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Genotyping of Sixteen Grapevine *(Vitis vinifera* L.) Cultivars in Palestine Using Microsatellite Markers

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Abstract

Cultivated grapevines (*Vitis vinifera* L. ssp. *Sativa*) are economically the second important fruit-trees after olives in Palestine with more than 20 cultivars covering about 7% of the total cultivated lands and an annual production of 55,000 tons. In this study, sixteen grapevine cultivars from Palestine were genotyped based on variations among thirteen microsatellite loci. A total of 27 alleles, ranging from 165-280bp in size were detected at 12 of the tested loci excluding Scu03vv locus which produced 10 alleles with an unexpected size range of 350-3000bp. The amplification products in 11 out of the 13 microsatellite loci showed polymorphism among the 16 grapevine cultivars. The two markers that showed no polymorphism were VVMD5 and Cc5sr5. The total number of alleles per locus ranged from 1 to 4 with the highest heterozygosity observed for loci VVS2 and VrZAG21 with 4 alleles per locus. In five of the loci, namely VVMD31, VVMD17, VrZAG62, VVMD27, and VrZAG79, only two alleles per locus were identified that were either homozygous or heterozygous in the different cultivars. At three other loci (CCSSR5, VVMD7, and VVMD5), only homozygous alleles were detected. In part of the cultivars, three alleles per each of the loci VVMC8A4, VVMD32, and VrZAG21were detected. A phylogram was established based on genetic similarities using the Neighbor-Joining method and the genetic relationships among the cultivars were further determined by the Principal Coordinates Analysis.

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The cultivars were resolved into two major groups encompassing a total of four clusters. The genetic relatedness among the cultivars was not always in accordance with their identification based on ampelographic properties.
For example, the cultivars Shami A and Shami B which have the same origin and share high morphological traits were resolved into two different clusters according to the studied loci. This study represents the first microsatellite-based genotyping of the grapevine varieties cultivated in Palestine.

Keywords: *Vitis vinifera* L., Simple Sequence Repeat, genotyping, Palestine.

Introduction:

Cultivated grapevines (*Vitis vinifera* L. ssp. *Sativa*) are economically the second important fruit-trees after olives in Palestine. There are more than 20 cultivars covering about 7% of the total cultivated lands and an annual production of 55,000 tons (Palestinian Central Bureau of Statistics, 2012). More than 75% of this annual production of grapes comes from the southern part of the West Bank (Hebron and Bethlehem). Grapes of most of these cultivars are consumed mainly as table-fruits while low-quality berries are processed into raisins, juice, jam, and molasses; a limited number of cultivars are used for wine.
Palestinian farmers and agronomists have always relied mostly on the morphological characteristics to differentiate among the different grapevine cultivars. In some cases, different names have been assigned for the same cultivar as in the case for Ballouti and Baitouni; in others, the same name has been used for two different cultivars as in the case of the name Albarín Blanco which has been used to call two different Spanish cultivars; Godello and Savagnin Blanc (Moreno-Sanz *et al.*, 2008). Genotyping locally cultivated grapevine cultivars would, therefore, assist in resolving naming problems and facilitate grapevine breeding programs in Palestine. Also, such genotypic profiles would be a helpful tool for grapevine growers and the horticultural authorities in discriminating among the various cultivars at earlier stages of development where the phenotypic traits are not well developed yet.
Microsatellites, or simple sequence repeats (SSR), are highly polymorphic co-dominant markers that are frequently and evenly distributed throughout most plant genomes (Belaj *et al.*, 2003; Senior *et al.*, 1998). They have been frequently utilized for studying genetic profiling and relatedness of many plants (Bowers *et al.*, 1996; Sefc *et al.*, 1997, 2000). The method involving PCR-amplification of microsatellite loci is fast, non-laborious and highly

reproducible compared to other methods such as RAPD and AFLP. For these reasons, microsatellite markers have been used to genotype grapevine varieties in Portugal (Pinto-Carnide *et al.*, 2003), Australia (Thomas and Scott, 1993), Brazil (Leao and Motoike, 2011), Romania (Gheţea *et al.*, 2010, a; b), Italy (Pellerone *et al.*, 2001), Iran, Russia, USA (Ramezani *et al.*, 2009), and several other countries. In this study, several microsatellite markers were amplified and analyzed to genotype 16 local Palestinian grapevine cultivars.

Materials and methods

Sample collection and DNA extraction

Sixteen grapevine cultivars were sampled at the beginning of the growth season 2011 from a field in Hebron, with terra rossa soil and an average annual rainfall of 400-500 mm. The names, origin and berry's color of these cultivars are shown in Table 1. The identity of each cultivar was verified according to Sultan (2005). DNA was extracted from young leaf material by using Qiagen DNeasy Plant Mini Kit. The DNA was eluted with 50µl of TE buffer and stored at -80°C till needed. The quality of the extracted DNA was assessed by gel- electrophoresis in 1% agarose gels, and the concentration of DNA was determined by means of spectrophotometry (NanoDrop ND-1000 spectrophotometer).

Table 1: Name, origin and berries' color of 16 grapevine varieties cultivated in Palestine.

Cultivar	Origin	Color	Cultivar	Origin	Color
Bairouti	Lebanon	White	Halawani	Lebanon	Red
Beauty Seedles	Israel / France	Black	Hamdani	Palestine	White
Baitouni	Palestine	Black	Marawi	Palestine	White
Dabouki (Jordan)	Jordan	White	Salti-khdari	Jordan	White
Dabouki (local)	Palestine	White	Shami (A)	France	Black
Darawishi	Syria	Black	Shami (B)	France	Black
Jandali (A)	Palestine	White	Sultanina	France / Turkey	White
Jandali (B)	Palestine	White	Zaini	Palestine	White

PCR amplification

The amplification was performed in 25 µl reaction volumes containing 50 ng of template DNA and 0.4 µM of each primer in *AccuPower*® PCR PreMix tube (Bioneer Corporation). Information about the names, sequences, targets and sources of these primers are shown in Table 2.

Table 2: The Microsatellite loci, their chromosomal localization, and the sequences of the primers used to amplify them

Locus	Ch*	Primer	Sequence (5`→3`)	Reference
VVMD5	16	VVMD5 [F] VVMD5 [R]	Ctagagctacgccaatccaa tataccaaaaatcatattcctaaa	*Bowers et al., 1996*
VVMD7	7	VVMD7 [F] VVMD7 [R]	Agagttgcggagaacaggat cgaaccttcacacgcttgat	*Bowers et al., 1996*
VVMD17	18	VVMD17 [F] VVMD17 [R]	Tgactcgccaaaatctgacg Cacacatatcatcaccacacgg	*Bowers et al,. 1996*
VVMD27	5	VVMD27 [F] VVMD27 [R]	Gtaccagatctgaatacatccgtaagt acgggtatagagcaaacggtgt	*Bowers et al., 1996*
VVMD32	4	VVMD32 [F] VVMD32 [R]	Tatgatttttttaggggggtgagg ggaaagatgggatgactcgc	*Bowers et al., 1996*
VVS2	11	VVS2 [F] VVS2 [R]	Cagcccgtaaatgtatccatc Aaattcaaaattctaattcaactgg	*Scott et al., 2000*
VrZAG62	7	VrZAG62 [F] VrZAG62 [R]	Ggtgaaatgggcaccgaacacacgc ccatgtctctcctcagcttctcagc	*Sefc et al., 1999*
VrZAG79	5	VrZAG79 [F] VrZAG79 [R]	Agattgtggaggagggaacaaaccg tgcccccattttcaaactcccttcc	*Sefc et al., 1999*
Scu03vv	ND	Scu03vv [F] Scu03vv [R]	Ttcggcacgaggttttag Attaggcagagaagagcgg	*Scott et al., 2000*
VMC8A4	10	VMC8A4 [F] VMC8A4 [R]	Caggtaaacttctcaacgga Ccgaagtcaaaagagcgatt	*Sefc et a.,l 1998*
VrZAG21	4	VrZAG21 [F] VrZAG21 [R]	Tcattcactcactgcattcatcggc Ggggctactccaaagtcagttcttg	*Sefc et al., 1999*
VVMD31	7	VVMD31 [F] VVMD31 [R]	Cagtggtttttcttaaagtttcaagg Ctctgtaaagaggaagagacgc	*Bowers et al., 1999*
CCSSR5	ND	CCSSR5 [F] CCSSR5 [R]	Tatcacgccagtgtttttgg Cctttcatccgacctcgtaa	Ranathunge, *et al., 2009*

*Ch: chromosome number, ND: not determined, F: forward primer, R: reverse primer.

PCR amplification was performed in a cycler (MJ research) as follows: an initial denaturation step for 4 min at 94°C followed by 36 cycles of 1 min at 94°C, 1 min at annealing temperatures ranging from 56°C to 60°C, 1 min at 72°C followed by a final step of 5 min at 72°C. The annealing temperatures were calculated for each primer pair according to the Amplifx software, version 1.5.4. Amplification products were resolved in 2% agarose gel stained with ethidium bromide run in 1X TBE buffer at 150 mA for 1 hour and visualized by UV gel documentation system (BioDoc-It imaging system, UVP). The molecular size of fragments was estimated by reference to the 100bp DNA ladder (Invitrogen). Each amplification reaction was conducted twice to confirm the obtained results.

Phylogenetic analysis

The cultivars were genotyped at 12 microsatellite loci excluding Scu03vv locus (Table 2), where only clearly identified bands were considered as potential polymorphic markers. The bands were scored depending on their presence or absence (1 or 0). Except for the locus VVMD5, where the repeat type was compound imperfect, all of the other studied loci contained a perfect repeat motif of either di- or trinucleotide (Table 3). A similarity matrix was calculated by the Nei method (Nei, 1972) and an unrooted phylogram was obtained by the neighbor-joining (NJ) (Saitou and Nei, 1987) using the NTSYSpc version 2.10e (Rohlf, 2002). Genetic relationships among the cultivars were further assessed by means of the Principal Coordinates Analysis (PCoA) using Jaccard's coefficient.

Results and Discussion

A total of 27 alleles, ranging from 165-280bp in size were detected at 12 of the tested loci excluding Scu03vv locus which produces 10 alleles with an unexpected size range of 350-3000 bp (Table 3). The amplification products in 11 out of the 13 SSRs loci showed polymorphism among the 16 grapevine cultivars.

Table 3: Sequences expected and observed allelic sizes of the microsatellite loci analyzed in this study.

Locus	Motif	Allele size	
		Expected	Observed
VVMD5	$(CT)_3AT(CT)_{11}$ $ATAG(AT)_3$	215-270bp	250-270bp
VVMD7	$(CT)_{14}$	180-270bp	240bp
VVMD17	(CT)n	212-236bp	220-240bp
VVMD27	(CT)n	165-210bp	180-220bp
VVMD32	(CT)n	239-273bp	170-200bp
VVS2	$(GA)_{19}$	123-165bp	150-200bp
VrZAG62	$(AG)_9$	181-220bp	190-230bp
VrZAG79	$(AG)_{19}$	230-270bp	250-280bp
Scu03vv	$(GA)_8$	125-205bp	350-3000bp
VMC8A4	(CTG)n	170-190bp	170-200bp
VrZAG21	(AG)n	190-214bp	200-250bp
VVMD31	(CT)n	196-224bp	165-175bp
CCSSR5	$(GT)_{10}$	241-260bp	250bp

The two markers that showed no polymorphism are VVMD5 and Cc5sr5. The total number of alleles per locus ranged from 1 to 4 with the highest heterozygosity observed for loci VVS2 and VrZAG21 (4 alleles per locus).

In five of the loci, namely VVMD31, VVMD17, VrZAG62, VVMD27, and VrZAG79, only two alleles per locus were identified that were either homozygous or heterozygous in the different cultivars. At three other loci (CCSSR5, VVMD7, and VVMD5), only homozygous alleles were detected. Three alleles per locus were detected in some cultivars. These loci are VVMC8A4, VVMD32, and VrZAG21.

Amplification of locus (Scu03VV) resulted in up to 10 bands in the various cultivars ranging from 350 to 3000bp. This falls outside of the range of the expected allele sizes (Table 3). Similar results at this locus were also obtained by Scott *et al.*, (2000) and it was excluded from the phylogenetic analysis for this reason. Although cultivated grapevine have diploid genomes (Bowers, *et al.*, 2003), recent reports indicated that they have originated from a hexaploid stock through duplication events (Jaillon *et al.* 2007). This means that part of the grapevine genome might have six copies with up to 12 alleles for some loci. This would explain the appearance of 10 bands for the Scu03VV locus. However, further assessment and study to determine the essence of these multiple bands at Scu03VV locus is need.

The sixteen cultivars formed two distinctive groups with each group containing clusters (Fig. 1). While the first cluster (C1) contained only one white berries cultivar (Bairouti), the second cluster C2, the largest, encompassed eight cultivars of which seven were white berries (Salti-Khdari, Zaini, Dabouki-local, Jandali A, Jandali B, Dabouki- Jordanian and Sultanina) and one black berry (Shami B). The third cluster (C3) was composed of one red berries cultivar (Halawani) and two black berries; ShamiA and Beauty Seedless (also called Black beauty). The fourth cluster (C4) contained two white berries (Marawi and Hamadani) and two black berries (Baitouni) and Darawishi.
A similar profile was also obtained with the Principal Coordinate Analysis (Fig.2).

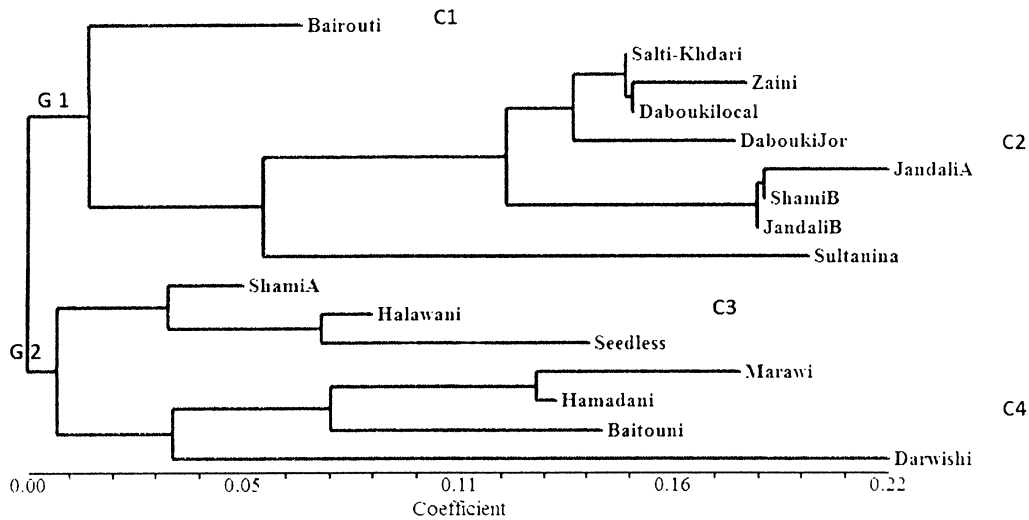

Figure 1. Phylogram generated by Neighbor –Joining cluster analysis using12 microsatellite markers for 16 grapevine varieties cultivated in Palestine. The tree was constructed based on genetic similarities among the cultivars according to Nei coefficient. C1, C2, C3, and C4 correspond to clusters 1, 2, 3, and 4, respectively. G1 and G2 correspond to groups 1 and 2, respectively

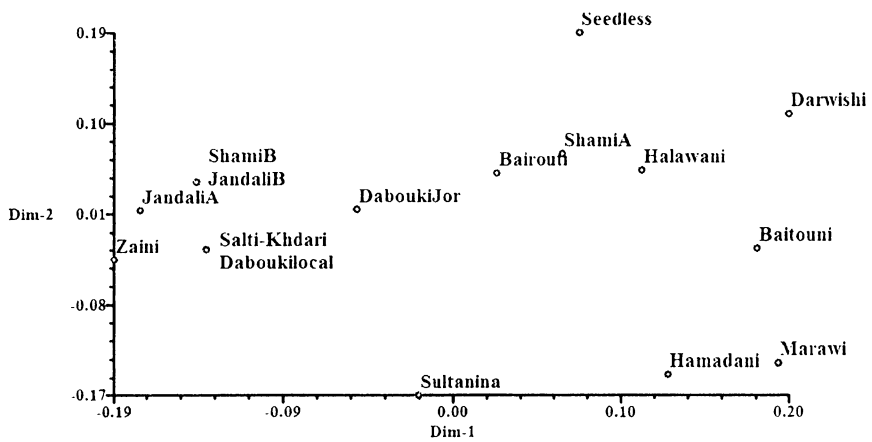

Figure 2: Genetic differentiation by principal coordinate analysis showing distinct clusters of cultivated grapevine in Palestine

The genetic relatedness among some of the cultivars was in accordance with their identification based on ampelographic properties. A study of the phenotypic characterizations of these cultivars showed that the white-berry cultivars Salti- Khdari, Zaini, Jandali A, and Dabouki were closely similar (Sultan, 2005) which is in agreement with the current genotyping results. It might be worth mentioning that these cultivars also share a common origin (Jordan). Another example where the phenotypic and genotypic similarities were in accordance is shown for the cultivars Jandali A and Jandali B, both of which originated in Palestine (Table 1 and Fig. 1). Moreover, the cultivars Sultanina and Darawishi showed not only the least genetic relatedness with the other members of clusters C2 and C4, respectively, (Fig. 1) but also the least morphological similarities (Sultan, 2005). On the other hand, the close genetic relatedness between Shami B and Jandali (Figs. 1 and 2) revealed in this study is not in accordance with their different origins and ampelographic characteristics (Sultan, 2005). A similar result was observed for cultivars Baitouni, Hamadani and Marawi, which joined the same cluster of the phylogram (Fig. 1), though phenotypically they are different from one other. Probably more microsatellite loci should be investigated to reveal the genetic differences among such cultivars.

The resolving power of this genotyping method was revealed for the cultivars Shami A and Shami B. Although they have the same origin (Table 1) and share high morphological traits, they still belong to two different clusters according to the studied SSR loci (Fig. 1). A similar trend was observed for Dabouki Jordan and Dabouki local, which have high morphological similarities. Although, these two cultivars belong to the same cluster, they share less genetic relatedness than with other members of the same cluster.

This is the first study of genotyping Palestinian grapevine cultivars using SSR markers. Although microsatellite loci proved to be a powerful tool to differentiate among grapevine cultivars based on their genotypic differences, this method revealed that the genotypic relatedness among most of the characterized cultivars was not in accordance with their classification according to their morphological features. This might be due to the fact that the SSR markers are not part of the genome coding for these morphological features. Further studies targeting more SSR loci as well as other genetic markers such as Inter Simple Sequence Repeats (ISSR) and Amplified

Fragment Length Polymorphism (AFLP) might further strengthen the genetic relatedness among the Palestinian cultivars of grapevine.

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