E

UPOV

BMT/9/11
ORIGINAL: English
DATE: June 1, 2005

INTERNATIONAL UNION FOR THE PROTECTION OF NEW VARIETIES OF PLANTS

GENEVA

WORKING GROUP ON BIOCHEMICAL AND MOLECULAR TECHNIQUES AND DNA PROFILING IN PARTICULAR

Ninth Session
Washington, D.C., June 21 to 23, 2005

A MICROSATELLITE-BASED SYSTEM FOR THE PROTECTION OF GRAPEVINE VARIETIES

Document prepared by experts from Spain

A MICROSATELLITE-BASED SYSTEM FOR THE PROTECTION OF GRAPEVINE VARIETIES

J. Ibáñez and M.D. Vélez

IMIDRA. Finca El Encín. Carretera A-2, PK 38,200. 28800 Alcalá de Henares. Madrid. Spain

Introduction

1. The purpose of this paper is to show very briefly the work that has been (and is being) carried out in IMIDRA to evaluate a system based on nine microsatellites that could be useful for all the legal issues related to grapevine varieties: variety identification, the DUS test, and the identification of putative Essentially Derived Varieties (EDVs).

Vitis vinifera L.: Grapevine

2. Grapevine is one of the oldest cultivated plants in the world. Their plants are woody, and asexually multiplied through cuttings. It is a particular crop, in the sense that there are a lot of varieties in the world (between 5,000 and 10,000) and many were being grown several centuries ago. Most are local varieties, and there are numerous synonyms (one variety having different names) and homonyms (different varieties having the same name) within and between countries.

3. There are two clearly distinguishable markets for grapevine: wine and table. The first one is much more important economically and is more stable with respect to the varieties used, because in many cases the wine producers of a given place must use certain varieties to obtain a “quality” label. Hence, breeding efforts are mainly focused on clone selection. The table market is much more dynamic and most of the varieties being cultivated now are different from those cultured 20 years ago. Crossbreeding is much more active for table grapes, and new interesting varieties are quickly spread to other producer countries. As a consequence, the issue of legal protection in grapevines affects mainly these table grape varieties.

Microsatellites

4. Microsatellites markers were first described in grapevine in 1993 (Thomas and Scott 1993) and have been broadly used all over the world to characterize the different varieties existing in the grapevine world: Australia, Central Europe, France, Greece, Italy, Spain, Portugal, USA, etc. Many groups have developed their own markers, but the only attempt to harmonize a microsatellite-based system for the identification of grapevine varieties was taken within the frame of the GENRES project, in Europe, and involved 10 different laboratories, including ours (This et al. 2004). Unfortunately, at the beginning of that project there was no information of map position of the microsatellites, and among the six markers chosen, two pairs were genetically linked. We were using those six markers, in addition to another three, but that discovery led us to consider the use of different markers. The criteria taken into account to make the new choice were:

- Availability (public)
- Map position (genetically independent)
- Polymorphism (high)

- Range of amplification (to allow multiplexing)
- 'Quality' (ease of amplification, absence of known null alleles, absence of alleles differing only in 1 bp)

5. Finally, 9 microsatellite markers were chosen: VVS2, VVMD5, VVMD27, VVMD28, ssrVrZAG29, ssrVrZAG62, ssrVrZAG67, ssrVrZAG83 and ssrVrZAG112. A system was optimized, including the design of a multiplex PCR with the nine markers, and capillary electrophoresis and fluorescence analysis in an automatic sequencer.

6. This system has been used to characterize more than 1300 accessions (2600 plants) of *Vitis vinifera* L. from the collection of grapevine varieties of "El Encín", in Madrid, including the key Spanish wine varieties (Ibáñez et al. 2003). Until now, all the varieties originating from different embryos (i.e., not arising from somatic mutations) have been clearly distinguished using the nine microsatellites.

Identification / EDVs

7. The first obvious practical use of the genotype table obtained was its use for identification of varieties, and some work was done. The most interesting work related to the defense of a protected variety for putative infringements on plant breeders' rights. Over several years, different samples from different producers and also from supermarkets were analyzed with microsatellites, and compared with a dedicated database. A match was always found between the samples and the protected variety. Then we used a forensic procedure for establishing the probability of such matches. A reference collection was chosen, and matching probabilities of grape microsatellite genotypes were calculated under the assumption of independent breeding programs. Even eliminating some microsatellites from the analysis, the matching probabilities were so low that the forensic procedure using microsatellites seemed to provide a sound basis for assessing infringements on plant breeders' rights for the case of grape (Ibáñez and Eeuwijk 2003). As an example, using the nine microsatellite described here, and a conservative, small reference collection, the likelihood ratio for a random match with a given protected variety is 1 in 156,000,000.

8. As already commented, grapevine is a woody plant, and so, the production of an EDV through repeated backcrossing is highly improbable. The usual methods for producing and EDV: selection of natural (or induced) mutants, and, in the near future, genetic transformation. In any case, regarding microsatellite analysis, EDVs will very probably have identical genotype that the initial varieties.

9. In other words, if we analyze the nine microsatellites from two different plants, and they match, we will conclude beyond doubt that they are of the same variety, or clones of the same variety, or synonyms, or one is an EDV of the other. The decision of which of the cases is the right one will rely on other characteristics (e.g., morphological, agronomical, etc.). From a practical point of view, in the case of protected grapevine varieties, it is improbable that neither synonyms nor clones exist, and so, the conclusion in the case of a match with nine microsatellites will be limited to two options: the same variety, or an EDV, which should be easily resolved.

10. If a new variety has been proved to be distinct from a protected variety and these nine microsatellites show no differences between the varieties, then a reversal of the burden of

proof should occur, and the breeder of the new variety should demonstrate that this new variety is not an EDV.

Distinctness

11. A variety may be considered to be clearly distinguishable if the difference in characteristics is: (a) consistent, and (b) clear. Regarding the microsatellite system developed, the consistency should not be a problem at all: by examining two or three plants it can be seen if the difference is consistent or is a technical artefact.

12. The distinctness, or the minimum distance, that should exist between two plant varieties so that they are considered distinct according to the UPOV Convention is a difficult question' (ISF Position Papers 2003). The key point when analyzing distinctness is to establish the minimum distance, and for that, we have to determine:

1) the lower number of different alleles between different varieties
2) the higher number of different alleles within a variety

13. If there is a clear border between those two numbers, we will be able to establish an acceptable minimum distance, using a fixed criterion, rather than the COYD or the 2 x p% criteria (Roberts 2004).

14. With respect to the point 1, we will use here a couple of examples of a limited number of varieties. Of course, a general database should be used, including varieties from the entire world, but one can expect more similarity between varieties of the same origin, or between relatives. Using the collection of key Spanish wine varieties (96), all the varieties differed in 4 or more alleles (of the total of 18 alleles), while using a very specialized collection of seedless grape varieties (45), which include parents, progenies, half sibs, full sibs, the lower number of different alleles was 3, between two full sibs. Again, the remaining pair-wise comparisons resulted in 4 or more different alleles.

15. On the other hand, within a variety, no differences are expected, as grapevine is vegetatively propagated. Nevertheless, microsatellites are highly polymorphic sequences and some cases of differences within a variety have been found. We found a difference in one allele between two synonyms of Black Corinth in the microsatellite VVMD7 (not included in the system of nine) (Ibáñez et al. 2000). More mutations have been found: in Pinot (Regner et al. 2000a), White Riesling (Regner et al. 2000b), Greco di Tufo, Muscat d'Alsace (Crespan 2004), etc, and even some cases of chimerism (Franks et al. 2002; Crespan 2004). Considering the nine microsatellites used here, the only one that appeared in any of those situations was VVS2, although all, or part, of the nine markers were analyzed in all the cases. The plants that differed in a higher number of alleles (of the nine microsatellites) within a variety were found in Pinot and in Greco di Tufo and differed in only 1 allele.

16. It is important to note that all the varieties with mutant alleles are very old (several centuries) and it is not expected that this phenomenon would be common during the period of protection of a new variety.

17. Anyway, considering the two extreme values obtained (three and one alleles), a minimum distance of 1 allele should be considered as an acceptable fixed criterion to

establish distinctness. This is supported by the work of Crespan, who estimated a general mutation rate per plant and microsatellite in grapevine as 8×10^{-5} (Crespan 2004). So, it would be very improbable to find in the same plant two independent mutations producing two allele differences. In practice, finding one or two allele difference between two grapevine samples is very rare, and it would be worthwhile to use additional microsatellites to confirm or reject the distinctness.

Uniformity and Stability

18. Microsatellites are highly polymorphic sequences, because of their higher mutation rate. This causes concern about their use for certain applications, especially those related with legal issues. For that reason, a considerable effort is being made in IMIDRA to evaluate the uniformity and stability of this set of microsatellites. The aim of a project started in 2002 is to study about 4,000 plants of 19 grapevine varieties with the nine microsatellites. Table 1 shows the varieties used, and the present situation of the analysis. The varieties were carefully selected, considering different uses of the grapes (wine and table), color of the skin of the berry (recommended in the UPOV Test Guidelines for grouping grapevine varieties), the presence or absence of seeds (very important trait in the new varieties), origin in time (ancient and more recent) and geographical (from different continents).

19. The project has not yet finished, and therefore, the conclusions are preliminary and must be treated with caution. These conclusions, of course, should consider what UPOV establishes in the Guidelines for the conduct of tests for distinctness, uniformity and stability in grapevine (Test Guidelines) for Vitis L. (TG/50/8): ‘For the assessment of uniformity […] In the case of a sample size of 10 plants, the maximum number of off-types allowed would be 1’.

20. To evaluate uniformity we have tried to study the nine microsatellites in 50 plants of 3 different plantations of each of the 19 varieties (2850 plants). The plant material used in all cases was young leaves, and came mainly from Spain, but was also supplied from South Africa (Cabernet sauvignon, Chardonnay, Sugraone, and Thompson seedless), and Chile (Cabernet sauvignon).

21. Grapevine is a woody plant, as it has been stated several times here. A controlled study of stability, multiplying plants of all the varieties was difficult and impractical, allowing the study of only one, or a very limited number of cycles of multiplication. For this reason, and considering that the same varieties are multiplied in different places, thereby inevitably producing plants that are from different cycles of multiplication, we decided to study stability by looking at plants of plantations from different origins, and when possible, of different ages.

22. To evaluate stability we have tried to study nine microsatellites in 5 plants of 10 different plantations (including the three studied for uniformity) of each of the 19 varieties (950 plants). The plant material used in all cases was young leaves, and came mainly from Spain, but was also supplied from Chile, France, Germany, Hungary, South Africa, and the USA.

23. In fact, the uniformity and stability are tested in both studies: if a change in one plant was found in a sample of 5 or of 50 plants, it could affect the conclusion on uniformity, while if we found a change in all the plants of a sample of 5 or of 50 plants it could affect the conclusion on stability. For this reason, the results are explained together.

24. Until now, we have completed (all the plants with all the microsatellites) the analysis of four varieties: Cabernet sauvignon, Chardonnay, Napoleon, and Sugraone. The analyses were carried out in an automatic sequencer, using a software (Genescan) that calculates the sizes of the amplified DNA fragments using an internal standard and a linear regression. For that reason, there is an experimental variation for the values obtained for a given allele. We have found no significant differences (less than one base pair (pb) between the maximum and the minimum value found for a given allele) in any of the microsatellites of Chardonnay, Napoleon, and Sugraone, and in seven out of nine microsatellites of Cabernet sauvignon (Table 2). Nevertheless we found significant differences in two microsatellites of Cabernet sauvignon: VVS2 and VVMD5. The differences were lower than 2 bp, and could correspond to a real mutation generating a new allele, or could be due to a technical artefact. To resolve this question, the PCR was repeated in the same conditions, using the DNAs that showed the more extreme values for each allele. The differences found were not significant. Furthermore, a more reliable polymerase than Taq, was used (Pyrobest DNA polymerase), and again the differences were not significant (Table 3). So, the differences observed were technical artefacts and not mutations. In conclusion, for these four varieties, the results show that the nine microsatellites are uniform and stable. If the conclusions of the complete study were identical, these nine microsatellites could be globally considered as a stable and uniform system for grapevine analysis.

25. We have not evaluated the plants morphologically, in order to establish any relationship between microsatellite and morphological uniformity or stability, but the results show that the use of this system of microsatellite analysis would not require any additional effort from the breeder to keep uniformity and stability in their new varieties.

Conclusions

26. Once the analyses have been completed, almost 7,000 plants will have been analyzed, and the conclusions about the convenience of using this set of nine microsatellite markers (or part of it) for legal applications will be definitive. Anyway, the results obtained until now are very promising, and in fact, we have already used the system for legal purposes many times. Even the Spanish Office for Plant Varieties has requested us to characterize their reference collection with this system.

27. For the near future, the remaining question is whether to include in the table of characteristics of the Test Guidelines for grapevine (Vitis L.) (TG/50/8) those microsatellites markers that, based on the global results, deserve to be there.

Acknowledgements

This work was financially supported by the projects VIN01-025 and RF99-009, INIA, Agriculture Ministry of Spain.

A lot of people, so numerous that would be impossible to name individually, have collaborated to obtain the samples for the Uniformity and Stability project. We are very grateful to them and to all the colleagues that have participated at any point in the project.

References

Crespan, M. (2004). "Evidence on the evolution of polymorphism of microsatellite markers in varieties of Vitis vinifera L." Theoretical and Applied Genetics 108(2): 231-237.

Franks, T. R., R. Botta, M. R. Thomas and J. Franks (2002). "Chimerism in grapevines: implications for cultivar identity, ancestry and genetic improvement." Theoretical and Applied Genetics 104(2-3): 192-199.

Ibáñez, J., M. T. d. Andrés and J. Borrego (2000). "Allelic variation observed at one microsatellite locus between the two synonym grape cultivars Black Currant and Mavri Corinthiaki." Vitis 39(4): 173-174.

Ibáñez, J., M. T. de Andres, A. Molino and J. Borrego (2003). "Genetic study of key Spanish grapevine varieties using microsatellite analysis." American Journal of Enology and Viticulture 54(1): 22-30.

Ibáñez, J. and F. A. v. Eeuwijk (2003). Microsatellite profiles as a basis for Intellectual Property protection in grape. Acta Horticulturae. E. P. Botos, E. Hajdu and É. Borbás. Leiden (The Netherlands), ISHS. 603: 41-47.

ISF Position Papers (2003). ISF View on Intellectual Property, Position Papers, Statements and Motions adopted by FIS, ASSINSEL and ISF.

Regner, F., A. Stadlbauer, C. Eisenheld and H. Kaserer (2000a). "Genetic relationships among Pinots and related cultivars." American Journal of Enology and Viticulture 51(1): 7-14.

Regner, F., E. Wiedeck and A. Stadlbauer (2000b). "Differentiation and identification of White Riesling clones by genetic markers." Vitis 39(3): 103-107.

Roberts, A. (2004). Statistical methods for testing Distinctness. Workshop on Data Handling, Beijing (China), UPOV.

This, P., A. Jung, P. Boccacci, J. Borrego, R. Botta, L. Costantini, M. Crespan, G. S. Dangl, C. Eisenheld, F. Ferreira-Monteiro, S. Grando, J. Ibáñez, T. Lacombe, et al. (2004). "Development of a standard set of microsatellite reference alleles for identification of grape cultivars." Theoretical and Applied Genetics 109(7): 1448-1458.

Thomas, M. R. and N. S. Scott (1993). "Microsatellite repeats in grapevine reveal DNA polymorphisms when analysed as sequence-tagged sites (STSs)." Theoretical and Applied Genetics 86: 985-990.

Table 1: Plant material used for the evaluation of uniformity and stability. Use (T: Table; W: Wine), Color (N: Noir, black, red; B: Blanc, white), Seeds (Y: yes; N: no), Nº plants (number of plants analyzed with the nine microsatellites, except those labeled with *)

NAME OF VARIETY	USE	COLOR	SEEDS	Nº PLANTS
CARDINAL	T	N	Y	86
CRIMSON	T	N	N	150
FLAME	T	N	N	92
ITALIA	T	B	Y	184
NAPOLEON	T	N	Y	192
OHANES	T	B	Y	138
RED GLOBE	T	N	Y	181
SUGRAONE	T	B	N	185
THOMPSON	T	B	N	97
AIREN	W	B	Y	185
CABERNET SAUVIGNON	W	N	Y	185
CHARDONNAY	W	B	Y	185
GARNACHA	W	N	Y	150
MERLOT	W	N	Y	135*
MONASTRELL	W	N	Y	125*
MUSCAT D'ALEXANDRIA	T/W	B	Y	78*
PALOMINO FINO	W	B	Y	173
SAUVIGNON BLANC	W	B	Y	182*
TEMPRANILLO	W	N	Y	183*

Table 2: Summary of Genescan data obtained for nine microsatellites in Napoleón (192 plants), Sugraone (185), Chardonnay (185), and Cabernet Sauvignon (185).

NAPOLEÓN	**ZAG67**		**VVMD27**		**VVMD5**		**ZAG29**		**ZAG62**		**ZAG112**		**VVS2**		**ZAG83**		**VVMD28**	
MAXIMUM VALUE	129,68	136,92	181,42	191,72	231,45	235,56	109,89		188,58	203,55	227,7	236,45	130,39	132,47	201,43		243,06	247,16
MINIMUM VALUE	129,19	136,49	180,44	191,13	230,76	234,82	108,9		187,73	203,12	227,18	235,87	129,77	131,81	201,11		242,33	246,32
DIFFERENCE	0,49	0,43	0,98	0,59	0,69	0,74	0,99		0,85	0,43	0,52	0,58	0,62	0,66	0,32		0,73	0,84
AVERAGE	129,45	136,73	180,68	191,36	231,11	235,16	109,16		188,01	203,30	227,46	236,18	130,05	132,09	201,28		242,69	246,70
STANDARD DEVIATION	0,1227	0,1081	0,1208	0,1141	0,1289	0,1325	0,1539		0,1272	0,0892	0,0836	0,0847	0,1404	0,1396	0,0553		0,1421	0,1469
SUGRAONE	**ZAG67**		**VVMD27**		**VVMD5**		**ZAG29**		**ZAG62**		**ZAG112**		**VVS2**		**ZAG83**		**VVMD28**	
MAXIMUM VALUE	123,71	136,97	176,92	178,98	222,95	232,93	109,41	111,37	185,93	188,05	232,84		115,69	132,21	189,8	195,48	246,86	
MINIMUM VALUE	123,41	136,67	176,45	178,47	222,48	232,03	108,74	110,76	185,45	187,55	232,02		115,13	131,75	189,39	195,2	245,9	
DIFFERENCE	0,30	0,30	0,47	0,51	0,47	0,90	0,67	0,61	0,48	0,50	0,82		0,56	0,46	0,41	0,28	0,96	
AVERAGE	123,55	136,81	176,69	178,77	222,68	232,74	109,09	111,04	185,69	187,81	232,18		115,41	131,99	189,59	195,33	246,45	
STANDARD DEVIATION	0,0664	0,0632	0,094	0,1154	0,08	0,1813	0,1438	0,1369	0,1093	0,1149	0,0770		0,1090	0,0863	0,072	0,0518	0,1703	
CHARDONNAY	**ZAG67**		**VVMD27**		**VVMD5**		**ZAG29**		**ZAG62**		**ZAG112**		**VVS2**		**ZAG83**		**VVMD28**	
MAXIMUM VALUE	136,92	150,74	178,94	186,7	231,32	235,26	109,16		188,12	195,63	238,21		134,25	140,55	189,85	201,67	216,78	226,83
MINIMUM VALUE	136,36	150,12	178,37	186,13	230,64	234,68	108,59		187,49	195,05	237,7		133,62	139,88	189,49	201,16	216,1	226,06
DIFFERENCE	0,56	0,62	0,57	0,57	0,68	0,58	0,57		0,63	0,58	0,51		0,63	0,67	0,36	0,51	0,68	0,77
AVERAGE	136,68	150,49	178,64	186,37	230,92	234,94	108,81		187,84	195,32	238,04		133,87	140,17	186,66	201,36	216,40	226,5
STANDARD DEVIATION	0,091	0,0945	0,1055	0,0969	0,1148	0,1152	0,1109		0,1257	0,1051	0,0716		0,1184	0,118	0,0701	0,0612	0,1314	0,1249
CABERNET SAUVIGNON	**ZAG67**		**VVMD27**		**VVMD5**		**ZAG29**		**ZAG62**		**ZAG112**		**VVS2**		**ZAG83**		**VVMD28**	
MAXIMUM VALUE	123,7	137,01	173,15	186,77	228,9	237,15	109,21		188,16	193,85	227,61	232,47	136,53	149,76	201,55		232,98	234,96
MINIMUM VALUE	123,05	136,53	172,16	185,92	227,28	235,41	108,23		187,24	193,05	227,05	231,91	135,47	148,57	201,17		232,00	234,03
DIFFERENCE	0,65	0,48	0,99	0,85	**1,62**	**1,74**	0,98		0,92	0,80	0,56	0,56	**1,06**	**1,19**	0,38		0,98	0,93
AVERAGE	123,36	136,72	172,55	186,24	228,17	236,26	108,64		187,67	193,45	227,37	232,14	135,78	148,96	201,33		232,45	234,44
STANDARD DEVIATION	0,1206	0,1074	0,2490	0,2057	0,4694	0,5282	0,2504		0,2290	0,1753	0,0951	0,1082	0,2134	0,247	0,0573		0,2467	0,2435

Table 3: Results obtained for microsatellites VVMD5 and VVS2 with Taq Polymerase and Pyrobest DNA Polymerase.

VVMD5	**Taq Polymerase**		**Pyrobest DNA Polymerase**	
Allele 1	**Value**	**Difference**	**Value**	**Difference**
Maximum	228,90	1,62	227,61	0,02
Minimum	227,28		227,63	
Allele 2	**Value**	**Difference**	**Value**	**Difference**
Maximum	237,15	1,74	235,85	0,03
Minimum	235,41		235,88	
VVS2	**Taq Polimerase**		**Pyrobest DNA polimerase**	
Allele 1	**Value**	**Difference**	**Value**	**Difference**
Maximum	136,53	1,06	134,75	0,01
Minimum	135,47		134,76	
Allele 2	**Value**	**Difference**	**Value**	**Difference**
Maximum	149,76	1,19	147,81	0,14
Minimum	148,57		147,95	

[End of document]