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Development and characterization of new microsatellite markers for grape

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Summary

Thirty five new grape microsatellite markers were developed under an international consortium involving AGROGENE. These loci were amplified in 41 Spanish cultivars of *V. vinifera*. Eleven of the markers were polymorphic and informative in *V. vinifera*. Twelve were monomorphic and of the remaining markers one was polymorphic but less useful because individuals amplified more than two bands and the rest had amplification problems. The number of alleles detected for the 11 informative markers ranged from 4 to 12, with heterozygosity values ranging from 0.6 to 0.8. Primer sequences are reported for these markers.

K e y w o r d s : Grape, *Vitis vinifera*, microsatellites, DNA polymorphism.

Introduction

Microsatellite markers (also called simple sequence repeat or SSR markers) are now widely used in grapevine genetic research for identification of cultivars (Sefc *et al.* 1999; Martín *et al.* 2003; Ibañez *et al.* 2003), parentage analysis (Bowers and Meredith 1997; Bowers *et al.* 1999 a), genome mapping (Doligez *et al*. 2002; Riaz *et al*. 2004) and genetic characterization of germplasm (Lopes *et al*. 1999, Sefc *et al.* 1999). The development of microsatellite markers is a costly and time-consuming procedure involving the construction and screening of genomic libraries and the design and optimization of PCR primers. In total, about 50 microsatellites markers have been developed in three different laboratories using the procedure mentioned above (Thomas *et al*. 1993; Bowers *et al.* 1996; 1999 b; Sefc *et al.* 1999; Lefort e*t al.* 2002). However, for the maker type to become of greatest use to the viticultural research community, more microsatellites are required. The International Vitis Microsatellite Consortium (VMC) including the private company Agrogene (France) and 21 research laboratories worldwide recently met the target of developing 333 new *Vitis* markers from a microsatellite- enriched library. Here we report the development of 35 new markers from the VMC, 11 of which are polymorphic and useful for *Vitis vinifera*.

Material and Methods

P l a n t m a t e r i a l : The plant material was obtained from the *Vitis* Germplasm Bank of El Encín, Instituto Madrileño de Investigación Agraria y Alimentaria, Consejería de Medio Ambiente, Spain. Total genomic DNA was isolated from young frozen leaves with the kit DNAeasy (Qiagen).

P C R c o n d i t i o n s : Microsatellite polymorphisms were detected radioactively. Forward primers were end-labeled by phosphorylation with δP^{33} ATP using T_4 Polynucleotide kinase. Polymerase chain reactions were carried out in 10 µl volume containing 25 ng template DNA, 200 µM of each dNTP (Larova Biochimie GmbH, Teltow, Germany), 0.4U of Taq DNA polymerase (Boehringer Mannheim, Germany), 1 µl 10X PCR buffer (100 mM Tris-HCl, 500 mM KCl, 20 mM $MgCl_2$), 5 µM of each primer, 1 µl of 50 % DMSO solution. T_m was the annealing temperature proposed with each primer. PCR amplification was performed with the following thermal cycles consisting of 15 cycles (denaturation, 30 s at 94 °C, annealing, 30 s at (T_m - 1) °C, the annealing temperature was reduced in each cycle by 0.2 °C during these 15 cycles, extension, 45 s at 72 °C) followed by 20 cycles (denaturation, 30 s at 94 °C, annealing, 30 s at (T_m - 1) °C - 3 °C, extension, 45 s at 72 °C).

After the PCR, samples were denatured by adding an equal volume of formamide buffer (98 % formamide, 10 mM EDTA pH 8.0, 0.05 % bromophenol blue, and 0.05 % xylene cyanol) and heated for 3 min at 94 ºC. Three µl of each sample were loaded on 6 % acrylamide/bisacrylamide 19:1, 7.5 M urea and 1X TBE gels and electophoresed at 90 W. After electrophoresis, gels were dried onto Whatman paper and exposed to X-ray film. Every sample was analysed at least twice to ensure genotype reproducibility. Polymorphic bands were scored by visual inspection of the resulting autoradiograms.

D a t a a n a l y s i s : All gels were scored visually at least two times. Allele sizes were initially determined by comparison to a sequencing reaction and in subsequent analysis by comparison to reference cultivars.

Results and Discussion

Polymorphisms for 35 nuclear SSRs loci were initially analysed against a sample set of 41 grapevine cultivars (*Vitis vinifera* L.) from Spain (Appendix). Only 11 of them were polymorphic. The new polymorphic microsatellite markers (VMC6G8, VMC6D12, VMC6B11, VMC6g10, VMC6C7, VMC6C10,VMC6E10, VMCNG2B7.2, VMCNG2G7, VMCNGH7, VMCNG2E8), with the assigned GeneBank accession numbers (BV209002; BV208992; BV208993; BV208994; BV208995; BV208996; BV208997; BV208998;

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Table 1

Characteristics of 11 polymorphic microsatellite markers in 41 *Vitis vinifera* cultivars

Locus	primers 5´-3´	Repeat	Allele size (bp)	Freq	Locus	primers 5´-3´	Repeat	Allele size (bp)	Freq
VMC6G8-F	GAGTGTCAGTCTCAAAATAAGGA	$(GA)_{15}$	109	0.15	VMC6C10-F	TTCCTGCGAATTCTAACCCCTT	$(GA)_{17}$	143	0.05
VMC6G8-R	CCCCTCATCTCTTCTCTATCTAA		105	0.17	VMC6C10-R	CCACTTCCATTCCCTCTCCTGT		130	0.22
			103	0.16				124	0.18
			101	0.26				120	0.20
			97	0.14				115	0.08
			89	0.08				109	0.14
			88	0.04				105	0.13
VMC6D12-F	CTCTCTTTTCCGAAATTGGGGT	$(TC)_{18}$	160	0.48	VMC6E10-F	CTAGGTGTGCCAAGAGATCAGA	$(GA)_{13}$	122	0.07
VMC6D12-R	ATTTTCCCTGGAAACAAAGTGG		153	0.07	VMC6E10-R	CATTTGTGGGTAGTTGTGAGGA		117	0.07
			148	0.08				116	0.12
			145	0.18				113	0.13
			141	0.05				110	0.16
			130	0.15				104	0.003
VMC6B11-F	TGATTATGGCAATAATCACACC	$(TC)_{20}$	116	0.09				108	0.14
VMC6B11-R	TTGCTTACCCATCAAAAAGAAA		109	0.18				100	0.07
			104	0.02				95	0.10
			100	0.12				98	0.06
			97	0.18				92	0.03
			92	0.24				90	0.04
			89	0.09	VMCNG2B7.2-F	TTTTGGAGTGAATAGAGACCCCT	$(GA)_{13}$	156	0.29
			85	0.05	VMCNG2B7.2-R	CAGAATTTGGCTCCATATTTGAA		154	0.10
			83	0.03				144	0.02
VMC6G10-F	CATCATTCATCCAAATTATGTAG	$(GA)_{14}$	180	0.03				142	0.13
VMC6G10-R	TTTAGTAGGTTAGGGATACCAGT		170	0.46				134	0.46
			168	0.14	VMCNG2G7-F	CAACAGAATTCAAATGAAATGGA	$(TC)_{18}$ $(TC)_{7}$	134	0.04
			167	0.08	VMCNG2G7-R	CAAACAGCATAAATACACAAGGA		118	0.10
			150	0.13				112	0.01
			131	0.16				110	0.09
VMC6C7-F	ACATATATCCGAAAGTGTGGGGC	$(GA)_{10}$	161	0.07				106	0.71
VMC6C7-R	CTTAAAGCTTGAAGCTTTTGGTGC		158	0.07				98	0.05
			157	0.36	VMCNG2H7-F	ACGTTAAATAGAACATGGTCCC	$(GA)_{16}$	178	0.13
			138	0.33	VMCNG2H7-R	CAACCTCTTTTTTGAGGTAGC		176	0.03
			132	0.14				172	0.65
			114	0.03				170	0.01
VMCNG2E8-F	CAGAGACAAAGGAAACGAGGCT	$(GA)_{29}$	208	0.10				168	0.14
VMCNG2E8-R	TGCCTACCTAGTGCCATTCAAA		206	0.07				150	0.04
			204	0.08					
			190	0.75					

Table 2

Charactersitic of SSR markers less useful

Locus	Primers 5´-3´	Comments
VMC6E9.2F	ACAAACACATGCGCATCACAC	No clear amplification
VMC6E9.2R	CGGGCACAATGGATATGAGAG	
VMC6F11F	ACAACTTTGTGCTGCCACTACC	More than two bands per individual
VMC6F11R	AGCCAGAGTTACTATGCTGCCA	
VMC16A1F	AATTAGTTTCTAATAATGCAGGA	Monomorphic
VMC16A1R	GTGAGAGAACAGGATGGTAA	
VMC16C1F	CGCATTACATATTCAATTTCCT	Monomorphic
VMC16C1R	TGAAGTGCTGTTTGAAGAGAGT	
VMC6A8F	TTGATTTTGGAGTTCTTTGGAC	Monomorphic
VMC6A8R	ACCAATTACCAAATTCTTGTTC	
VMCNG2E7F	AGAGTGATGAGGTGAAAAGGAG	No clear amplification
VMCNG2E7R	TTATGAGGAATGTGGAAAGGAG	
VMCNG2B8F	GGGAATTCATGGAAGGAAAGA	Monomorphic
VMCNG2B8R	AGACAATCACCGTGTATTGCTG	
VMCNG2C12.1F	ACTTACGCCCTCGTTTCGCT	No clear amplification
VMCNG2C12.1R	GCGCAGTCTGCTGAATTCTGTAT	
VMCNG2G8F	AGAGGCTTGTTAAGGCGAGGTT	Monomorphic
VMCNG2G8R	GTCACATGCGAGTGAGCTTTTC	
VMCNG2A9F	TCCGCAGTAGCGCTCAGA	No clear amplification
VMCNG2A9R	TTCGCGACACTTCCCCTT	
VMCNG2B9.2F	GACTGAAGAGAGTGCCTTTGCC	Monomorphic
VMCNG2B9.2R	CTTCCTGCCCTGCTGTTACC	
VMCNG2A10F	TTTCCACCGGTGTAACACCC	No clear amplification
VMCNG2A10R	TTGCCATCCCCCACAC	
VMCNG2H10F	AATCTGACACTGTATTTCTGGCCA	Momomorphic
VMCNG2H10R	TTGGAAAAAAAGGGAAAAGAGAGA	
VMCNG2A11F	CTGAAGGAGGATAAAGGGGTAA	No clear amplification
VMCNG2A11R	GGTATGCATGAAAAGGAACAAC	
VMCNG2B11F	GTGCCTTCATCTGGATATGTCT	Monomorphic
VMCNG2B11R	ATGTATCTGTGAGCTGTGGGTA	
VMCNG2E11F	TGCATCCGAGTTCGAATACC	Monomorphic
VMCNG2E11R	CTCTGCAACTGGCTCCTGTC	
VMCNG2H11F	GAAAGGAGGAAGAATAGCACGA	Monomorphic
VMCNG2H11R	TCCAGACACAAATCCACTATGG	
VMCNG2A12F	CGTAACAGTAACAATCGCCAGA	No clear amplification
VMCNG2A12R	ATGGTAGCTGATGAACCAGAGG	
VMCNG2E12F	CTATGTACGCCGTGGACTGA	No clear amplification
VMCNG2E12R	GCATGTGCACCATATGGACC	
VMCNG2G12F	AAGTATTCTGCTGACTGGCTCC	Monomorphic
VMCNG2G12R	ATCGCTTTCTACATCATTTCCG	
VMCNG2H12F	TCATCTCGCAAGATGCATTACC	Monomorphic
VMCNG2H12R	GCGCTCTTGTCACTTTCTGTCC	
VMCNG2F12F	TCGCTGGAGAGATAGATGCCTT	No clear amplification
VMCNG2F12R	AGGCCACCGGATCAAAACT	
VMCNG2D11F	GAGTTTCCAAACAGGTGGCATC	More than two bands
VMCNG2D11R	CAGCCATTCCGTTTTCCATCTA	
VMCNG2G9F	TGCAATCTCATCCACTGGACG	No clear amplification
VMCNG2G9R	GGATCGAAGACTCTTTTTCTCGC	

BV208999; BV209000; BV209001) were characterised and analysed in 41 traditionally grown wine and table cultivars. The markers are polymorphic in *V. vinifera* and produce unambiguous results. Primer sequences and genetic information for these markers are shown in Tab. 1. Amplified products ranged in size from 90 to 208 base pairs (bp). The number

Appendix

Spanish grapevine cultivars analysed in this study

1. Graciano
2. Malvasía (Vitoria)
3. Rojal (Logroño)
4. Moscatel de grano menudo
5. Turruntés (Rioja)
6. Malvasía (Logroño)
7. Turruntés (Haro)
8. Moscatel de Cadiz
9. Malvasía (Navarra)
10. Tempranillo (Rioja)
11. Moscatel (Cordoba)
12. Laíren
13. Torrontés (Cordoba)
14. Jaén Negro
15. Zalema
16. Listán Blanco
17. Moscatel Negro
18. Moristel
19. Alcañon
20. Parraleta
21. Vidadillo
22. Garnacha Peluda
23. Garnacha Blanca
24. Derechero de Muniesa
25. Malvasía (Las Palmas)
26. Moscatel Blanco (Gran Canaria)
27. Malvasía blanca (Lanzarote)
28. Malvasía (Tenerife)
29. Ondarrabi Beltza
30. Cariñena
31. Pansa rosada
32. Malvasía de Sitges
33. Xarello
34. Subirant Parent
35. Parellada
36. Macabeo
37. Garnacha tintorera
38. Roja blanco
39. Rojal (Albacete)
40. Albillo (Madrid)
41. Rojal (Cuenca)

of alleles observed per locus ranged from 4 to 12. At least 70 % of the cultivars were heterozygous at each locus. Allele frequencies were generally similar in wine and table grapes. All the cultivars were distinguished by the 11 loci. The level of polymorphism found at the 11 polymorphic loci were similar to other studies with Spanish cultivars (MARTÍN *et al.* 2002, IBAÑEZ *et al.* 2003). Mendelian inheritance of these alleles has been demonstrated in parentage analysis (CABEZAS *et al*. 2003) and in a segregating mapping population (CABEZAS *et al.*, unpubl.). Because the allele sizes of some of these markers do not overlap, their amplification products can be combined in a single polyacrylamide gel.

One of the markers (VMC6F11) produces more than two bands per individual. Nevertheless, these markers may be useful for genome mapping in some populations and for studies of other *Vitis* species.

Twelve markers were monomorphic in the 41 cultivars of *V. vinifera* and thus can not be used for variety identification in that species. They may, however, be polymorphic in other *Vitis* species. Data and primer sequences for these markers are shown in Tab. 2.

References

BOWERS, J. E.; BOURSIQUOT, J. M.; THIS, P.; CHU, K.; JOHANSSON, H.; MEREDITH, C. 1999: Historical Genetics: The parentage of Chardonnay, Gamay, and other wine grapes of Northeastern France. Science **285**, 1562-1565.

BOWERS, J. E.; DANGL, G. S.; MEREDITH, C. P; 1999 b: Development and characterization of additional microsatellites DNA markers for grape. Am. J. Enol. Vitic. **50**, 243-246.

BOWERS, J. E.; DANGL, G. S.; VIGNANI, R.; MEREDITH, C. P.; 1996: Isolation and characterization of new polymorphic simple sequence repeat loci in grape (*Vitis vinifera* L.). Genome **39**, 628-633.

BOWERS, J. E.; MEREDITH, C. P.; 1997: The parentage of a classic wine grape, Cabernet Sauvignon. Nat. Genet. **16**, 84-87.

CABEZAS, J. A.; CERVERA, M. T.; ARROYO-GARCÍA, R.; IBÁÑEZ, J.; RODRÍGUEZ-TORRES, I.; BORREGO, J.; CABELLO, F.; MARTINEZ-ZAPATER, J. M. 2003: Garnacha and Garnacha Tintorera: Genetic relationships and origin of teintourier varieties cultivated in Spain**.** Am. J. Ernol. Vitic. **54**, 237-245.

DOLIGEZ, A.; BOUQUET, A.; DANGLOT, Y.; LAHOGUE, F.; RIAZ, S.; MEREDITH, P.; EDWARDS, J.; THIS, P. 2002: Genetic mapping of grapevine (*Vitis vinifera* L) applied to detection of QTLs for seedlessness and berry weight. Theor. Appl. Genet. **105**, 780-795.

IBAÑEZ, J.; DE ANDRES, MT.; MOLINO, A.; BORREGO, J.; 2003: Genetic study of key spanish varieties using microsatellites analysis. Am. J. Enol. Vitic. **54**, 22-30.

LOPES, M. S.; SEFC, K. M.; EIRAS DIAS, E.; 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

MARTIN J. P.; BORREGO, J.; CABELLO, F.; ORTIZ, J. M.; 2003: Characterization of Spanish grapevine cultivar diversity using sequence-tagged microsatellite site markers. Genome **46**, 10-18.

RIAZ, S.; DANGL, G. S.; EDWARDS, K. J.; MEREDITH, C.; 2004: A microsatellite marker based framework linkage map of *Vitis vinifera* L. Theor. Appl. Genet. **108**, 864-872.

SEFC, K. M.; REGNER, F.; TURETSCHEK, E.; GLÖSSL, J.; STEINKELLNER, H.; 1999: Identification of microsatellite sequences in *Vitis riparia* and their applicability for genotyping of different *Vitis* species. Genome **42**, 367-373

THOMAS, M. R.; SCOTT, N. S.; 1993: Microsatellite repeats in grapevine reveal DNA polymorphisms when analysed as sequence-tagged sites (STSs). Theor. Appl. Genet. **86**, 985-990.

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