Bulletin UASVM Horticulture, 70(1)/2013, 131-136
Print ISSN 1843-5254; Electronic ISSN 1843-5394

Molecular Characterisation of Romanian Grapevine Cultivars Using Nuclear Microsatellite Markers

Monica HÂRŢA, Doru PAMFIL

Faculty of Horticulture, University of Agriculture Sciences and Veterinary Medicine, 400372, Cluj-Napoca, 3-5, Mănăştur Street, Romania; monica_bodea@hotmail.com

Abstract. In the present study we use six nuclear SSR loci (SS2, MD5, MD7, MD27, ZAG 62 and ZAG 79) to characterize four autochthonous grapevine cultivars (Băbeasca neagră, Feteasca regală, Frâncuşa, and Grasa de Cotnari), including four international comparative genotypes (Cabernet Sauvignon, Chardonnay Blanc, Riesling Italian and Merlot Noir). The DNA microsatellite analysis was used to construct a barcode system. The advantage of this mode of grouping data is a visual representation of the number and size of alleles, allowing easy detection of genotypic differences between analysed cultivars. Our results shows that this system of data grouping can be useful for characterisation of Romanian cultivars at molecular level. The integration of such DNA barcodes into nationally and internationally coordinated databases could increase the accuracy with which grapevine genetic resources are managed in Romania.

Keywords: *Vitis vinifera*, cultivars, SSR , DNA barcodes

INTRODUCTION

Romania have a multimillenary tradition in grapevine cultivation and wine production. In recent years, the concerns of some research teams have focused on developing a national research direction for the characterization of grape germplasm fund, as response to the European Union initiative for inventory and conservation of genetic resources. In this context, the European Union has developed international research projects having as main aim colecting the data, scientific colaboration between the involved researchers and improving knowledge regarding conservation and sustainable use of *Vitis* genetic resources in Europe (Gheţea *et al.*, 2012).

Identification of genetic polymorphism of grape varieties that make up the national germplasm funds of some countries no longer constitute an element of novelty. Some of them (Greece, Hungary, Croatia, Czech) have grouped the scientific informations into national grape databases. Another countries (Germany, France, Italy) are partners in coordination the European *Vitis* Database.

In Romania some studies regarding molecular characterisation of grape cultivars (Bodea *et al.*, 2009; Butiuc *et al.*, 2010; Coste *et al.*, 2010; Gheorghe *et al.*, 2008; Motoc *et al.*, 2010; Pop *et al.*, 2005; Pop, 2008) suggest that DNA fingerprinting techniques represent efficient and reliable tools for the accurate characterization of autochthonous and newly created grapevine cultivars, completing the classical methods of identification based on ampelographic description. Identifying of specific genetic profiles using molecular markers, especially SSR, is a real passport that certifies authenticity of cultivars and represents a guarantee for further preservation of Romanian grapevine cultivars with scientific and economic value (Gheţea *et al.*, 2012). Valuable gene resources must be inventoried for a complete genetic characterization, to facilitate the registration of Romanian cultivars in the European *Vitis* Database and also to have an efficient management of data.

In agreement with this purpose, an efficient mode of data grouping can be usefull for designing the genetic profil of analysed cultivars.

In the present study we used six nuclear SSR loci to characterize four autochthonous grapevine cultivars, including four international comparative genotypes and the microsatellite analysis was used to construct a barcode system. From our knowledge, this is the first report on the SSR characterization of Romanian grapevine cultivars through this system of data grouping.

MATERIALS AND METHODS

Young grapevine leaves from *V. vinifera* cultivars Băbeasca neagră, Feteasca regală, Frâncuşa, Grasa de Cotnari, Cabernet Sauvignon, Chardonnay Blanc, Riesling Italian and Merlot Noir were collected from experimental vineyard of the University of Agriculture Sciences and Veterinary Medicine "Ion Ionescu de la Brad, Iaşi, Romania (ROM 022 holding institution). DNA isolation from leaves was carried out according to Lodhi *et al*. (1994) protocol, modified by Pop *et al*. (2003) and adapted to our lab conditions. The concentration and purity of DNA were cuantified using a Nanodrop ND-1000 Spectrophotometer (Thermo Scientific).

Six SSR loci proposed by OIV (Organisation Internationale de la Vigne et du Vin): VVMD5, VVMD7 (Bowers *et al*., 1996), VVMD 27 (Bowers *et al*. 1999), ZAG62, ZAG79 (Sefc *et al*., 2000), VVS2 (Thomas and Scott, 1993) were used for microsatellite analysis.

PCR reactions were performed in 96 Well Gradient Palm - Cycler (Corbet Research) in a 10 µl vol. containing 1 x Taq buffer collor less, 1.5mM $MgCl_2$, 100µM dNTP mix, 0.5 U Go Taq Polymerase, Nuclease free water (Promega, USA), 0,25 µM of each primer (Integrated DNA technologies, USA) and 2 µl DNA (20 ng/µl) isolated from leaves. In each PCR reaction, predenaturation was conducted at 95^0C for 1 min, followed by 20 cycles: denaturation - 95^0C/ 30s, annealing between 50 and 56^0C/ 1 min, depending of each primers set, and extension at 72^0C for 30 s. The final extension step was conducted for 5 min at 72^0C. The forward primer in each pair, was labelled with 5' WellRed ^{TM} fluorescent dyes (D2, D3, and D4). Tab. 1 shows the optimal primer annealing temperature, according to the results obtained for each primer pair in the temperature gradient PCR and allele size range (bp) cited in the literature (The Greek *Vitis* Database).

Tab.1

Description of analysed SSR loci

Locus	Primer sequence	T^0 annealing	Allele size range (bp)
VVS2	F 5'- /5D2/ CAGCCCGTAAATGTATCCATC-3' R 5'- AAATTCAAAATTCTAATTCAACTGG-3'	53.5 ^{0} C	129-155
VVMD5	F 5'- /5D3/ CTAGAGCTACGCCAATCCA-3' R 5'- TATACCAAAAATCATATTCCTAAA-3'	55.5 ^{0} C	226-246
VVMD7	F 5'- /5D4/ AGAGTTGCGGAGAACAGGAT-3' R 5'- CGAACCTTCACACGCTTGAT-3'	52 ^{0} C	233-263
VVMD27	F 5'- /5D4/GTACCAGATCTGAATACATCCGTAAGT-3' R 5'- ACGGGTATAGAGCAAACGGTGT-3'	56 ^{0} C	173-194
ZAG 62	F 5'- /5D4/GGTGAAATGGGCACCGAACACACG-3' R 5'- CCATGTCTCTCCTCAGTTCTCAGT-3'	50.5 ^{0} C	185-203
ZAG 79	F 5'- /5D2/ AGATTGTGGAGGAGGGAACAAACCG-3' R 5'- TGCCCATTTTCAAACTCCCTTCC-3'	50.5 ^{0} C	236-260

After amplification, PCR products were checked by electrophoretic migration in a 2% agarose gel, in 1 x TAE buffer at 0.29 Volts/cm^2 for 2 hours. Gels were visualized using UV light Biospectrum AC Imaging System (UVP BioImaging Systems) after staining with 0.5 μg/μl ethidium bromide, for 15 minutes.

In order to determine the number and size of alleles/ primer, one microlitre from each labeled PCR products was mixed (D2-D3, D2-D4) and also diluted with sample loading solution (30 μl). A volume of 0.25 μl of Genome DNA Standard Kit-400 (Beckman Coulter, Fullerton, CA, USA) was added prior to electophoretic migration in the CEQ 8800TM capillary DNA analysis system (Beckman Coulter, Fullerton, CA, USA). Allele sizes were determined for each SSR locus using the CEQ fragment analysis software. DNA barcodes were constructed using the Microsoft Excel 2003 software.

RESULTS AND DISCUSSIONS

SSR analysis allowed us to determine the size (bp) of each amplicon obtained for six microsatellite loci. Analysed grape cultivars showed, at a certain SSR locus, a homozygotic (the presence of a single allele/ locus), or a heterozygotic (two allele/ locus) condition. Based on the number and size of the amplicons generated by the six SSR markers, a genetic profile was obtained for each of the analysed cultivars (Tab. 2).

Tab. 2

SSR genetic profile obtained at analysed grape cultivars

Cultivar	Analysed SSR loci					
	SS2	MD5	MD7	MD27	ZAG 62	ZAG 79
Băbeasca neagră	133: 133	228: 228	243: 243	181: 195	202: 202	256: 256
Feteasca regală	133: 133	238: 242	247: 249	183: 191	194: 204	248: 248
Frâncuşa	143: 143	228: 236	247: 247	185: 195	188: 204	248: 248
Grasa de Cotnari	133: 145	228: 240	239: 255	179: 195	196: 204	236: 250
Cabernet Sauvignon	139: 151	232: 240	239: 239	175: 189	188: 194	246: 246
Chardonnay blanc	137: 143	236: 240	239: 243	181: 191	188: 196	244: 246
Riesling italian	135: 151	226: 238	247: 257	185: 189	194: 196	248: 248
Merlot noir	139: 151	226: 236	239: 247	187: 191	194: 194	258: 258

As it can be seen in Tab. 2 all of the detected alleles are within the size interval cited in the literature and specified in Tab. 1 for each analysed SSR locus. Our results regarding the number and size (base pairs) of alleles at Feteasca regală, Grasa de Cotnari, Cabernet Sauvignon, Chardonnay blanc, Riesling italian (Welschriesling) and Merlot noir cultivars are in accordance with those published in *Vitis* International Varieties Catalogue (VIVC) Database. The difference in one up to four base pairs is accepted between laboratories and is due to different analytical and rounding methods (This *et al*., 2004).

In case of Frâncuşa cultivar we identified two alleles (188: 204) at ZAG 62 locus in comparision with VIVC Database data (196: 196). An explanation of this difference in our study can be due by high variability in results observed at ZAG locus, at grapevine cultivars of different gene pools, in different countries (Ghețea *et al.*, 2012).

In this study, the highest and lowest variability in allele size were obtained at loci MD27 (8 different allele sizes) and ZAG 62 (5 different allele sizes), respectively (Tab. 2).

Genetic profile obtained at Băbeasca neagră with six primers used, offers data on the molecular characterization of this Romanian cultivar, which may be subsequent introduced in international databases.

International cultivars (Cabernet Sauvignon, Chardonnay blanc, Riesling italian and Merlot noir) were used to compare the generated genetic profile in this study with international database sources and also to obtain the molecular DNA fingerprinting data, since these varieties are grown in Romania, in different parts of the country. Even though most of the used biological material is the result of clonal selection, as a result of mutation in certain environmental conditions, may occur differences highlighted at the molecular level.

Identification by SSR markers of allele size had the advantage that can be subjected to pair-wise comparision to detect genotypic differences (Galbacs *et al.,* 2009).

The resulting numerical data can be converted to real fingerprints by the construction of barcodes (Jeffrey *et al.,* 1985).

The barcode system is a visual representation of the data and can facilitate an easy detection of genotypic differences. In situation that an overlap occurs in the allele sizes representation (two or more markers have the same allele sizes), the bar can be marked by an index showing those diferences (Galbacs *et al.,* 2009).

In this study, we converted the SSR results to DNA barcodes according to Galbacs *et al*. (2009) method, by separating the allele size from each SSR locus and then sorting the allele size data from lowest to highest. The Fig. 1 shows the allele size bars drawn to a linear scale for all of the analysed cultivars.

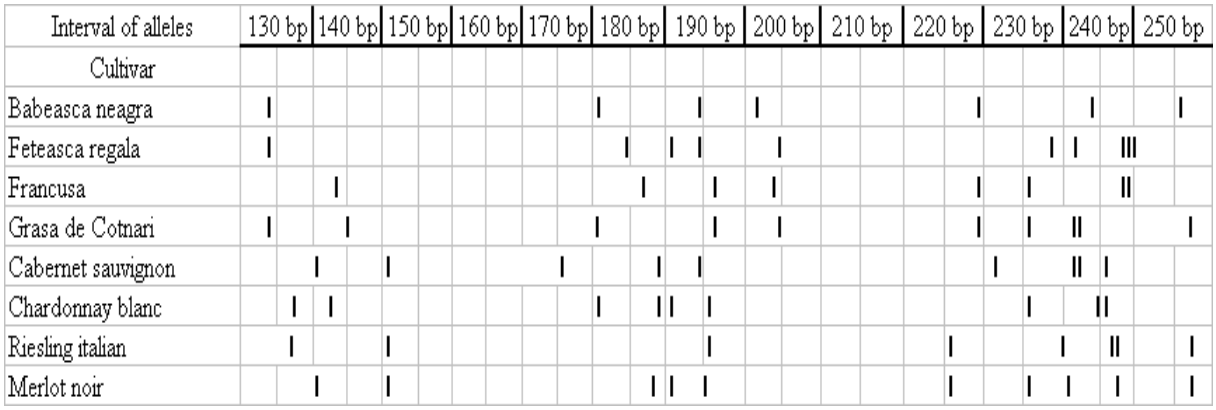

Fig. 1. DNA barcode of analysed grapevine cultivars

From our knowledge, this is the first report on the SSR analysis of Romanian grapevine cultivars through this system of grouping data and can be useful for further studies concerning characterisation of Romanian grapevine cultivars at molecular level. The integration of such DNA barcodes into nationally coordinated database could increase the precision with which grapevine genetic resources are managed in Romania.

CONCLUSION

Our results concerning the allele sizes (base pairs) at Feteasca regală, Grasa de Cotnari, Cabernet Sauvignon, Chardonnay blanc, Riesling italian and Merlot noir cultivars are in accordance with those published in *Vitis* International Varieties Catalogue (VIVC) Database.

Genetic profile obtained at Băbeasca neagră with the primers: SS2, MD5, MD7, MD27, ZAG 62 and ZAG 79 (proposed by OIV and VIVC for grapevine molecular characterisation), offers data to this Romanian cultivar which may be subsequent introduced in international databases.

Microsatellite analysis based on DNA barcode represented a useful mode of grouping data, with visual representation of the number and size of alleles, allowing a facile detection of genotypic differences between analyzed cultivars. The integration of such DNA

barcodes into nationally and internationally databases may increase the accuracy with which grapevine genetic resources are characterized in Romania.

Acknowledgments. The authors thank Lecturer Dr. Bogdan Nechita (University of Agricultural Sciences and Veterinary Medicine „Ion Ionescu de la Brad" Iaşi) for providing the biological material used in this study. This research work has been financed by the Romanian Research Ministry, through the Second National Plan for Research, Development and Innovation, grant no. 51-003 (2007-2010) and Internal Competition for Research Grants of USAMV Cluj-Napoca, Human Resources-ISI, Contract no. 1357 /08.02.2013.

REFERENCES

1. Bodea, M., D. Pamfil, R. Pop and I.F. Pop (2009). Use of Random amplified polymorphic DNA to study genetic diversity anong Romanian local vine (*Vitis vinifera* L.) cultivars. Bul. USAMV-CN, seria Horticulture. 66(1):17-22.

2. Bowers, J.E., G.S. Dangl, R. Vignani and C.P. Meredith (1996). Isolation and characterization of new polymorphic simple sequence repeat loci in grape (*Vitis vinifera* L.). Genome 39:628-633.

3. Bowers, J.E., G.S., Dangl and C.,P., Meredith (1999). Development and characterization of additional microsatellite DNA markers for grape. Am. J. Enol. Vitic. 50(3):243-246.

4. Butiuc-Keul A.L., C. Craciunas, A. Coste and M. Fagaro (2010). Discrimination and genetic polymorphism in several cultivars of grapevine. Rom. Biotech. Lett. 15(1):102-110.

5. Coste, A., D. Postolache, F. Popescu and A.L. Butiuc-Keul (2010). Authentification of valuable grapevine varieties from Romania through molecular markers. Rom. Biotech. Lett. 15(1):3-11.

6. Galbacs, Z., S. Molnar, G. Halasz, P. Kozma, S. Hoffmann, L. Kovacs, A. Veres, Z. Galli, A. Szoke, L. Heszky and E. Kiss (2009). Identification of grapevine cultivars using microsatellite-based DNA barcode. Vitis 48(1):17-24.

7. Gheorghe, R.N, C.F. Popescu, D. Pamfil, A.E. Pauchnecht (2008). Genetic diversity evaluation of some autochthonous grapevine varieties by RAPD markers. Lucr. St. Seria Horticultura, Anul LI. 51:73-76.

8. Gheţea, L.G., R.M. Motoc, C.F. Popescu, N. Bărbăcar, L.E. Bărbării, C.M. Constantinescu, D. Iancu, T. Bătrânu, I. Bivol, I. Baca and G. Savin (2012). Assessment of diversity in grapevine gene pools from Romania and Republic of Moldova, based on SSR markers analysis, Horticulture, Prof. Alejandro Isabel Luna Maldonado (Ed.), ISBN: 978-953-51-0252-6. InTech.

9. Jeffrey, A.J., V. Wilson and S.L. Thein (1985). Hypervariable 'minisatellite' regions in human DNA. Nature. 314:67-73.

10. Lodhi, M. A., Z. F. Guang- Ning, N.F. Weeden, B.I. Reisch (1994). A simple and efficient method for DNA extraction from grapevine cultivars, *Vitis* species and *Ampelopsis*. Plant Mol. Bio. Rep. 12(1):6-13.

11. Motoc, R.M., L.G. Gheţea, C.F. Popescu, N. Bărbăcar, G. Savin, D. Iancu, C. Constantinescu and L.A. Bărbării (2010). Genetic variability revealed by sequencing analysis at two microsatellitic loci, in some grapevine cultivars from Romania and Republic of Moldavia. Rom. Biotech. Lett. 15(2):122-125.

12. Sefc, K.M., M.S. Lopes, F. Lefort, R. Botta, K.A. Roubelakis-Angelakis, J. Ibanez, I. Pejic, H.W. Wagner, J. Glössl and H. Steinkellner (2000). Microsatellite variability in grapevine cultivars from different European regions and evaluation of assignment testing to assess the geographic origin of cultivars. Theor. Appl. Genet. 100:498-505.

13. This, P., A. Jung, P. Boccacci, J. Borrego, R. Botta, L. Constantini, M. Crespan, G.S. Dangl, C. Eisenheld, F. Ferreira-Monteiro, S. Grando, J. Ibanez, T. Lacome, V. Laucou, R. Magalhaes, C.P. Meredith, N. Milani, E. Peterlunger, F. Regner, L. Zulini and E. Maul (2004). Development of a standard set of microsatellite reference alleles for identification of grape cultivars. Theor. Appl. Genet. 109:1448-1458.

14. Thomas, M..R. and N.S. Scott (1993). Microsatellite repeats in grapevine reveal DNA polymorphisms when analysed as sequenced-tagged sites (STSs). Theor. Appl. Genet. 86:985-990.

15. Pop, R., M. Ardelean, D. Pamfil and I.M. Gaboreanu (2003). The efficiency of different DNA isolation and purification protocols in ten cultivars of *Vitis vinifera*. Bul. USAMV-CN, 59:259-261.

16. Pop, R., M. Ardelean, I.M. Gaboreanu, D. Pamfil, M.I. Cordea, P. Raica, M. Bodea, M. Cantor (2005). RAPD analyses used in revealing molecular polymorphism of several grape varieties from vineyards of Romania, XL Croatian Symposium on Agriculture. 229.

17. Pop, R. (2008). Studiul variabilităţii somaclonale la viţa de vie cu ajutorul markerilor moleculari, Ed. Bioflux, Cluj-Napoca. 59-108.