DeGrassette	0.12	0.31	0.42	0.30	0.54	0.33	0.18	0.36	0.30	1.00					
V. parviflora	0.33	0.33	0.33	0.09	0.23	0.36	0.22	0.27	0.44	0.10	1.00				
Male hybrid	0.16	0.14	0.33	0.05	0.23	0.15	0.57	0.27	0.20	0.20	0.43	1.00			
H-144	0.28	0.46	0.27	0.07	0.27	0.50	0.40	0.31	0.25	0.27	0.44	0.30	1.00		
H-516	0.25	0.38	0.28	0.27	0.20	0.42	0.30	0.45	0.15	0.08	0.33	0.20	0.50	1.00	
Perlette	0.28	0.31	0.31	0.08	0.21	0.45	0.33	0.25	0.40	0.09	0.83	0.37	0.56	0.44	1.00

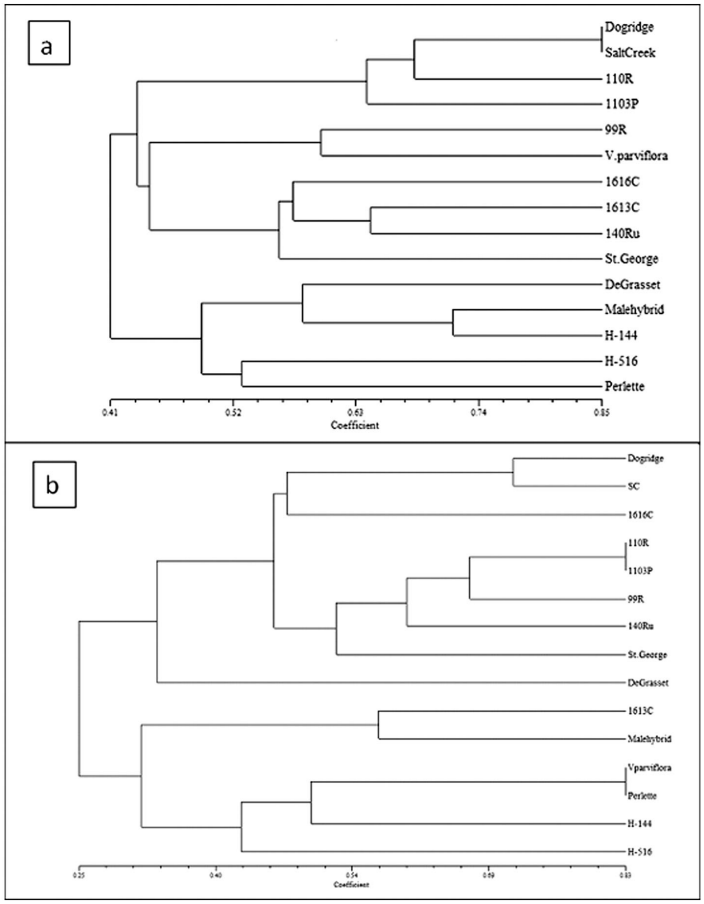


Fig. 1. Dendrogram of genetic relationship of stress tolerant grape genotypes: (a) ISSR and (b) SSR analysis.

The dendrogram based on ISSR similarity indices as shown in Fig. 1a, separated the 15 grape genotypes into two main clusters. Except in a few cases, the rootstock and *vinifera* species having same parentage or species were grouped into same cluster. The first cluster was further divided into three sub-clusters. Group I include Dogridge, Salt Creek into main group, 110 Richtier,1103 Paulsen deviated further from the main group. Group II had 99 Richtier and *Vitis parviflora*, while group III had 1616C,1613C,140 Ruggeri and St. George. The second cluster was grouped into two sub-clusters containing DeGrasette, Male hybrid, and H-144 into one group and H-516 and Perlette in other sub-cluster. The dendrogram based on SSR similarity matrices (Fig. 1b) separated the 15 grape genotypes into two main clusters. The cluster I included all grape rootstock species and interspecific hybrids expect 1613 C, while cluster II

contained the *Vinifera* species and hybrids. The cluster I was further grouped into three sub-clusters. Group I includes the rootstock having the same species status, i.e., Dogridge and Salt Creek into one group and 1616C, which is a multiple species hybrid in the other group. Group II involved all inter-specific hybrids representing *riparia* or *berlandiari* as a parent expect St. George (*rupestris*), which formed a separate sub-group. DeGrasette was placed in a separate group in the cluster I. The second cluster was further grouped into two sub-clusters. The interspecific hybrids 1613C and Male hybrid formed into one group and remaining genotypes and hybrids, *viz.*, *V. parviflora*, Perlette, H-144 and H-516 formed into another group. Several investigations advocated the combined analysis concept to be advantageous compared to single marker systems (Brighurst et al. 1981, Abdel-Tawab et al. 2004). In addition, present results are in aggrement with the findings of Choudhary et al. (2014). Earlier, Aruradha et al. (2007) also reported that the average similarity index for SSR analysis of grape rootstocks ranged from 0.0 to 0.80 and dendrogram obtained from SSR analysis grouped the grape rootstock genotypes into two main clusters, which either represent same species or shared common parentage.

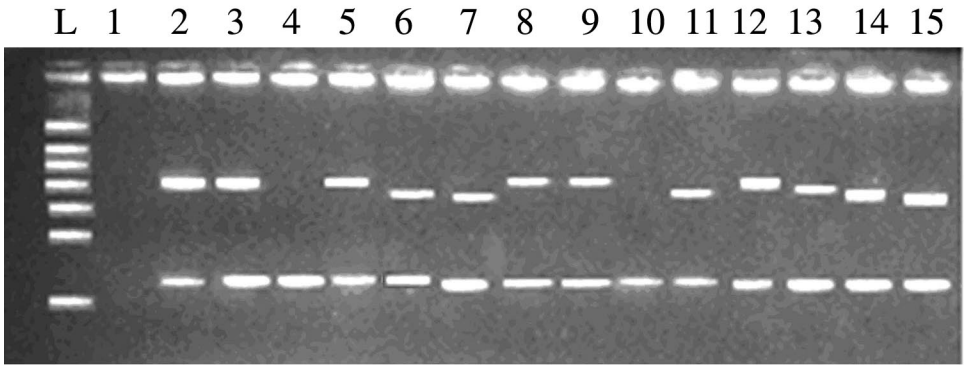
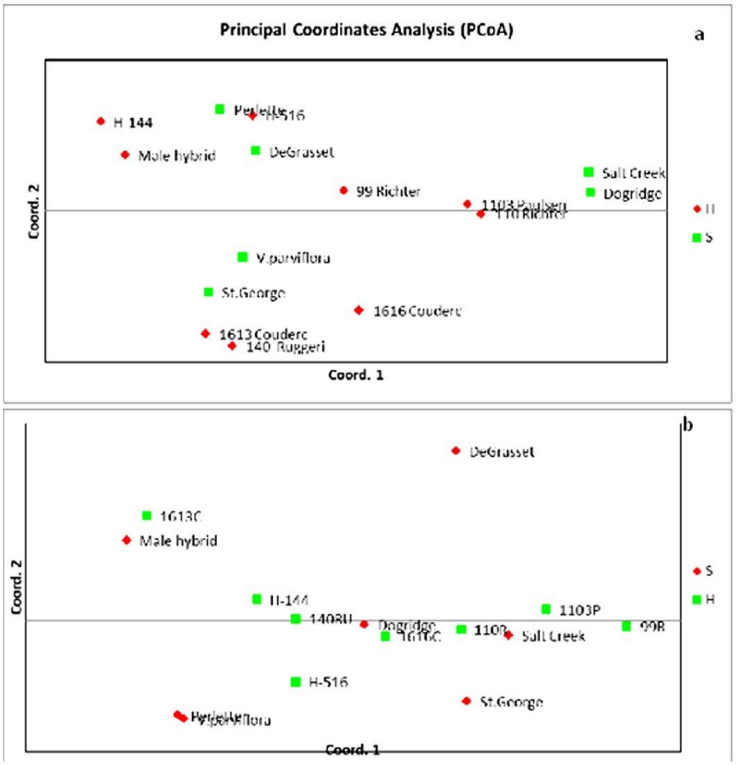


Fig. 2. SSR profile generated for grape genotypes by primer VVMD27, where L = 50 bp DNA ladder. (Lane 1. Dogridge, 2. Salt Creek, 3. 110 R, 4. 99R, 5. 1103P, 6. 1616C, 7.1613C, 8. 140 Ru, 9. St. George, 10. Degrassette, 11. *Vitisparviflora*, 12. Male hybrid, 13. H-144, 14. H-516, 15. Perlette).

The principal coordinate analysis (PCA) further helped in depicting the variability among species and hybrid grape genotypes in three dimentional modes. Clustering based on PCA resulted in two clusters, which were more or less similar to that of dendrogram clustering (Fig. 3a, b). In case of ISSRs, the first three coordinates accounted to 51.92% total variability with the first, second and third accounts for 21.56, 17.54 and 12.82%, respectively, while in case of SSRs, the first, second and third principal coordinate accounted for 28.22, 17.42 and 14.05% of overall variation, i.e. which accounted for 59.70% of total variation. Present observations were in conformity with the findings of Choudhary et al. (2014)



Species	Interspecific hybrid	
Dogridge	110R	140Ru
Salt Creek	99R	Male Hybrid
St. George	1103Paulsen	H-144
Vitis parviflora	1616C	H-516
De Grassette	1613C	
Vitis vinifera cv perlette		

Fig. 3. Three-dimensional plots of Principal Coordinate Analysis(PCA) based on (a) ISSR and (b) SSR analyses.

who also got similar clustering pattern based on PCA of grape suggesting total variability of 93.77%.In another study, Anuradha et al. (2007) reported the total variability of 38% in the first three coordinates among the grape rootstock genotypes. In the present study too the molecular markers (ISSR and SSR) could successfully characterized the grape rootstocks and scion types. The information generated will be helpful in exploiting the genetic information for grape rootstock improvement, and also applied in practice for assessment of clonal fidelity of planting material from nurseries.

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