The Bioscan 14(1):063-068, 2019
AN INTERNATIONAL QUARTERLY JOURNAL OF LIFE SCIENCES
www.thebioscan.com

ANALYSIS OF GENETIC DIVERSITY IN GRAPE (*Vitis vinifera* L.) CULTIVARS BY ISSR (Inter-Simple Sequence Repeats) Markers

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KEYWORDS
Vitis
ISSR markers
Genetic relationship
Diversity

Received on :
24.10.2018

Accepted on :
27.01.2019

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ABSTRACT
The genomic DNA isolated from 19 grape cultivars (*Vitis vinifera* L.) were subjected to PCR amplification using 34 ISSR primers. Of 34 primers, 22 showed polymorphism and produced 182 bands, of which 149 were polymorphic with an average of 81.86 % polymorphism, each primer thus produced on an average 6.77 polymorphic bands. The size of amplified product was ranging from 171.31 bp to 2154.67 bp. The number of amplicons generated by each primer varied from 3 bands (ISSR 828) to 16 bands (ISSR 890).
The similarity coefficient between the genotypes varied from 0.51 to 0.96 indicating low to moderate diversity among the genotypes. The UPGMA based cluster analysis using dice similarity coefficient grouped nineteen grape cultivars into two major clusters. The cluster A had 13 cultivars, while in cluster B there were 6 cultivars. The dendogram generated based on UPGMA method of cluster analysis using ISSR marker data revealed little different but similar grouping of genotypes into two major clusters *viz*., cluster A and cluster B. The distribution of the cultivars in the dendogram was mostly consistent with the known pedigree information and the morphological attributes. Overall results indicate that ISSR markers appears to be reliable and efficient for the assessment of genetic relationship among grape cultivars.

INTRODUCTION

Grapes (*Vitis vinifera* L.) belongs to the family Vitaceae and is one of the most widely grown fruit crop in the world. The majority of commercial grape cultivars around the world belong to vinifera species. . In India, it is grown over an area of around 111 thousands ha. with production of 1235 thousands MT mainly in the states of Maharashtra, Karnataka, Andrapradesh and Tamilnadu (Anon, 2013). The wide biodiversity of grapevine germplasm provides invaluable resources to breeders.

Grape 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 Mbp and has a number of unique features including novel shoot architecture and non-climactric fleshy fruit produced from a perennial deciduous woody vine .

The variability in plant and fruit characteristics has been noticed in Vinifera genus due to heterozygous nature of the crop and thus becomes very difficult to maintain these genotypes without proper documentation. There is an urgent need for accurate documentation and proper identification of grape genotypes.

Usually, the maintenance and evaluation of germplasm is based on phenotypic features such as morphological, physiological or horticultural descriptions. However, descriptions have limited value, as the plant grown at different locations, as only specific developmental stages are suitable for screening and detection of hybridization and pedigree determination. The other problems like influence of environment, human virtual judgement and less genome coverage limit the use of phenotypic markers. Furthermore, the actual identity of some cultivars is still a question, because such cultivars grown in different areas often have various names. Such limitations can be largely avoided by means of molecular characterization *i.e.* DNA profiling.

Grape cultivars are propagated vegetatively, each elite cultivar represents a unique, usually highly heterozygous genotype (Singh and Singh, 2011). Vegetative propagation over several centuries has resulted in the accumulation of genetic erosion. These variations are responsible for the frequent appearance of bud sports, somatic mutants or clones (Upadhyay *et al*., 2011). Cultivation of the same cultivars for long period of time may lead to the genetic erosion confining the subsequent breeding programmes. It is necessary to characterise grape cultivars and introduce new variability in to plant germplasm as well. With passing time several accessions have been lost and there is possibility of mis-nomenclature during material transfer from one locality to another. In this regards, characterization descriptors are comprised of highly heritable qualitative traits that can be equally expressed in all conditions (IPGRI,1997).

Genetic analysis techniques based on molecular markers are able to provide objective information on the genetic potential of a species, because of their simplicity and ease of handling. Thus, helping in effective characterization and exploitation of germplasm in modern agriculture. Molecular markers such as RFLP, RAPD, microsatellites or SSR, ISSR and AFLP are in use for characterization of grape varieties, parentage analysis, identification of clones, studying genetic relationships, genetic maps and marker-assisted selection (Upadhyay *et al*.,2010 and Bahurupe et al. 2013). Inter-Simple Sequence Repeat (ISSR) polymorphic DNA is a fast technique for generating genetic

markers (Choudhary, et al.2014 and Hassan, *et al*.,2011). ISSR are applied in many aspect of genetic research such as cultivar identification, analysis of genetic diversity (Roose, *et al*., 2000). In grapes, ISSR approach has been applied so far to analyses a limited of varieties (Moreno, *et al*.,1998 and Herrera, *et al*., 2002).

Therefore, for improvement, identification and conservation of genetic quality of grape germplasms, the present investigation was undertaken with the objectives to reveal ISSR-based genetic diversity among different grape cultivars along with polymorphism level.

MATERIALS AND METHODS

Plant material

The young primordial leaf samples of nineteen grape cultivars were used present study. For evaluation, nineteen cultivars of grape were obtained from the germplasm collection block of AICRP on Grape, M.P.K.V., Rahuri; NRC on Grape, Pune, ARI, Pune and from some progressive farmers. List of varieties is given in Table 1.

Isolation of genomic DNA from young leaves

The isolation of genomic DNA from fresh young leaves of the grape was carried out by using Genei Pure™ Plant Genomic Purification Kit (Ms. Genie MERCK Chemicals, Ltd. Chennai).

DNA quantification and purity analysis

Quantification of purified DNA was performed using UV visible spectrophotometer (Nanodrop, ND-1000 USA) at 260 and 280 nm wavelengths. The ratio of absorbance 260/280 was calculated. 2 µl of all DNA extracts were subjected to electrophoresis (Bio Rad sub cell model 96 USA) in 0.8 % (w/v) on agarose gel containing 0.5 µg/ml ethidium bromide in 1X TBE buffer. The samples were diluted to a final concentration of 50ng/ì l.

DNA amplification by ISSR Primers

PCR amplification of all 19 grape DNA samples was performed with 34 custom synthesized ISSR primers (Table 2) amplified at different temperatures to standardize the annealing temperature *i.e.* gradient.

PCR amplification was performed in a 0.2 ml PCR tubes having 25 µl reaction volume as described by Sabir *et al*. (2009) with some modifications. PCR amplification reaction mixture contained 1µl genomic DNA, 2.5l of 10X Taq Buffer F (Tris without MgCl2), 1.5 µl each of 25 mM MgCl2 and 10mM dNTP Mix, 1 µl of Primer (ISSR), 0.33µl of 3U/µl TaqDNA Polymerase and 17.17 µl quanity of distilled water.

PCR amplification was carried out using thermal cycler (Perkin Elmer 9600) as

Initial denaturation for 1 min. at 94ºC, 40 cycles of PCR were performed (1 min. at 94ºC denaturation, 1 min at primer-specific annealing temperature (Tm between 40-55ºC), 1 min at 72ºC extension) followed by 7 min at 72ºC final extension stage.

Agarose gel electrophoresis of amplified PCR products
Aliquots of PCR products were electrophoressed in 1.2% Agarose gel with 1x TBE buffer. A 100 bp ladder was used as size standard. The gels were stained with ethidium bromide and photographed by UV transilluminater in gel documentation system (Flour Chem. TM Alpha innotech, USA).

Molecular Data Analysis

The bands in each gel were scored manually for their presence (1) and absence (0) in the ISSR data sheet and Dice similarity coefficients were calculated. The genetic associations between cultivars were evaluated by calculating the Jaccard's similarity coefficient for pair wise comparisons based on the proportions of shared bands produced by primers (Jaccard, 1908). Jaccard similarity matrix was constructed by computerised NTSYSpc 2.02i (Rohalf, 1998) software. Based on similarity matrix, dendogram showing the genetic relationships between genotypes constructed using the Unweighted Pair Group Method Using Arithmetic Averages (UPGMA).

RESULTS AND DISCUSSION

Polymorphism

Data obtained from the analysis of genomic DNA isolated from 19 grape cultivars were subjected to PCR amplification using 34 ISSR primers in order to reveal and verify genetic relationship. Of which 22 ISSR primers amplified and showed polymorphism which are listed in Table 2 and were selected for genetic diversity analysis by considering the repeatability, sharpness and intensity of amplicons.

A total of 182 bands were generated by amplification with 22 polymorphic primers, each primer thus produced on an average 6.77 polymorphic bands. The number of bands produced by each primer varied from 3 (ISSR 828) to 16 (ISSR 890).The range of amplicon size and average number of loci reproduced in the present investigation were in comparison with the earlier studies done by Dhanorkar *et al*. (2005) and Tamhankar *et al*., (2008).

The size of amplified product was ranging from 171.31 bp to 2154.67 bpof which 149 were polymorphic with an average of 81.86 % polymorphism.

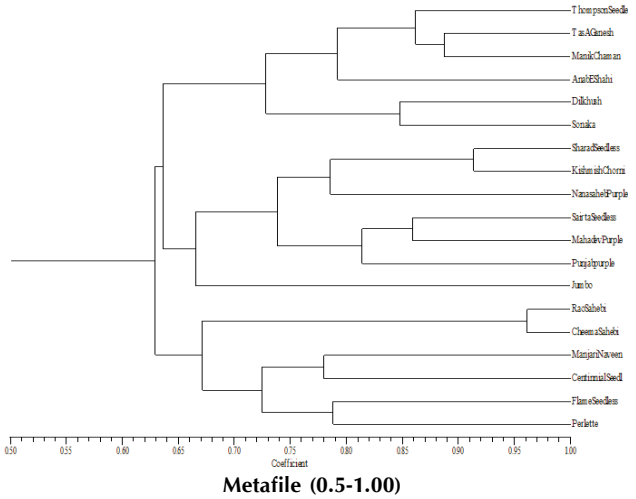
Among the ISSR primers, ISSR 890 produced maximum number of bands (16) followed by ISSR 891 (13 bands) and ISSR 827 (12 bands). However, least number of bands were amplified by ISSR 828 (3 bands).The highest (100%) polymorphism was shown by ISSR 12,ISSR 807, ISSR 828, ISSR 855, ISSR 857, ISSR 890 and NAGPURSSR 8932804 primer, while ISSR 8081 primer showed minimum *i.e.*, 40 % polymorphism.

Table 1 : Grape cultivars for characterization

Sr. No.	Cultivar	Sr. No.	Cultivar
1	Thompson Seedless	11	Nanasaheb Purple
2	Tas-A-Ganesh	12	Mahadev Purple
3	ManikChaman	13	Jumbo
4	Anab-E-Shahi	14	Punjab Purple
5	RaoSahebi	15	Manjari Naveen
6	CheemaSahebi	16	Centinial Seedless
7	Dilkhush	17	Perlette
8	Sonaka	18	Flame Seedless
9	Sharad Seedless	19	KishmishChorni
10	Sarita Seedless		

Table 2: List of ISSR primers and the degree description of the polymorphism obtained among 19 grape varieties

Sr. No.	Primer	No. of bands generated	Poly-morphic bands	Mono-morphic bands	Unique bands	% poly-morphic bands	Fragment size (bp)
1	ISSR 12	10	10	0	0	100	307.39-1874.78
2	ISSR 13	8	6	0	2	75	326.33-1331.27
3	ISSR 8	5	4	0	1	80	454.20-875.94
4	ISSR 807	8	8	0	0	100	283.82-1303.05
5	ISSR 8081	5	2	2	1	40	268.79-980.41
6	ISSR 812	5	4	1	0	80	312.86-874.13
7	ISSR 826	9	5	1	3	55.55	280.84-1408.58
8	ISSR 827	12	9	2	1	75	171.31-1479.56
9	ISSR 828	3	3	0	0	100	716.32-1502.85
10	ISSR 834	11	6	3	2	54.54	360.74-1641.06
11	ISSR 8386	7	6	1	0	85.71	195.09-589.95
12	ISSR 841	6	5	1	0	66.66	303.54-785.24
13	ISSR 855	5	5	0	0	100	331.83-984.44
14	ISSR 857	8	8	0	0	100	472.90-2154.67
15	ISSR 891	13	12	1	0	92.3	262.43-1746.73
16	ISSR 890	16	16	0	0	100	282.05-1736.51
17	NAGPURSSR 8932801	8	7	1	0	87.5	225.66-1212.07
18	NAGPURSSR 8932803	6	3	3	0	50	273.27-1582.84
19	NAGPURSSR 8932804	9	9	0	0	100	272.08-999.10
20	NAGPURSSR 8932806	9	8	1	0	88.88	273.51-1149.42
21	NAGPURSSR 8932808	10	6	0	4	60	172.91-1893.73
22	NAGPURSSR 8932811	9	7	1	1	77.77	392.73-1944.12



Metafile (0.5-1.00)

Figure 1: UPGMA cluster analysis (Dendogram) of 19 grape cultivars by ISSR markers

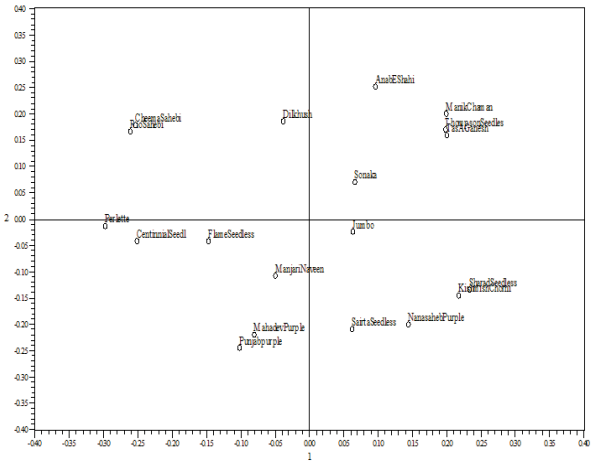


Figure 2: Cluster diagram showing grouping of 19 grape cultivars using ISSR markers

Tamhankar *et al*. (2008) reported that, of 94 ISSR primers used for characterization. Only 16 primers showed polymorphism and used to generate DNA amplification profiles. Total 119 bands were obtained with the number of bands per primer varied from 3-28. The extent of polymorphism in this study was higher as compared to the earlier reports by Dhane *et al*.(2006), Seyedimoradi *et al*. (2012) and Zeinali *et al*. (2012) in ISSR primers, Upadhyay *et al*. (2010) in AFLP markers and Nagaty *et al*. (2011) in RAPD markers for grape cultivars.

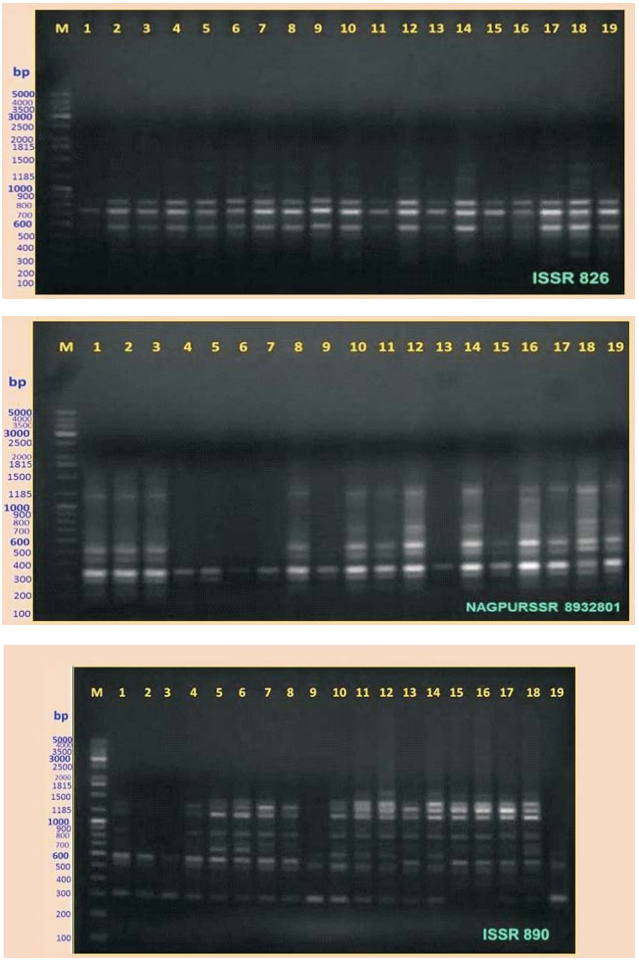
Genetic cluster analysis

Genetic diversity is calculated by genetic distance or genetic similarity where there is either similarity or dissimilarity. Based on band data obtained, the similarity matrix was calculated using Dice coefficient and UPGMA algorithm.

The Dice similarity coefficient ranged from 0.51 to 0.96 (Table 3). This range showed that, cultivars are genetically less or more divergent. Minimum Dice similarity coefficient 0.51 was recorded in between cv. Anab-E-Shahi and Nanasaheb Purple indicating that cv. Anab-E-Shahi is more divergent from Nanasaheb Purple. Maximum Dice coefficient 0.96 was present in between cv. Rao Sahebi and Cheema Sahebi, indicating that these cultivars are less divergent *i.e.* closely related to each other than other cultivars. Similar findings were reported by Dhanorkar *et al*. (2005). He reported the Dice similarity coefficient ranged from 0.65 to 0.96 in grape cultivars by using ISSR primers. Sabir *et al*. (2008) reported that, the dice similarity coefficient ranged from 0.42 to 0.91 for table grape cultivars using ISSR primers. The minimum range in similarity coefficient indicated the presence of low genetic

Table 3: Dice similarity coefficient values based on ISSR marker data of 19 grape cultivars

	Thompson Seedless	Tas-A-Ganesh	Manik Chaman	Anab-E-Shahi	Rao Sahebi	Cheema Sahebi	Dilkhush	Sonaka	Sharad Seedless	Sairta Seedless	Nanasaheb Purple	Mahadev Purple	Jumbo	Punjab purple	Manjri Naveen	Centennial Seedless	Flame Seedless	Perlette	Kishmish Chernyi
Thompson Seedless	1																		
Tas-A-Ganesh	0.88	1																	
Manik Chaman	0.84	0.88	1																
Anab-E-Shahi	0.77	0.77	0.82	1															
Rao Sahebi	0.58	0.62	0.58	0.67	1														
Cheema Sahebi	0.58	0.59	0.57	0.67	0.96	1													
Dilkhush	0.68	0.7	0.67	0.78	0.74	0.74	1												
Sonaka	0.74	0.79	0.72	0.72	0.7	0.67	0.84	1											
Sharad Seedless	0.61	0.68	0.63	0.59	0.53	0.52	0.57	0.67	1										
Sairta Seedless	0.66	0.67	0.62	0.63	0.67	0.65	0.68	0.75	0.79	1									
Nanasaheb Purple	0.6	0.62	0.56	0.51	0.56	0.55	0.58	0.71	0.78	0.82	1								
Mahadev Purple	0.61	0.62	0.57	0.6	0.7	0.68	0.67	0.68	0.67	0.85	0.74	1							
Jumbo	0.64	0.66	0.58	0.59	0.62	0.61	0.66	0.73	0.64	0.68	0.73	0.65	1						
Punjab purple	0.57	0.6	0.54	0.57	0.66	0.63	0.65	0.71	0.66	0.78	0.69	0.84	0.63	1					
Manjri Naveen	0.57	0.62	0.56	0.58	0.61	0.57	0.59	0.7	0.6	0.68	0.68	0.64	0.75	0.71	1				
Centennial Seedless	0.57	0.59	0.58	0.6	0.71	0.68	0.67	0.66	0.52	0.68	0.57	0.69	0.62	0.75	0.78	1			
Flame Seedless	0.58	0.6	0.61	0.62	0.65	0.65	0.66	0.67	0.56	0.71	0.63	0.71	0.64	0.67	0.72	0.75	1		
Perlette	0.55	0.57	0.55	0.57	0.74	0.73	0.68	0.66	0.53	0.66	0.57	0.72	0.6	0.7	0.65	0.75	0.78	1	
Kishmish Chernyi	0.645	0.7	0.68	0.59	0.55	0.55	0.6	0.72	0.91	0.84	0.78	0.71	0.64	0.69	0.61	0.57	0.59	0.57	1



1 Thompson Seedless 2.Tas-A Ganesh 3.Manik Chaman 4 Anab E-Shahi 5Rao Sahebi
6Cheema Sahebi 3.Dilkhush 4.Sonaka 9 . S h a r a d Seedless 10.Sarita Seedless
11. Nanasaheb Purple Purple 12. Mahadev Purple 13. Jumbo 14. Punjab 15.Manjari Naveen
16. Centennial Seedless Chomi 17.FLame Seedless 18.Perlette 19.Kishmish

Plate 6: ISSR Polymorphism of greape cultivars

diversity in material studied and hence need to be wide the germplasm with exploration of the other parts of India for characterization.

Genetic linkage among 19 grape cultivars was generated by UPGMA based dendogram cluster analysis diagram. The cultivars under study were grouped into two major clusters 'A' and 'B'. Cluster A was divided into two sub-clusters *viz*., A1 and A2, whereas cluster B was divided into two sub-clusters B1 and B2. Sub-cluster A1consisted 6 green cultivars and in cluster A2, 7 black-purple cultivars. Sub-cluster B1 consisted green two cultivars, while sub-cluster B2 consisted four green coloured cultivars.

Several subgroups based on their colour, flavour and seeds were observed in this cluster indicating the diverse nature of these varieties. Sub-cluster A1consisted 6 green cultivars, of which Anab-E-Shahi and Dilkhush are seeded. In cluster A2, 7 black-purple cultivarswere grouped, among these cv. Jumbo separated initially from them into an individual cluster. The

cvs. Thompson Seedless, Tas-A-Ganesh, Manik Chaman and Anab-E-Shahi were grouped into subcluster A1(a). Among these, all are seedless except Anab-E-Shahi. Sub cluster A1(b) consisted only two cultivars *viz*., Sonaka and Dilkhush. The sub-cluster A2 (a) consisted 6 cultivars, *viz*. Kishmish Chernyi, Sharad Seedless, Sarita Seedless, Nanasaheb Purple, Mahadev Purple, Jumbo and Punjab Purple, all were black-purple in colour. Sub-cluster B1 consisted cvs. Rao Sahebi and Cheema Sahebi, while Sub-cluster B2 consisted seedless, green coloured cultivars *viz*., Manjri Naveen, Centennial Seedless and Perlette except the cv. Flame Seedless which was rose-pink in colour.

Dhanorkar *et al*. (2005) reported that Kali Sahebi reddish purple variety grouped in subgroup with yellow-green varieities, while Motia, Foster Seedling and Thompson Seedless grouped with black-purple varieties. Dhane *et al*. (2006) also found similar results in case of green-yellow varieties, that three varieties Italia, Queen of Vineyard and ThompsonSeedless having yellowish green berries were also grouped with Red-black varieties.

The Dice similarity coefficient ranged from 0.51 to 0.96. Minimum Dice similarity coefficient 0.51 was recorded in between cv. Anab-E-Shahi and Nanasaheb Purple indicating that cv. Anab-E-Shahi is more divergent from Nanasaheb Purple. Maximum Dice coefficient 0.96 was present in between cv. Rao Sahebi and Cheema Sahebi, indicating that these cultivars are very less divergent *i.e*. closely related to each other than other cultivars.

The Dice similarity coefficient of cv. Thompson Seedless with Tas-A-Ganesh was 0.88 and with Manik Chaman was 0.84. This shows that cvs. Tas-A-Ganesh and Manik Chaman are less divergent from Thompson Seedless. Also, Dice similarity coefficient between cv. Thompson Seedless and Sonaka was 0.74, indicating that these cultivars are more divergent. Because, cvs. Tas-A-Ganesh, Manik Chaman and Sonaka are selection from Thompson Seedless. Dilkhush is selected from Anab-E-Shahi and Dice similarity coefficient 0.78 was recorded in between them, indicating that these two cultivars are more divergent.

The cvs. Kishmish Chernyi and its bud sport Sharad Seedless had 0.91 Dice coefficient showed that these two cultivars are closely similar with each other. The Dice similarity coefficient of cv.Sharad Seedless with its clonal selection *viz*., Sarita Seedless, Nanasaheb Purple, Mahadev Purple and Jumbo was 0.79, 0.78, 0.67 and 0.64 observed, respectively. These Dice similarity results indicating that these cultivars are more divergent. The cvs. Centennial Seedless and its selection Manjri Naveen had 0.78 Dice coefficient showed that these two cultivars are more divergent from each other. The cv. Punjab Purple showed maximum Dice similarity coefficient *i.e*. 0.84 with cv. Mahadev Purple, indicating that these cultivars are less divergent.

From the Dice similarity coefficient values varietal combinations are recorded with two values as < 0.60 and > 0.85. The maximum varietal combinations with Dice similarity coefficient < 0.60 are found in cv. Manik Chaman *i.e*. eight combinations followed by cvs. Thompson Seedless, Cheema Sahebi and Nanasaheb Purple (each of 7 combinations), while least varietal combinations with Dice similarity coefficient < 0.60 are found in cv. Mahadev Purple (one varietal combination) followed by Jumbo (2 varietal combinations). With Dice similarity coefficient > 0.85 are found in cv. Tas-A-Ganesh *i.e*. two combinations, while in cvs. Thompson Seedless, Rao Sahebi, Cheema Sahebi, Sharad Seedless, Sarita Seedless and Kishmish Chernyi only one varietal combination was recorded.

From the consensus tree (dendogram), it is observed that distant genetic similarity was found between different grape cultivars. Cultivars Rao Sahebi and Cheema Sahebi had close similarity followed by in between Sharad Seedless and Kishmish Chernyi. The cv.Anab-E-Shahi was far divergent from Nanasaheb Purple. From the Dice coefficient and dendogram, it is revealed that these cultivars fall in different clusters and sub-clusters may results in obtaining heterosis in relation to various characters.

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