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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

1. The BMT agreed that, where agreed by the relevant experts, the presentations made at the meeting should be made available in the BMT document section of the UPOV website, as addenda to the relevant documents. This document contains a copy of the presentation made by Mr. Javier Ibañez (Spain), based on document BMT/9/11.

A microsatellite-based system for the protection of grapevine varieties

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BMT 9. Washington D.C., June 21 to 23, 2005

- Woody, asexually multiplied
- Very old culture
- Very high number of varieties
- Two different markets: wine and table





Sequence-Tagged Microsatellite Sites Grapevine

- Broadly used for characterization
- About 300
- GENRES 081 project


 **Sequence-Tagged Microsatellite Sites**
GENRES 081

- 6 loci, 10 laboratories
- Proposal of a standard set of alleles / varieties
- This et al. (TAG 2004)



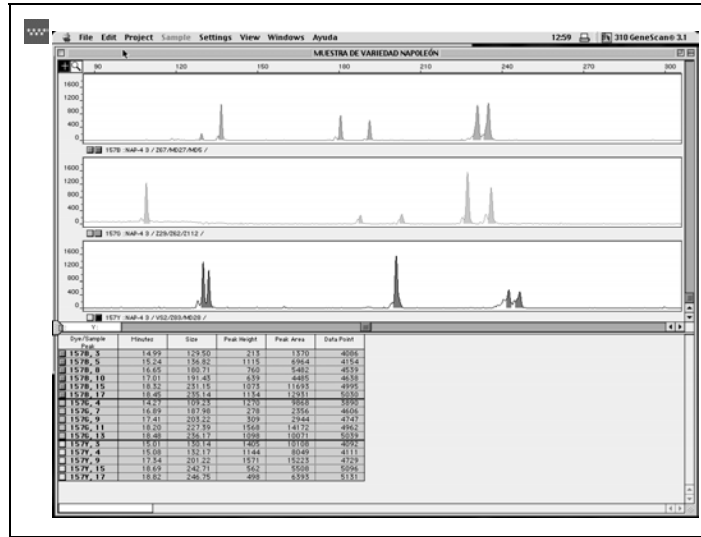
 **Selection of Microsatellites**
Criteria

- Availability (public)
- Map position (genetically independent)
- Polymorphism (high)
- Range of amplification (that allow multiplexing)
- ‘Quality’ (ease of amplification and scoring, absence of known null alleles, absence of alleles differing only in 1 bp)

 **Selection of Microsatellites**

ZAG67	VVMD5	VVMD27
ZAG29	ZAG62	ZAG112
VVS2	ZAG83	VVMD28

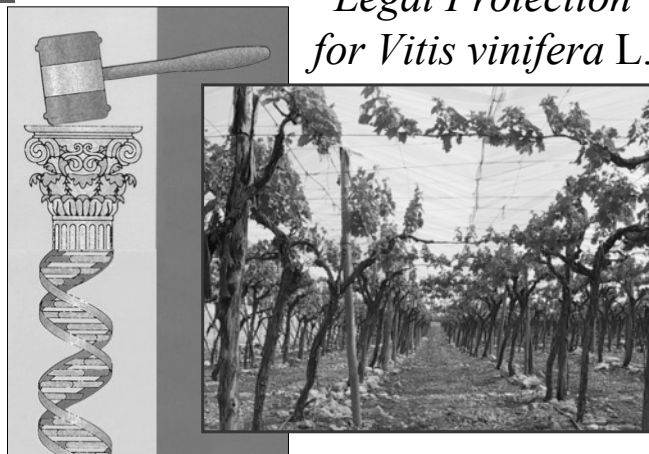
Multiplex PCR of 9 microsatellites



Characterization of grapevines with 9 microsatellites

- More than 1,300 accessions (2 plants / accession)
- Until now, all varieties arising from different embryos have been distinguished
- Clones and sports cannot be distinguished

Legal Protection for Vitis vinifera L.



(From Evett & Weir, 1998)



Role of microsatellites in the legal Identification

- Non protected varieties:
 - Mistakes and frauds from nurseries
 - Authorized by Origin Denomination Councils
 - List of commercial varieties
- Protected varieties:
 - Identification of illegal plants
 - Identification of EDVs



Possible role of microsatellites in the legal Protection

- DUS tests: Rapid screening against the reference collection (previous to the morphological exam?)
- Faster resolution to the objections to applications.



Role of microsatellites in the legal Identification

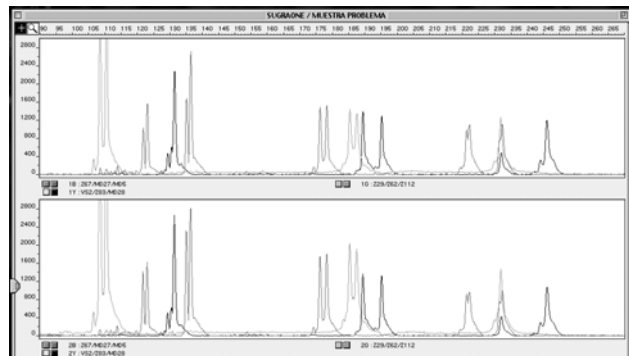
Forensic approach:

- Database of a Reference collection
- Analysis of microsatellites in the sample
- Mathematical analysis of the match (if proceed)

Database (9 Microsatélites)

- ❖ World seedless table grape collection (45 varieties).
- ❖ World table grape collection (267 varieties).
- ❖ Spanish wine grape collection (96 varieties).
- ❖ All the varieties can be distinguished unequivocally.

Identity analysis




Likelihood ratio

$$LR = \frac{P(R / V)}{P(R / NV)}$$


P(R/V): probability of the match if the sample belongs to the variety.

P(R/NV): probability of the match if the sample does NOT belong to the variety.



Identity analysis

- ❖ 6 microsatellites. LR = 1 in 87.000
- ❖ 9 microsatellites. LR = 1 in 156.000.000




Role of microsatellites in the legal Identification

Grapevine protected variety

Total Identity
9 Microsatellites

Variety EDV Clone Synonym

Morphologically Distinct?
Reversal of the burden of proof



Role of microsatellites in the legal Identification

2 or more different alleles
9 Microsatellites

Different Variety

Role of microsatellites in the legal Identification


1 different allele
9 Microsatellites

Analyze more microsatellites

Possible role of microsatellites in the legal Protection

Evaluation of microsatellites markers for:


- ❖ Distinction
- ❖ Uniformity
- ❖ Stability



Possible role of microsatellites in the legal Protection

Evaluation of microsatellites markers for:

- ❖ Distinction
- ❖ Uniformity
- ❖ Stability



Possible role of microsatellites in the legal Protection: Distinction

Difference:

- Consistent
- Clear

→ MINIMUM DISTANCE

Possible role of microsatellites in the legal Protection: Distinction

- Lower number of different alleles between varieties
- Higher number of different alleles within varieties

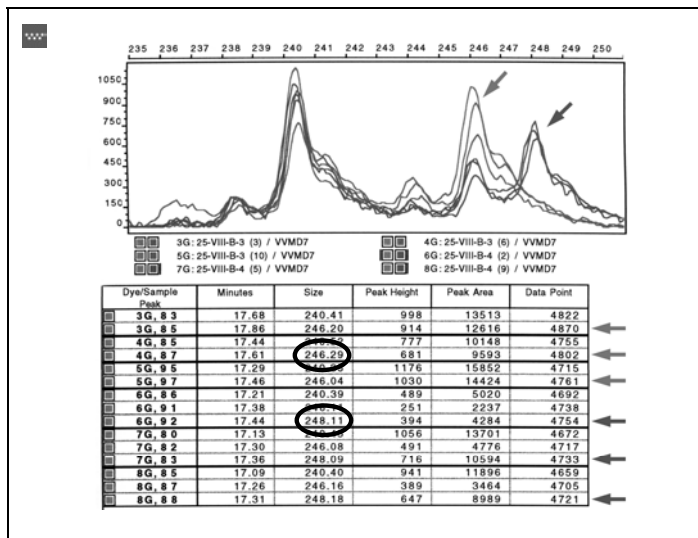
→ MINIMUM DISTANCE

Possible role of microsatellites in the legal Protection: Distinction

- Lower number of different alleles between varieties
 - Spanish wine varieties (96): 4 alleles
 - World seedless table grape varieties (45): 3 alleles (including parents, progenies, half-sibs, full-sibs, etc.)
 - World table grape varieties (267): 2 alleles (including parents, progenies, half-sibs, full-sibs, etc.)

Possible role of microsatellites in the legal Protection: Distinction

- Higher number of different alleles within varieties



Possible role of microsatellites in the legal Protection: Distinction

- Higher number of different alleles within varieties
 - Pinot meunier (1 allele in VVS2, chimerism)
 - Greco di Tufo (1 allele in VVS2)
 - Chasselas (1 allele in ZAG83)
 - Mutation rate in grapevine per microsatellite and plant: 8×10^{-5}

Possible role of microsatellites in the legal Protection: Distinction

- Lower number of different alleles between varieties
- Higher number of different alleles within varieties

The diagram consists of two circles, one containing the number '2' and the other containing '1'. An arrow points from the '2' circle to the '1' circle. To the right of the arrow is a rectangular box containing the text 'MINIMUM DISTANCE'. Below this box is a smaller black square containing the number '2'.

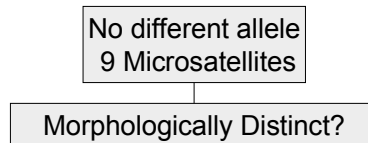
Possible role of microsatellites in the legal Protection: Distinction

A flowchart with a rectangular box at the top containing the text '2 different alleles' and '9 Microsatellites'. A vertical line connects this box to a second rectangular box below it containing the text 'Distinct Variety'.

Possible role of microsatellites in the legal Protection: Distinction

A flowchart with a rectangular box at the top containing the text '1 different allele' and '9 Microsatellites'. A vertical line connects this box to a second rectangular box below it containing the text 'Analyze more microsatellites'.

Possible role of microsatellites in the legal Protection: Distinction



Possible role of microsatellites in the legal Protection

Evaluation of microsatellites markers for:

- ❖ Distinction
- ❖ Uniformity
- ❖ Stability



Evaluation of 9 microsatellites for Uniformity and Stability

Project VIN01-025 (2002/2004)

- 9 microsatellites and 19 varieties (wine and table)
- Plant material from Spain, Europe, Africa and America
- About 4,000 plants

Evaluation of 9 microsatellites for Uniformity and Stability

- Uniformity:
 - 3 plantations / variety
 - 50 plants / plantation
- Present DUS tests in grapevine: 10 plants (1 off-type allowed)

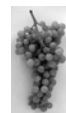


Evaluation of 9 microsatellites for Uniformity and Stability

- Stability:
 - 10 plantations / variety
 - 5 plants / plantation
- Different plantations are probably in different cycles of propagation

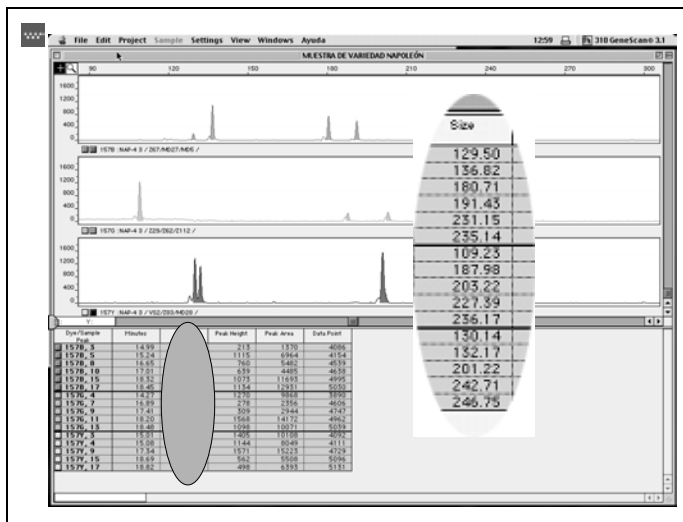
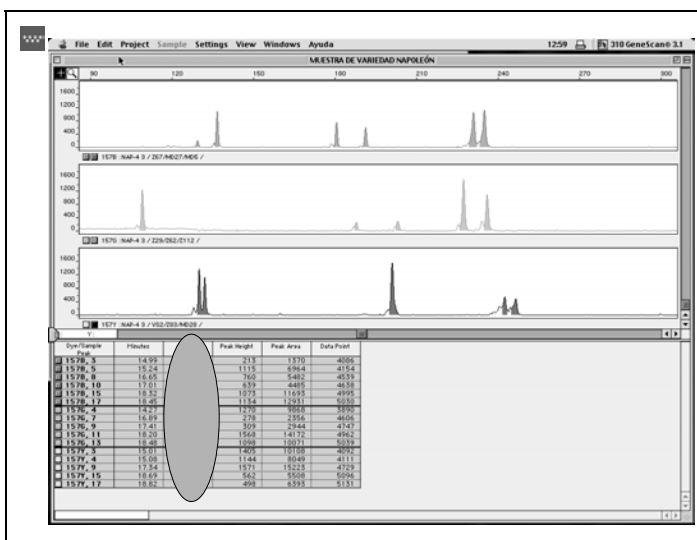


NAME OF VARIETY	USE	COLOR	SEEDS	N° PLANTS
CARDINAL	T	N	Y	86
CRIMSON SEEDLESS	T	N	N	150
FLAME SEEDLESS	T	N	N	92
ITALIA	T	B	Y	184
NAPOLEON	T	N	Y	192
OHANES	T	B	Y	138
RED GLOBE	T	N	Y	181
SUGRAONE	T	B	N	185
THOMPSON SEEDLESS	T	B	N	97
AIREN	W	B	Y	185
CABERNET SAUVIGNON	W	N	Y	185
CHARDONNAY	W	B	Y	185
GARNACHA	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*



Plant Material

VARIETY	Nº PLANTS	RANGE YEAR PLANTATION	COLLECTING COUNTRIES
NAPOLEÓN	192	(1800-1997)	Spain
SUGRAONE	185	(1987-2000)	Chile, South Africa, and Spain
CHARDONNAY	185	(1988-2000)	Chile, Hungary, Germany, South Africa, and Spain
CABERNET SAUVIGNON	185	(1965-2001)	Chile, Hungary, Germany, South Africa, and Spain



**Analysis of GENESCAN values for
Napoleón**

	Z67		MD27		MD5		Z29		Z62	
AVERAGE	129,45	136,73	180,68	191,36	231,11	235,16	109,16	0	188,01	203,30
DIFFERENCE (max-min)	0,49	0,43	0,98	0,59	0,69	0,74	0,99	0	0,85	0,43
STANDARD DEVIATION	0,1227	0,1081	0,1208	0,1141	0,1289	0,1325	0,1539	0	0,1272	0,0892

	Z112		VS2		Z83		MD28	
AVERAGE	227,46	236,18	130,05	132,09	201,28	0	242,69	246,70
DIFFERENCE (max-min)	0,52	0,58	0,62	0,66	0,32	0	0,73	0,84
STANDARD DEVIATION	0,0836	0,0847	0,1404	0,1396	0,0553	0	0,1421	0,1469

**Analysis of GENESCAN values for
Sugraone**

	Z67		MD27		MD5		Z29		Z62	
AVERAGE	123,55	136,81	176,69	178,77	222,68	232,74	109,09	111,04	185,69	187,81
DIFFERENCE (max-min)	0,30	0,30	0,47	0,51	0,47	0,90	0,67	0,61	0,48	0,50
STANDARD DEVIATION	0,0664	0,0632	0,094	0,1154	0,08	0,1813	0,1438	0,1369	0,1093	0,1149

	Z112		VS2		Z83		MD28	
AVERAGE	232,18	0	115,41	131,99	189,59	195,33	246,45	0
DIFFERENCE (max-min)	0,82	0	0,56	0,46	0,41	0,28	0,96	0
STANDARD DEVIATION	0,0770	0	0,1090	0,0863	0,072	0,0518	0,1703	0

**Analysis of GENESCAN values for
Chardonnay**

	Z67		MD27		MD5		Z29		Z62	
AVERAGE	136,68	150,49	178,64	186,37	230,92	234,94	108,81	0	187,84	195,32
DIFFERENCE (max-min)	0,56	0,62	0,57	0,57	0,68	0,58	0,57	0	0,63	0,58
STANDARD DEVIATION	0,091	0,0945	0,1055	0,0969	0,1148	0,1152	0,1109	0	0,1257	0,1051

	Z112		VS2		Z83		MD28	
AVERAGE	238,04	0	133,87	140,17	186,66	201,36	216,40	226,5
DIFFERENCE (max-min)	0,51	0	0,63	0,67	0,36	0,51	0,68	0,77
STANDARD DEVIATION	0,0716	0	0,1184	0,118	0,0701	0,0612	0,1314	0,1249

Analysis of GENESCAN values for Cabernet sauvignon

	Z67		MD27		MD5		Z29		Z62	
AVERAGE	123,36	136,72	172,55	186,24	228,17	236,26	108,64	0	187,67	193,45
DIFFERENCE (max-min)	0,65	0,48	0,99	0,85	●	●	0,98	0	0,92	0,80
STANDARD DEVIATION	0,1206	0,1074	0,2490	0,2057	0,4694	0,5282	0,2504	0	0,2290	0,1753

	Z112		VS2		Z83		MD28	
AVERAGE	227,37	232,14	135,78	148,96	201,33	0	232,45	234,44
DIFFERENCE (max-min)	0,56	0,56	●	●	0,38	0	0,98	0,93
STANDARD DEVIATION	0,0951	0,1082	0,2134	0,247	0,0573	0	0,2467	0,2435


Differences in Cabernet sauvignon

Differences in VVMD5 and VVS2 :

¿Mutation or technical artefact?

Mutation

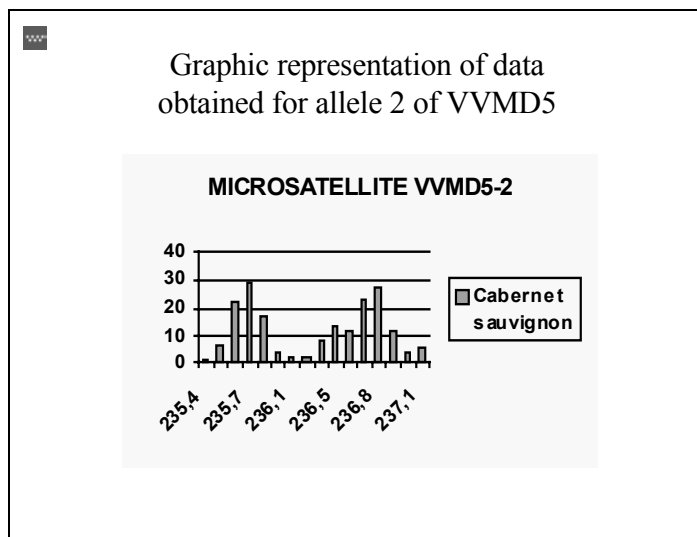
Technical Artefact



Lack of stability

'+A' Peaks (Taq)

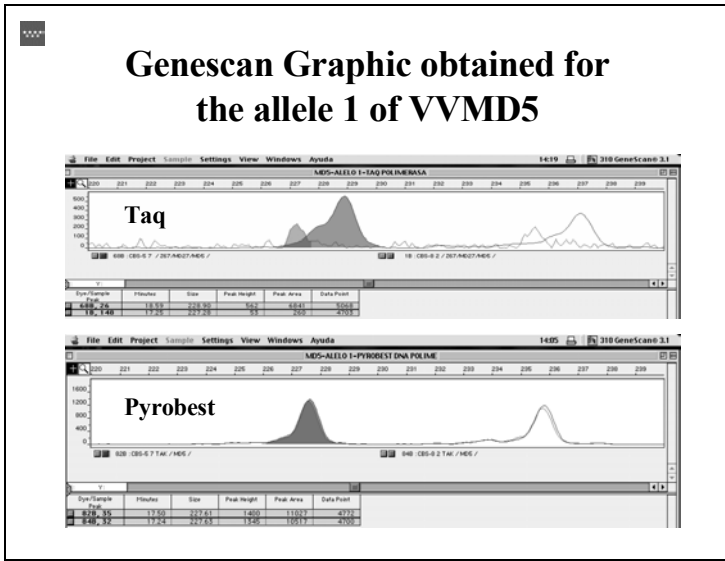
Electrophoresis



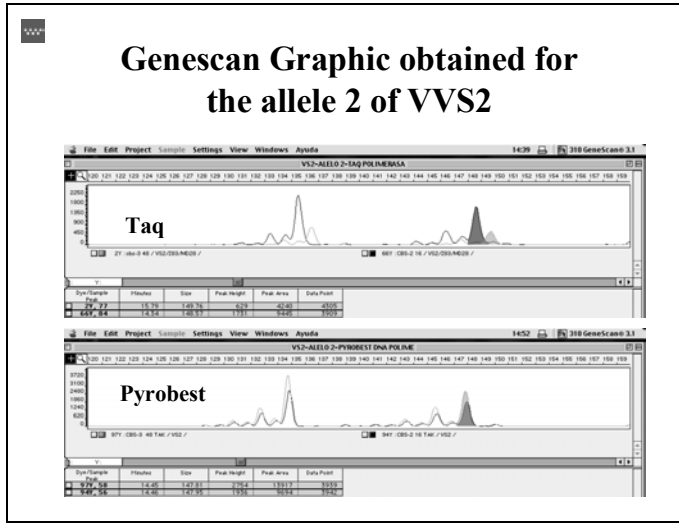
Data obtained for allele 1 of VVS2

135,47	135,52	135,57	135,64	135,68	135,82	135,88	135,93	136,00	136,19
135,48	135,52	135,58	135,64	135,68	135,82	135,89	135,93	136,01	136,20
135,48	135,52	135,58	135,65	135,69	135,82	135,89	135,93	136,02	136,21
135,49	135,52	135,59	135,65	135,71	135,83	135,89	135,94	136,03	136,38
135,50	135,52	135,59	135,65	135,71	135,83	135,89	135,94	136,06	136,53
135,50	135,52	135,61	135,65	135,72	135,84	135,89	135,94	136,07	
135,50	135,52	135,62	135,66	135,72	135,84	135,90	135,95	136,07	
135,50	135,52	135,62	135,66	135,72	135,85	135,90	135,97	136,07	
135,50	135,53	135,62	135,66	135,73	135,86	135,90	135,97	136,07	
135,50	135,53	135,62	135,66	135,74	135,86	135,91	135,97	136,10	
135,50	135,53	135,62	135,66	135,76	135,86	135,91	135,97	136,11	
135,50	135,53	135,63	135,66	135,76	135,86	135,91	135,97	136,11	
135,50	135,53	135,63	135,66	135,76	135,87	135,91	135,98	136,11	
135,51	135,53	135,63	135,66	135,77	135,87	135,92	135,98	136,13	
135,51	135,53	135,63	135,66	135,78	135,87	135,92	135,98	136,13	
135,51	135,53	135,63	135,66	135,78	135,88	135,92	135,98	136,14	
135,51	135,53	135,63	135,67	135,78	135,88	135,92	135,99	136,14	
135,51	135,54	135,64	135,67	135,78	135,88	135,92	136,00	136,17	
135,51	135,54	135,64	135,67	135,80	135,88	135,93	136,00	136,18	
135,51	135,56	135,64	135,67	135,81	135,88	135,93	136,00	136,18	

- Differences in Cabernet
sauvignon
- Repetition in the same conditions, using Taq polymerase
 - Check using Pyrobest DNA polymerase (proof reading activity, no nucleotidil-transferase activity: no '+A' peaks)



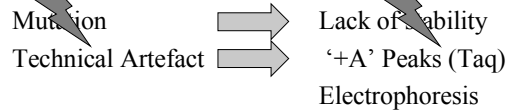
Genescan Graphic obtained for the allele 2 of VVS2



Differences in Cabernet sauvignon

Differences in VVMD5 and VVS2 :

¿Mutation or technical artefact?



Conclusions based on present data

- Distinction: Minimum distance: 2 alleles
- Uniformity and stability: no additional efforts for breeders
- Inclusion in the Test Guidelines for Grapevine (TG/50/8)

**A microsatellite-based system for
the protection of grapevine varieties**



Thank you for
your attention

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