Genetic Diversity among Grapevine (Vitis Vinifera L.) Cultivars of Tartous Province (Syria) using Microsatellite Markers

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Abstract

Genetic variation among 32 grapevine (Vitis vinifera L.) accessions belongs to 23 cultivars from Tartous province were analysed using 10 SSR primers (Simple Sequence Repeat). The ten SSR loci generated (44) alleles, of which (32) were polymorphic (72.72%). The number of SSR markers per primer pair ranged from 3 (VVMD8 and SCU10VV) to 7 (VVS5) with an average of 4.4 alleles/primers. Two loci (VVS1 and VVMD8) gave 100% polymorphic alleles.

Cluster analysis separated the investigated cultivars in six major groups with a genetic variance of 0.29. High level of genetic diversity obtained in the collection may be attributed due to continuous seed propagation by birds, natural hybridization between local and introduced cultivars and human selection. Levels of polymorphism detected within the studied accessions proved to be sufficiently genetically diverse and therefore these accessions would be useful in grapevine breeding programs.

Keywords - *Grapevine, Genetic diversity, SSR technique, cluster analysis.*

I. INTRODUCTION

Vitis vinifera L. belongs to the family Ampelidaceae [1]. However, Vitaceae is the most recent and common classification of the family. The Vitaceae contains 17 genera, the genus *Vitis* is the most important one within the family, and its divide into two subgenera: *Muscadinia* Planch. Includes two or three species and *Vitis* Planch. With more than 60 species distributed in the temperate regions of Asia, America and Europe ([1], [2]).

All *cultivated* grapevines belong to the genus *Vitis* [1]. The European vine species *V. vinifera subsp. Sativa* is considered one of the most popular and widely used species, while the European wild grape *V. vinifera subsp. Sylvestris* which is used in breeding programs and genetic improvement of planted grapevine. Recently, phylogenetic analysis using plastid DNA markers of the two taxa subsp. *sylvestris* and subsp. *vinifera* suggests at least two origins for cultivated grapevine, one in the Near East and a second in the Iberian Peninsula [3].

Grapes (*Vitis* spp.) are one of the world's most economically valuable fruit crops. It's believed to be originates from the area around the Caspian Sea (between Asia and Europe) or the area between the South Black Sea and the Caucasian Sea in Asia Minor [4]. The ancestor of cultivated grapevine, later spread over a relatively large area ranging from Spain and North Africa in the west, over the Central European rivers and beyond the Caucasus in the east, and cultivated later in Australia and southwestern America [4]. According to the annual agricultural statistical group 2016, grape takes up the fourth place in terms of importance among fruitful trees, where the number of cultivars is estimated to be about 100 [5]. This large number of planted species provides clear evidence of the large size of the genetic base of grapevine in Syria. Therefore, it is important to expand classification studies, both in their morphological and molecular parts, in order to preserve and multiply the most desirable and genetically different cultivars. Recently, the development of DNA-based markers has provided more objective and reliable alternatives for species and cultivar identification.

SSRs (Simple Sequence Repeat) have become a favorite type of DNA marker for molecular identification studies because they are highly informative, codominant, multi-allele genetic markers that are experimentally reproducible and transferable among related species [6]. In particular, it is a useful marker in studies of diversity and genetic distance, gene flow and crossing over rates, evolutionary studies, as well as genetic relations.

[7] first investigated the use of SSR marker for identifying grapevine cultivars. They showed that microsatellite sequences were very informative for identifying *V. vinifera* cultivars. [8] analyzed six SSR loci to evaluate the genetic variability and cultivar relatedness in 25 *Vitis vinifera varieties* from Peru and Argentina. The results show high level of polymorphic information content ranged from 0.70 to 0.88 which indicating that the SSRs were highly informative and could be used to detect genetic variation within grapevine varieties.

Ten SSR loci were analyzed to evaluate genetic variation within 190 grapevine including varieties

from Europe, America and some hybrids, the results show 67 different genotypes, from which 27 were described for the first time [9]. Most of the Iranian collection grape cultivars were analyzed using 23 SSR loci, the number of alleles obtained ranged between (2-15) with an average of 8.65 alleles/locus, five SSR markers showed high degree of polymorphism with PIC values greater than 0.80 which could be used for rapid fingerprinting of many grape genotypes [10].

Eight SSR loci were used to determine the genetic variance among 40 wild grape species and grape cultivars, the SSR genotyping showed that wild grape species are more highly diverse than cultivated grapes [11].

Two studies on grape cultivar characterization based on SSRs have been performed in Syria. [12] used 20 SSR markers to characterize and determine genetic variation in six Phylloxera resistance grape rootstocks. While [13] analyzed a sample of 5 accessions from which three were local cultivars and two well-known international grape cultivars using twenty pairs of primers, The SSR markers were able to amplify 36 alleles of which 24 were polymorphic. Cluster analysis grouped the studied cultivars into three clusters, the local cultivar Zeiny formed the first group, Alfons Lafalieh formed the second one, while the third cluster includes two local cultivars Aswad Aady and Aswad Qarry, and the second sub cluster and the interred cultivar Mosqat Italy.

Despite the importance of grape in Syrian agriculture economy, and the long-standing tradition of grape cultivation, no clear characterization has been undertaken until recently. In this study, SSR markers were employed for characterizing genetic variation and cultivar relatedness in most important grape varieties cultivated in Tartous province, Syria. The integration of the obtained results with ampelographic data allow to univocally identify the studied cultivars and could become a significant tool in cultivation and breeding programs of grape.

II. MATERIALS AND METHODS

A. Plant materials

A total of 32 grapvine accessions belong to twenty-three local and interred grape cultivares were taken from eight vineyards in Tartous Province (coastal city of Syria) were analyzed in the present study. The cultivars used in this study are listed in (Table 1).

TABLE 1: The list of grape (*Vitis vinifera* L.) cultivars used for SSR analysis, area of cultivation and altitude.

N	Accession	Cultivar-common name	Area of cultivation	Altitude
1	D1	Black	Aldenbeh	360
2	K1	Red Halwani	Kaf Al–hammam	380
3	K2	Red Halwani	Kaf Al–hammam	580
4	K3	Black	Kaf Al–hammam	580
5	K4	Black Turkish	Kaf Al–hammam	475
6	H1	Black Jbee	Al–delibe	650
7	H2	Red	Al–delibe	650
8	H3	Early Black	Al–delibe	650
9	H4	Red Halwani	Al–delibe	650
10	R1	Red June	Khirbet Mahasin	650
11	L1	Al–rakmi	Black	L1
12	G1	Bstan Al–seagull	Jbee	G1
13	G2	Bstan Al–seagull	Jbee	G2
14	G3	Bstan Al–seagull	Khomeini	G3
15	G4	Bstan Al–seagull	Early Red	G4
16	G5	Bstan Al–seagull	Early Black	G5
17	G6	Bstan Al–seagull	Zine	G6
18	G7	Bstan Al–seagull	Jbee	G7
19	G8	Bstan Al–seagull	Halwani	G8
20	G9	Bstan Al–seagull	Early Halwani	G9
21	S1	Sergis	Black June	S1
22	S2	Sergis	Compact Jbee	S2
23	S3	Sergis	Black	S3
24	S4	Sergis	French Halwani	S4
25	S5	Sergis	Heart of the bird	S5
26	S6	Sergis	Compact Halwani	S6
27	S7	Sergis	Compact French Halwani	S7
28	S8	Sergis	Turkish Halwani	S8
29	S9	Sergis	Black Turkish	S9
30	E1	Qali'a	Apple	E1
31	E2	Qali'a	Halwani	E2
32	E3	Qali'a	Green Halwani	E3

B. Genomic DNA Isolation and selection of SSR primers:

DNA was prepared by grinding 5 g of young Silica-gel dried leaves from each plant in liquid nitrogen, 2 ml of preheated CTAB extraction buffer added with 1% PVP (40000). Samples were incubated at 65°C for 30-45 min in a water bath. Subsequently, the mixture was treated twice with 600 µL chloroform-isoamyl alcohol (24:1) and centrifuged for 10 min at 14000 rpm. DNA precipitation was performed following the method described by [14]. The quality of the DNA was estimated on an agarose gel (0.1%). Gels were stained with ethidium bromide,

visualized with a UV trans illuminator (CAMAG Reprostar3 - Switzerland).

A set of 10 SSR primers (Metabion, Germany) were screened initially for polymorphism within the studied grapevine cultivars. The SSR markers were selected depends on former studies Fand considered as suitable loci for grapevine analysis (Table 2).

Table 2. SSR loci used for grapevine identification

Primer	Altitude	Annealing Temp.	Ref.
VVS1	F:ACAATTGGAAACC GCGTGGAG	55	[7]
	R:CTTCTCAATGATA TCTAAAACCATG		
VVS3	F:TGCCCTATCAATTA GTTCACCTA	55	[7]
	R:TCGACTTTGATAT ATTGATGATT		
VVS5	F:ATTGATTTATCAA ACACCTTCTACAT	55	[7]
	R:TAGAAAGATGGAA GGAATGGTGAT		
VVMD5	F:CTAGAGCTACGCC AATCCAA	56	[15]
	R:TATACCAAAAATC ATATTCCTAAA		
VVMD7	F:AGAGTTGCGGAGA ACAGGAT	52	[15]
	R:CGAACCTTCACAC GCTTGAT		
VVMD8	F:TAACAAACAAGAA GAGGAAT	54	[15]
	R:AGCACATCCACAA CATAATG		
SCU10VV	F:TACCCCCACAACC CTTTTTCCC	56	[16]
	R:TTCTCCGCCACCTC CTTTTCAC		
SCU12VV	F:GAATTCGGCACGA GGAACTA	50	[16]
	R:ACAGTGGAGAGGT GAATGCA		
SCU14VV	F:CTGCACTTGAATA CGAGCAGGTC	54	[16]
	R:TGTTATATGATCCT CCCCCTCCTC		
SCU15VV	F:GCCTATGTGCCAG ACCAAAAAC	54	[16]
	R:TTGGAAGTAGCCA GCCCAACCTTC		

C. PCR amplification and electrophoresis of SSR markers

Amplification was performed using PCR – Flexi gene (Germany) in a reaction volume of 20 µl containing 2 µL of genomic DNA ((20ng/µL)), 2.5 µL buffer (10X), 1.5 mM MgCl2 (25mM), 2.5 mM of each dNTP, 1.25 µM both primers and 0.2 unit of DNA Polymerase (5U).

Amplification condition were performed as initial DNA denaturation at 94 °C for 3 min., followed by 38 cycles of 30 s denaturing at 94 °C, 30 s annealing at 52 - 56 °C (temperature varied for individual locus) and 1 min extension at 72 °C. The last cycle of the extension was prolonged to 5 min and the amplification products were then stored at 4 °C.

PCR products were separated by electrophoresis in 2% agarose gels with 1x TBE (90 mM Trizma base, 90 mM boric acid, 2 mM EDTA) buffer, using a voltage range of 80 V for 2.5 h. The gel was stained with 0.3 mg/ml ethidium bromide and visualized on a UV trans illuminator and photographed by Biometra, GelDoc-Analyze (Germany).

D. Data analysis

In our molecular analysis using SSR markers, the polymorphic bands were scored as either present (1) or absent (0). A matrix of genetic distances was constructed for the 32 accessions and interred into the Numerical Taxonomy and Multivariate Analysis System (NTSYS) [17].The level of similarity between accessions was established as the percentage of polymorphic bands, and a matrix of genetic similarity was compiled using Jaccard's similarity coefficient (JSI). Similarity coefficients were used to construct the dendrogram using the unweighted pair group method with arithmetic average (UPGMA) and the sequential hierarchical and nested clustering routine in the NTSYS pc program, representing genetic relationship between the studied grapevine verities.

III. RESULTS AND DISCUSSION

Results showed that all SSR primer-pairs gave good amplification and variable patterns among the studied grapevine cultivars. The total number of detected alleles were 44 from which were 32 polymorphic (57.14%). The number of alleles detected per locus ranged from three for both VVMD8 and SCU10VV to 7 for VVS5, with an average of 4.4 alleles/primers. The number of alleles per locus ranged from 2 to 5. Two loci (VVS1 and VVMD8) gave 100% polymorphic alleles, while the lowest percentage of detected alleles (50%) resulted from the loci VVS3, The numbers of bands generated by each primer is given in Table 3, the ISSR profiles of the amplification products of two primers are shown in Fig. 1.

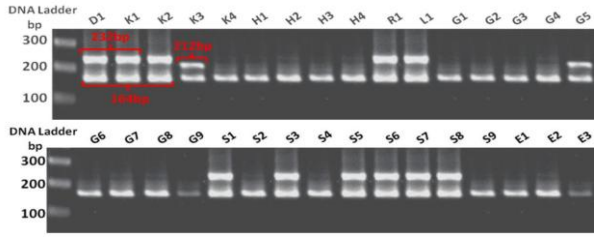
Unique DNA bands with different sizes were detected in the cultivars, Some of the primers produced polymorphic bands specific to a set of cultivars such as K1 and K2 (Red Halwani), G1 and G2 (Jbee), but no specific bands were detected in particular cultivar.

TABLE 3: Genetic parameters of 10 SSR loci used in the grape cultivars.

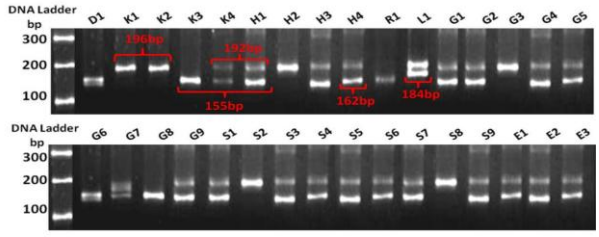
No	Primer code	NE*	ND*	NV*	PV*	AS*
1	VVS1	8	5	5	100	155-196
2	VVS3	4	4	2	50	210-219
3	VVS5	12	7	5	71.42	188-220
4	VVMD5	8	5	3	60	248-226
5	VVMD7	11	6	4	66.66	263-220
6	VVMD8	6	3	3	100	141-

						180
7	SCU10VV	2	3	2	66.66	205-268
8	SCU11VV	1	4	3	75	220-245
9	SCU14VV	2	4	3	75	168-188
10	SCU15VV	3	3	2	66.66	164-232
Sum		57	44	32	72.72	141-268

*Number of expected allels (NE), number of detected alleles (ND), effective number of polymorphic allele (NV), Percent of variant alleles (PV), and allele size base pair (AS).



SCU15VV



VVS1

Figure 1: Examples of tested primers used in the present study, primer SCU15VV produced three alleles (232, 212, 164bp) displayed in the cultivars, from which two were polymorphic (232, 212bp); primer VVS1 produced five polymorphic bands.

In the dendrogram generated from the matrix of pairwise distances, based on microsatellite data, six main groups were distinguished with a genetic distance of 0.29 (Figure 2). The first group included the largest number of studied grapevine accessions (11 accession) that were scattered in two branches. The first branch included seven accessions, five of which are belongs to black cultivars (3 black, 2 Turkish black), While the second branch included four accessions belongs to three cultivars (one red (H2), two early blacks (H3, G5), in addition to the only shrub representing the apple cultivar in the current study (E1).

The second group include three accessions belongs to the Red-Halwani cultivar (E2, K2, K1), the same number of accessions were observed in the third group but belongs to three different varieties (S1) Black June, (S5) Bird Heart and (S8) Turkish Halwani.

The fourth group was subdivided in two subgroups with a genetic distance of 0.23 at the cut point, The first subgroup was composed by seven accessions belongs to five cultivars Halawani, early Halawani, Green Halwani, Red June and the only accession representing cultivar Ziene in our study (G6). The second subgroup included three accessions (L1, G3, G7) belonging to three cultivars respectively (Black, Khomeini, Jbee).

Group 5 is composed only by two accessions, H1 Gbai and S2 Compact Gbai, whereas the sixth

The rest of the studied accessions clustered in group 6. All belongs to cultivar Halawani (S4 French Halwani, S6 compact Halwani, S7 French compact Halwani).

The closest genetic relationship was observed between the two accessions K1 and K2 (0.00) both belongs to Red Halwani cultivar, followed by G4 (Early Red) and G9 (Early Halwani) (0.04), while the largest genetic distance was observed between the cultivars S4, S6, S7 and all other varieties (0.29).

Our study is the first genetic diversity analysis of the grapevines in Tartous province of Syria. The study revealed a large genetic diversity within the grapevine cultivars. Furthermore, the accessions belongs to one cultivar are not clustering in ways concordant with geographic origin. A high level of gene diversity detected in grape cultivars could be inherited from their wild ancestors and preserved due to the practice of vegetative propagation. In contrast to [15] who found low levels of genetic stability in vineyards of cv. 'Italy' cultivated in six different Brazilian regions and indicated vineyards with less genetic stability as a possible source of somatic mutants and genetic variation.

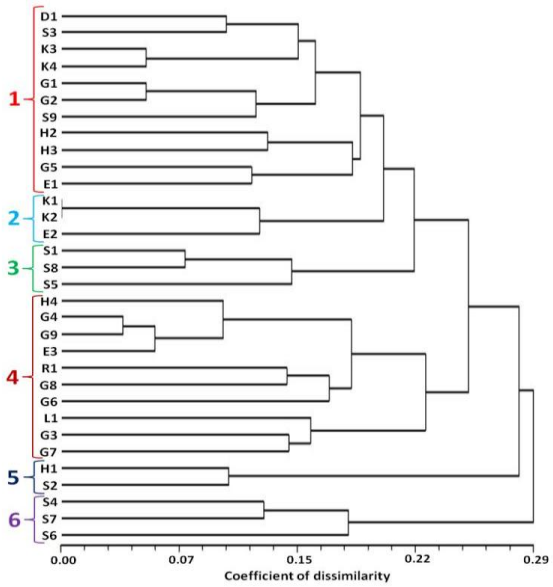


Figure. 2: Cluster analysis of 32 grapevine accessions by the UPGMA clustering, using the arithmetic complement of weighted index based on ten microsatellite markers.

In this study, the general dissimilarity ratios among the cultivars were found to be between 0.00 and 0.29, showing a parallelism with other SSR analysis performed by other researchers ([5], [10], [11]). Due to the high discriminative power of SSR markers to detect genetic variances between different genotypes and species even within plants propagated in tissue

culture technique (Sefc et al., 1998), the finding of identical genotypes in two different plants (as Red Halwani K1, K2) is strong evidence that these two accessions were propagated from the same plant.

in the present study, No single allele was specific to an individual genotype, but In most of the genotypes belongs to the same Cultivar such as Gibai (G1, G2), Red Halwani (K1, K2) and French Halwani (S4, S7), unique alleles with different sizes were detected, but not in the others. The UPGMA dendrograms constructed from the SSR marker showed that, most of the shrubs of the same variety were clustered in one group or closely related groups. [10] found similar results in their study of different grape cultivars (table, wine, juice, drying) cultivated in a small geographical area in Iran. They detected that the grapevine classified as table-grape with white- Raspberry fruit colored were included in one group in the cluster analysis in spite of their genetic variation.

Low levels of polymorphism found for the studied varieties at 10 SSR loci compared to that reported for other *V. vinifera* germplasms assessed with SSR markers. However, factors such as number and nature of genotypes analyzed ([18], [19], [20]), the number of SSR loci and repeat types have been reported to influence allelic differences. e.g. [18] reported 6–11 alleles per locus in 77 studied cultivars, [21] resolved 4–16 alleles with an average of 11.4 alleles per locus, in 62 varieties of Iranian grapevine collection. [22] Profiled 136 Iranian genotypes together with 36 European cultivars at nine SSR loci, they detected 6–12 alleles with an average of 9.33 alleles per locus. While the mean number of alleles detected per locus in our study ranged from 3 to 7, with a mean value of 4.4 alleles per locus, is high when compared to the study of [16] which include only two grapevine cultivars.

IV. CONCLUSIONS

In past decade, SSR markers have been extensively used for molecular characterizations of grape. The studied accession revealed high polymorphism levels, which may be attributed due to continuous seed propagation by birds, natural hybridization between indigenous and introduced plants and human selection. The study confirms the microsatellites are very useful tool for the identification and characterization of Syrian grapevine cultivars.

REFERENCES

[1] M. G. Mullins, A. Bouquet, and L. E. Williams, “Biology of grapevine,” Cambridge University Press, Cambridge, pp. 251, 1992.

[2] C. Arnold, A. Schnitzler, A. Douard, R. Peter, and F. Gillet, “Is there a future for wild grapevine (Vitis vinifera subsp silvestris) in the Rhine Valley?,” Biodiversity and Conservation, vol. 14 (6), pp. 1507-1523, 2005.

[3] R. Arroyo- García, L. Ruiz- García, L. Bolling, R. Ocete, M. A. López, and C. Arnold, “Multiple origins of cultivated grapevine (Vitis vinifera L. ssp. sativa) based on chloroplast DNA polymorphisms,” Molecular Ecology, vol. 15, pp. 3707–3714, 2006.

[4] M. H. K. Nazif, M. I. Atef, I. Mohamed, and A. H. Abdul-Fattah, “Grapes (cultivation, care, and production). El maaref Establishment Alexandria,” pp. 455, 1990.

[5] F. Abdul Rahman, B. M. Muhammad, and F. Hamed, “Molecular characterization of some Vitis vinifera L. Grape varieties Localized and involved in Suweida Governorate using SSR Technology,” Journal of Al-Baath university, vol. 39(16), 2017.

[6] A. S. Mason, SSR Genotyping. In: J. Batley, editor, “Plant Genotyping,” Springer, New York, pp. 77–89, 2015.

[7] M. R. Thomas, and N. S. Scott, “Microsatellite repeats in grapevine reveal DNA polymorphisms when analysed as sequence tagged sites (STSs),” Theor. Appl. Genet, vol. 86, pp. 985–990, 1993.

[8] L. E. Martinez, P. F. Cavagnaro, R. W. Masuelli, and M. Zuniga, “SSR-based assessment of genetic diversity in South American Vitis vinifera varieties,” Plant Science, vol. 170, pp. 1036–1044, 2006.

[9] M. R. Schuck, F. M. Moreira, M. P. Guerra, J. A. Voltolini, M. S. Grando, and A. L. da Silva, “Molecular characterization of grapevine from Santa Catarina, Brazil, using microsatellite markers,” Pesq. agropec. Bras, vol. 44(5), pp. 79-84, 2009.

[10] H. Doulati-Baneh, S. A. Mohammadi, and M. Labra, M, “Genitic structure and diversity analysis in Vitis vinifera L. cultivars from Iran using SSR markers,” Scientia Horticulturae, vol. 160, pp. 29-36, 2013.

[11] N. Goto-Yamamota, A. Azuma, N. Mitani, and S. Kobayashi, “SSR Genotyping of Wild Grap Species and Grap Cultivars of Vitis vinifera and V. vinofera x V. labrusca,” J. Japan. Soc. Hort. Sci, vol. 28(2), pp. 125-130, 2013.

[12] W. A. Al-yousef, W. I. Mohsen, A. F. Al-Tahir, and A. S. R. Abu al-Jadayel, “Molecular characterization of some grape origins published in Syria using molecular indicators SSR,” Jordanian journal of Agricultural sciences, vol. 8(3), 2013.

[13] F. Abdel Rahman, B. Muzher, and F. Hamed, “Molecular characterization for some local and introduced grape cultivars Vitis vinifera .L in Sweida governorate using SSR technique,” Albaath University Magazine, vol. 39(16), pp. 45-65. 2017.

[14] G. Khal, “The Dictionary of Gene Technology: genomics, transcriptomics, proteomics,” Wiley-VCH, Weinheim, pp. 941, 2001.

[15] J. E. Bowers, G. S. Dangl, R. Vignani, and C. P. Meredith, “Isolation and characterization of new polymorphic simple sequence repeat loci in grape (Vitis vinifera L.),” Genome, vol. 39, pp. 628-633, 1996.

[16] K. D. Scott, P. Eggler, G. Seaton, M. Rossetto, E. M. Ablett, L. S. Lee, and R. J. Henry, “Analysis of SSRs derived from grape ESTs,” Theor Appl Genet, vol. 100, pp. 723-726, 2000.

[17] F. J. Rohlf, “Numerical taxonomy and multivariant analysis system. NTSYS version 2.11a.Applied Biostatistics Inc.,” New York, Stoy Book. N.Y. USA, pp. 231, 2002.

[18] S. H. Zeguim Maria, S. Oliveira-Collet, C. A. Manolin, and M. F. Machado, “Differential genetic stability in vineyards of the cultivar ‘italy’ (vitis vinifera L.) Cultivated in different regions of southern and southwestern Brazil,” Ciência Téc. Vitiv, vol. 33(1), pp. 66-77, 2018.

[19] S. A. Tamhankar, S. G. Patil, and V. S. Rao, “Assessment of the genetic diversity of some important grape genotypes in India using RAPD markers,” Vitis, vol. 40, pp.157-161, 2001.

[20] P. C. Souza-Leäo, and S. Y. Motoike, “Genetic diversity in table grapes based on RAPD and microsatellite markers,” Pesq. agropec. bras, Brasília, vol. 46(9), pp.1035-1044, 2011.

[21] R. Fatahi, A. Ebadi, N. Bassil, S. A. Mehlenbacher, and Z. Zamani, “Characterization of Iranian grapevine cultivars using microsatellite markers,” Vitis, vol. 42(4), pp.185- 192, 2003.

[22] J. Najafi, L. Alipanah, B. Ghareyazie, S. A. Mohammadi, A. Hagh-Nazari, and P. This, “Genetic diversity of Iranian and some of European grapes revealed by microsatellite markers,” Iran. J. Biotech, vol. 4, pp. 36–44, 2006.