Optimization of PCR Conditions to Amplify Microsatellite Loci in Grapevine (*Vitis vinifera* L.) Genomic DNA

Sana GHAFFARI [1 *], Nejib HASNAOUI [2], Ali FERCHICHI [1]

[1.] Laboratoire d'Aridoculture et Cultures Oasiennes, Institut des Régions Arides (IRA) de Médenine, 4119, Médenine, Tunisie.

[2.] Institut Supérieur de Biologie Appliquée de Médenine (ISBAM), 4119, Médenine, Tunisie.

(*) Auteur correspondant Adresse e-mail: sana.ghaffari@yahoo.fr

Abstract: A total of three different primer pairs were optimized for polymerase chain reaction (PCR) to amplify microsatellite loci in total genomic DNA of grapevine (*Vitis vinifera* L.). Different concentrations of $MgCl_2$, DNA and different regimes of annealing temperature were optimized. For all the primer pairs, 2.5 mM $MgCl_2$ concentration was found optimum. For DNA concentration, 100 ng in the final reaction volume was suitable for good amplification. Annealing temperatures 56°C, 61°C and 58°C were found optimum to amplify with primer pairs VVMD5, scu04vv and VMC8E6, respectively. The other reagents used in PCR and temperature regimes (denaturation and extension temperature) were kept constant. The protocol has been successfully applied producing scorable and clear amplicons in all cultivars studied. These loci can be used to evaluate the genetic variability and cultivar relatedness in autochthonous *Vitis vinifera* cultivars from Tunisia.

Key Words: DNA, Grapevine (*Vitis vinifera* L.), Microsatellites, PCR.

Résumé : Un total de trois différents couples d'amorces ont été optimisés pour amplifier les loci microsatellites dans l'ADN génomique total de la vigne (Vitis vinifera L.). Différentes concentrations de $MgCl_2$ et d'ADN et différents régimes de températures d'hybridation ont été optimisées. Pour tous les couples d'amorces, 2,5 mM en $MgCl_2$ ont été constatés optimales. Concernant l'ADN, une concentration de 100 ng/volume réactionnel final est appropriée pour avoir une bonne amplification. Des températures d'hybridation de 56°C, 61°C et 58°C ont été trouvés optimales pour amplifier, respectivement, avec les paires d'amorces VVMD5, scu04vv et VMC8E6. Les autres réactifs utilisés dans la PCR et les régimes de température (température de dénaturation et d'extension) ont été maintenus constants. Le protocole a été appliqué avec succès pour la production des amplimères aisément interprétables chez tous les cultivars étudiés. Ces loci peuvent être utilisés pour évaluer la variabilité génétique et la parenté des cultivars dans les cultivars Vitis vinifera autochtones de la Tunisie.

Mots Clés: ADN, Vigne (*Vitis vinifera* L.), Microsatellites, PCR.

1. Introduction

Grapevine (*Vitis vinifera* L.) is one of the oldest and most important perennial crops in the world. In Tunisia grapevine was introduced by the Phoenicians. Afterwards viticulture products were exported from Minor Asia and Northern Africa until 1000 BP. After the defeat of Carthago vine production became compromise by the cultivation of other cultures, mainly maize. With the Muslims and the Ottomans table grape production and also wine grape developed and new varieties were introduced by the French, being the most important Grenache and Mourvèdre. However cultivation of grapevine in Tunisia suffers from the same problems as other countries, namely bacteria and viruses, leading to the abandonment of its production. Therefore the risk of erosion of these genetic resources is very high. Tunisia has a very rich germplasm of autochthones cultivars, from North to South, adapted to different local climatic conditions (Ben Salem-Fnayou et al., 2005). Some of these already disappeared and others are reduced to a small number of plants. In order to avoid the loss of these genetic resources correct identification and genetic characterisation urges either for conservation and selection.

Microsatellite markers are codominants, hypervariables, neutrals and reproducible. Therefore they represent the best suited markers for genetic diversity studies. Nuclear SSR markers for grapevine have been developed by different groups (Thomas and Scott, 1993; Bowers et al. 1996; Pellerone et al. 2001; Lefort et al. 2002). One of the major applications of SSR markers in grapevines has been the identification and discrimination of cultivars in order to facilitate management of cultivar collections and control trade of plant material (Sefc et al., 2000). These markers have also been used in several parentage studies in grapevine (Bowers and Meredith, 1997; Bowers et al., 1999; Lopes et al., 1999; Piljac et al., 2002; Sefc et al., 1997; Sefc et al., 1998). Although nuclear microsatellite markers allow the identification of parent/progeny relationships the male and female contributions are impossible to determine unless other markers are used, such as chloroplast microsatellites. Chloroplast microsatellites are maternally inherited allowing the determination of geneflow direction.

This study describes an optimised protocol suited specifically for DNA amplification via SSR-PCR for *Vitis vinifera* L. species.

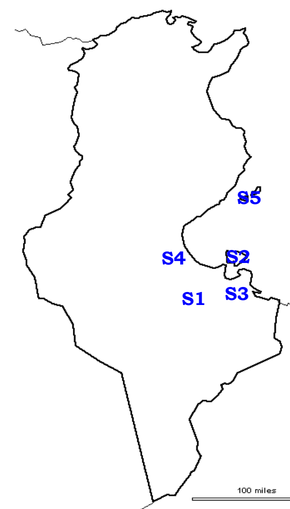
2. Materials and Methods

2.1. Plant material

The plant material used in the study consisted of 26 grapevine *Vitis vinifera* L. genotypes. The grapevine genotypes were mostly collected from their centre of origin (Table 1).

Table 1. Cultivar names, sample codes, and collection sites of 26 Tunisian grapevine samples

Cultivar name	**Sample code**	**Collection site**
Dalia	DALm	Medenine (33°20' N, 10°29' E), S1
Beldi	BLDd	Djerba (33°48' N, 10°50' E), S2
Siper abiadh	SABd	Djerba (33°48' N, 10°50' E), S2
Superieur Italie	SITd	Djerba (33°48' N, 10°50' E), S2
Muscat d'Italie	MITd	Djerba (33°48' N, 10°50' E), S2
Meski	MESd	Djerba (33°48' N, 10°50' E), S2
Cardinal	CARd	Djerba (33°48' N, 10°50' E), S2
Bazzoul kalba	BAKd	Djerba (33°48' N, 10°50' E), S2
Mguargueb	MGBd	Djerba (33°48' N, 10°50' E), S2
Arbi	ARBd	Djerba (33°48' N, 10°50' E), S2
Akhal	AKHd	Djerba (33°48' N, 10°50' E), S2
Akhal tawil	AKTd	Djerba (33°48' N, 10°50' E), S2
Superior seedless	SPSd	Djerba (33°48' N, 10°50' E), S2
Bazzoul kalba	BAKz	Zarzis (33°30' N, 11°06' E), S3
Muscat d'Alexandrie	MEAz	Zarzis (33°30' N, 11°06' E), S3
Razzegui	RAZz	Zarzis (33°30' N, 11°06' E), S3
Meski	MESz	Zarzis (33°30' N, 11°06' E), S3
Cardinal	CARz	Zarzis (33°30' N, 11°06' E), S3
Bazzoul kalba	BAKg	Chénini Gabès (33°51' N, 10°03' E), S4
Meski	MESg	Chénini Gabès (33°51' N, 10°03' E), S4
Medina	MEDg	Chénini Gabès (33°51' N, 10°03' E), S4
Korkobbi	KORg	Chénini Gabès (33°51' N, 10°03' E), S4
Mlouhi mkarkeb	MMKg	Chénini Gabès (33°51' N, 10°03' E), S4
Saoudi	SADg	Chénini Gabès (33°51' N, 10°03' E), S4
Razzegui	RAZk	Kerkenah (34°39' N, 11°04' E), S5
Tounsi	TONk	Kerkenah (34°39' N, 11°04' E), S5



Map of Tunisia showing the sites of collection.

Sites:

S1 = Medenine,
S2 = Djerba,
S3 = Zarzis,
S4 = Chénini Gabès,
S5 = Kerkenah.

2.2. DNA extraction

Total plant DNA for PCR was isolated from fine powdered dry young expanding leaves according to a modified CTAB method following the procedure described by Lefort and Douglas (1999) (Ghaffari et al., submitted). DNA concentration was determined by spectrophotometer (Perkin Elmer, USA) and was checked for integrity on a 1% agarose gel with a 100 bp Jules™ ladder (Qbiogene).

2.3. PCR condition optimisation

The effects of Taq polymerase concentrations, concentrations of $MgCl_2$, template DNA concentrations, and different periods of time and temperatures during the annealing stage of amplification were optimised for the three SSRs. However, the concentration of dNTPs was kept constant.

Finally, the PCR-SSR reaction was carried out in a final volume of 20 µl of reaction mixture containing 2 x PCR buffer (Qbiogene), 2.5 mM $MgCl_2$ (Qbiogene), 0.3 mM of dNTPs (Qbiogene), A unit of AmpliTaq DNA polymerase (Qbiogene), 0.6 µM of primer, 100 ng of template DNA. The mixture was covered with 20 µL of mineral oil. DNA amplifications were performed in a GeneAmp®PCR System 9700 thermocycler (PE Applied Biosystems), with the following PCR cycle: 4 min at 94 °C, followed by 35 cycles of 20 sec at 94 °C, 1 min at the primer's specific melting temperature (Tm) (Table 2), 2 min at 72 °C, and a final extension step of 5 min at 72 °C. Reaction mixture, wherein

template DNA replaced with distilled water was used as negative control. Three primers were used to check the fidelity of amplification: VVMD5 (Bowers et al., 1996), scu04vv (Scott et al., 2000) and VMC8E6 (VMC consortium; Adam-Blondon et al., 2004). Amplified products were resolved on 3% agarose gel in Tris-Borate-EDTA buffer (1 x), run at a constant voltage of 80 V approximately 1 h, then stained with ethidium bromide, visualized under ultraviolet light and photo graphed. A 100 bp Jules™ ladder (Qbiogene) was used as a molecular size marker. Photographs of SSR results were taken with a BIO-PRINT system (KAISER, Germany).

Table 2. Set of grapevine microsatellite markers (SSR) used

Primers	Primer Sequence (5' to 3')	Tm
VVMD5	CTAGAGCTACGCCAATCCAA (F)	60.4
	AAATCCTTATACTAAAAACCATAT (R)	52.61
scu04vv	TGTCCTCTTTCCCTCTCCCAAC (F)	64.54
	CCGATGTACCAGTCTACTGTCTGAC (R)	66.22
VMC8E6	AAGGGGTTCATTTGATTGAGAG (F)	58.94
	AGATTCGACATTCCTACCTACTTC (R)	61.15

3. Results

The microsatellite markers are PCR based that makes them attractive for use in genetic diversity studies and as genetic markers in the construction of genetic maps (Dalbo et al., 2000) which requires only tens of the nanograms of DNA or even crude DNA extracts. Template DNA is easy to obtain. Moreover, presence of easily scorable, unique alleles and/or allele combinations, make them an ideal system for cultivar identification (Thomas and Scott, 1993; Bowers et al., 1996; Sefc et al., 2000; Pellerone et al., 2001; Lefort et al., 2002).

Before conducting any genetic studies, optimization of concentration of different reagents and temperature regimes used in polymerase chain reaction (PCR) is necessary. However, annealing temperature and concentration of $MgCl_2$ are important parameters, which need optimization. Two genomic DNA was used to optimize the PCR conditions. Amount of template DNA strongly influences the outcome of the reaction. More than 30 ng/25 µL give the premium amplification (Henegariu *et al*., 1997). However in the present studies, 100 ng/20 µL was found optimum. Optimization of $MgCl_2$ is an important factor for precise amplification. In these experimental studies, 2.5 mM of $MgCl_2$ was found optimum in 20 µL final volume.

The Mg ions binds tightly to the phosphate sugar backbone of nucleotides and nucleic acids, and variation in the $MgCl_2$ concentration has strong effects on nucleic acid interactions. Variations in $MgCl_2$ concentration below 4 mM can improve performance of PCR by affecting specificity (higher concentrations lower the specificity, lower concentrations raise specificity) (Blanchard *et al*., 1993).

Moreover, concentration of dNTPs in reaction mixture is also strongly correlated to the Mg ions concentration due to the interaction between mononucleotides and the Mg^{2+}. A higher concentration of Mg^{2+} allows amplification with a higher concentration of dNTPs, that is not seen at lower Mg^{2+} concentrations (Blanchard *et al*., 1993).

In PCR, 2-2.5 units of Taq Polymerase are normally used in 100 µl final volume. Higher Taq Polymerase concentration (above 4 units/100 µL) can generate nonspecific products and may reduce the yield of the desired product (Saiki, 1989). However, in the present study, 1 unit/20 µL reaction was used to amplify the loci without non-specific products.

Annealing temperature is one of the most important parameters that need adjustment in the PCR. The normal range of annealing temperature is 36-75°C. The annealing temperatures 56°C, 61°C and 58°C were found optimum to amplify with primer pairs VVMD5, scu04vv and VMC8E6, respectively. The optimised method was found suitable to amplify the 26 V. vinifera cultivars (Figure 1).

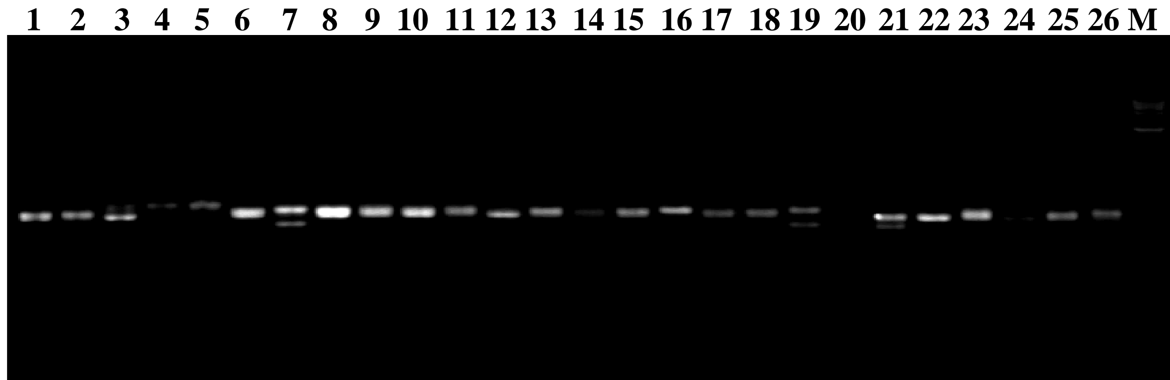


Figure 1. SSR profile of 26 *V.vinifera* DNA obtained by the primer VMC8E6, using a 3% agarose gel. M: 100 bp DNA ladder.

4. Conclusion

It is hoped that the SSR optimized conditions for PCR and reagents described here will help in characterisation and genetic diversity studies regarding *Vitis vinifera* L. Application of the method to other species may require adaptation and possible further specific validation.

5. References

Adam-Blodon AF, Roux C, Claux D, Butterlin G, Merdinoglu D, This P. 2004. Mapping 245 SSR markers on the *Vitis vinifera* genome: a tool for grape genetics. *Theor Appl Genet*, **109**, 1017-1027.

Ben Salem-Fnayou A, Hanana M, Fathalli N, Souid I, Zemni H, Bessisr, Ghorbel A. 2005. Caractères anatomiques adaptatifs de la feuille de vigne dans le sud tunisien. *J Int Sci Vigne Vin*, **39**(1), 8.

Blanchard MM, Tailon-Miller P, Nowotny P, Nowotny V. 1993. PCR buffer optimization with a uniform temperature regimen to facilitate automation. *PCR Methods Applic*, **2**, 234-40.

Bowers J, Boursiquot JM, This P, Chu K, Johansson H, Meridith C. 1999. Historical genetics: the parentage of Chardonnay, Gamay, and otherwine grapes of northeastern France. *Science*, **285**, 1562-1565.

Bowers JE, Meredith CP. 1997. The parentage of a classic wine grape. Cabernet Sauvignon. *Nature Genet*, **16**, 84-86.

Bowers JE, Dangl GS, Vignani R, Meredith CP. 1996. Isolation and characterization of new polymorphic simple sequence repeat loci in grape (*Vitis vinifera* L.). *Genome*, **39**, 628-633.

Dalbo MA, Ye GN, Weeden NF, Steinkellner H, Sefc KM, Reish BI. 2000. A gene controlling sex in grapevines placed on a molecular marker-based genetic map. *Genome*, **43**, 333-340.

Henegariu O, Heerema NA, Dlouhy SR, Vance GH, Vogt PH. 1997. Multiplex PCR: Critical parameters and step-by-step protocol. *Biotechniques*, **23**, 504-511.

Lefort F, Douglas GC. 1999. An efficient micro-method of DNA isolation from mature leaves of four hardwood tree species Acer, Fraxinus, Prunus and Quercus. *Ann For Sci*, **56**, 259-263.

Lefort F, Kyvelos CJ, Zervou M, Edwards KJ, Roubelakis-Angelakis KA. 2002. Characterization of new microsatellite loci from *Vitis vinifera* and their conservation in some Vitis species and hybrids. *Mol Ecol Notes*, **2**, 20-21.

Lopes MS, Sefc KM, Eiras Dias JE, Steinkellner H, Laimer da Câmara Machado M, da Câmara Machado A. 1999. The use of microsatellites for germplasm management in a Portuguese grapevine collection. *Theor Appl Genet*, **99**, 733-739.

Pellerone FI, Edwards KJ, Thomas MR. 2001. Grapevine microsatellite repeat: isolation, characterization and use of genotyping grape germplasm from Southern Italy. *Vitis*, **40**, 179-186.

Piljac J, Maletic E, Kontic JK, Dangl GS, Pejic I, Mirosevic N, Meredith CP. 2002. The parentage of *Poˇsip bijeli*, a major white wine cultivar of Croatia. *Vitis*, **41**, 83-87.

Saiki RK. 1989. The design and optimization of the PCR. In: Erlich, H.A. (Eds.), PCR Technology - Principles and Applications for DNA Amplification, Stockton Press, New York, pp. 7-16.

Scott KD, Eggler P, Seaton G, Rossetto M, Ablett EM, Lee LS, Henry RJ. 2000. Analysis of SSRs derived from grape ESTs. *Theor Appl Genet*, **100**, 723-726.

Sefc KM, Lopes MS, Lefort F, Botta R, Roubelakis-Angelakis KA, Ibanez J, Pejic I, Wagner HW, Glossl J, Steinkellner H. 2000. Microsatellite variability in grapevine cultivars from different European regions and evaluation of assignment test to assess the geographic origin of cultivars. *Theor Appl Genet*, **100**, 498-505.
Sefc KM, Regner F, Glossl J, Steinkellner H. 1998. Genotyping of grapevine and rootstock cultivars using microsatellite markers. *Vitis*, **37**, 15-20.
Sefc KM, Steinkellner H, Wagner HW, Glossl J, Regner F. 1997. Application of microsatellite markers to parentage studies in grapevine. *Vitis*, **36**, 179-183.
Thomas MP, Scott NS. 1993. Microsatellite repeats in grapevine reveal DNA polymorphisms when analysed as sequenced-tagged sites (STSs). *Theor Appl Genet*, **86**, 985-990.