Clonal preselection of grapevine cultivars of the appellation "Cangas Quality Wine" (Asturias, Spain)

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Abstract

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The Asturian vineyard is composed mostly of autochthonous minority cultivars only present in the northwest of Spain. This vineyard is characterized by its antiquity, confusion as to the identity of certain cultivars, lack of certified plant material, and a great number of mixed cultivars in each individual vineyard. With the aim of restoring this viticulture after years of abandonment and improving the quality of its wines, old plants belonging to four red grapevine cultivars (Albarín tinto, Carrasquín, Verdejo tinto, and Mencía) presenting a good sanitary state and adequate yield were selected for clonal preselection. Agronomic and enological data were collected over three years. Those plants exhibiting above average values of probable alcohol content and yield for their respective vineyards were tested for viruses so as to discard unhealthy individuals. Six loci microsatellites were analysed to verify the identity of the selected plants. A final number of 62 clones belonging to these cultivars were selected for planting in a plot for their subsequent study under homogeneous conditions.

Keywords: autochthonous cultivar; microsatellite; virus; *Vitis vinifera* L.; wine quality

At the present time, there is an increasing interest in grape growing regions in differentiating their wines from those from other regions and this is mainly accomplished through the development of minority cultivars. Some problems arose when restoring vineyards with these cultivars, such as uncertain identity or the misnaming of some of them, their sanitary state or the lack of certified plant material. Clonal selection is the tool used for grapevine improvement. This methodology takes into account the genetic variability within cultivars and their sanitary state. Genetic variability within cultivars may be explained by their polyclonal origin and the progressive accumulation of genetic mutations over time (Rives 1961; Ulanovsky et al. 2002; Sefc et al. 2009). As the purpose of clonal selection is to provide grape growers with healthy grapevine clones possessing varietal authenticity and a potentially good quality for producing grapes, it is currently being undertaken in all the grape producing regions of the world (Audeguin et al. 2000; Stefanini et al. 2000; Muñoz et al. 2001; Maigre et al. 2003).

The Organisation Internationale de la Vigne et du Vin, Paris, France (O.I.V.) defines a clone as the vegetative descendant of a vine selected for its indisputable identity, its phenotypic characteristics and health status (Walter 1998). The clonal selection process consists in prospecting clones in the field, studying their agronomic and enological performances, sanitary state, and varietal identity. The healthy and more interesting clones are then selected, attempting to maintain intravariability as far as possible. These clones are planted under homogeneous conditions to study their capacity to produce a quality

grape with the aim of certifying and subsequently distributing these clones to grape growers.

A drawback of clonal selection is the excessive uniformity of vineyards and wines, in addition to genetic erosion. The availability of a wide range of selected clones is therefore important so as to enable a good response to natural selection pressure (new pests, climate changes, etc.), to enhance the quality and complexity of wines and to maintain genetic variability within cultivars. The best method for this purpose is to perform the preselection on old vineyards, placing more emphasis on the number of vineyards than on the number of plants per vineyard, thereby achieving a higher variability in selected phenotypes (Lacombe 2004).

The region of Asturias is located in the northwest of Spain. The cultivation of grapevines goes back a long time, being documented since the 9th century, and is based mainly on autochthonous cultivars. In 1858, vineyards occupied an area of 5,493 ha (Feo Parrondo 1986). However, the phylloxera, mildew and oidium crises and subsequently the mining boom, which led to young people migrating to the cities, led to the abandoning of grapevine cultivation and the extinction of some of these cultivars.

At the end of the 20th century, vine growers led the recovery of this old culture, obtaining the region-specific wine appellation "Cangas Quality Wine" in 2008. This crop currently occupies an area of around 100 ha of old vineyards on mountain slopes. The vineyards, most of which are small in extension (occupying as little as 1 ha), have low productivity as a result of the presence of viruses, a lack of uniformity in plant material, and the mixing of cultivars with the subsequent differences in maturing times. This results in low economic profit at each vineyard. There is no certified material for most of the regulated cultivars in this appellation. The material for new plantations is obtained from old vineyards with a deficient sanitary state, the result of fungal diseases caused by the damp climate and virus infections. Moreover, problems of misnamings for some cultivars are commonplace (Moreno-Sanz et al. 2008).

In order to improve both the viticulture and viniculture of this region and the quality and performance of typical local wines, in 2003 the Regional Government of Asturias Agrifoods R&D Service (Servicio Regional de Investigación y Desarrollo Agroalimentario, SERIDA), Asturias, Spain began clonal preselection of the most interesting red cultivars of the "Cangas Quality Wine" appellation: Albarín tinto, Carrasquín, Verdejo tinto, and Mencía. The results of this work are presented in this paper.

Fig. 1. Map of the location of Asturias in Spain. Areas prospected are indicated in black color
CN – Cangas del Narcea; IB – Ibias; P – Pesoz

MATERIAL AND METHODS

Plant material

Four red cultivars regulated within the "Cangas Quality Wine" appellation were chosen for clonal selection: Albarín tinto, Carrasquín, Verdejo tinto, and Mencía.

The search for plants of these cultivars was carried out throughout 2003 and 2004 in 11 vineyards in Cangas del Narcea, Ibias, and Pesoz (Fig. 1; Table 1), boroughs which include more than 95% of the total surface area of vineyards. The vineyards under study were chosen for their age (more than 50 years old) and their general sanitary state (good control of fungal diseases and no symptoms of virus infections). For each cultivar, plants presenting a good sanitary state, and an adequate yield on the basis of visual inspection were selected for the study. Over a three-year period, probable alcohol content (%, v/v), yield (kg), and titratable acidity (g/l tartaric acid) at harvest were measured for every vine. Other interesting data, such as fungal infections or problems in the fruit set, were likewise noted.

ELISA test

In accordance with Spanish regulations for the certification of grapevine material, which stipulate ob-

Table 1. Characteristics of the vineyards and number of vines studied on each

Vineyard	Borough	Slope	Orientation	Training system	Fertilization	Altitude (m)	Vines of each cultivar studied			
							AT	CR	M	VT
1	CN	medium	east	simple Guyot	No	430	19	9	8	8
2	CN	low	east	simple Guyot	Yes	434	5	10	9	3
3	CN	medium	east	simple Guyot	Yes	469	13	6	7	0
4	CN	low	east	simple Guyot	Yes	381	0	2	5	11
5	CN	high	south	simple Guyot	Yes	474	15	23	12	24
6	CN	high	south	simple Guyot	Yes	393	9	33	3	3
7	CN	medium	southeast	simple Guyot	No	412	4	0	0	2
8	CN	low	southwest	simple Guyot	No	503	9	0	11	20
9	CN	high	east	simple Guyot	No	471	9	0	3	0
10	IB	low	south	bilateral cordo	No	293	1	0	0	2
11	P	low	south	head training	Yes	317	4	0	5	0

CN – Cangas del Narcea, IB – Ibias, P – Pesoz; AT – Albarín tinto, CR – Carrasquín, M – Mencía, VT – Verdejo tinto

ligatory tests for viruses of *Vitis vinifera* clones, the following analyses were performed: *Grapevine leafroll-associated virus* 1 and 3 (GLRaV-1, GLRaV-3), *Grapevine fanleaf virus* (GFLV), *Grapevine fleck virus* (GFkV, obligatory until 2006), and the *Arabis mosaic virus* (ArMV). Additionally, *Grapevine leafroll-associated virus* 2 (GLRaV-2) was also included in the analysis. A total of 97 individual vines were evaluated by ELISA (Enzyme-Linked Immunosorbent Assay) in 2005. The analyses were repeated in 2006 on the negative vines, together with 53 new individuals. Subsequent analyses were repeated only on the negative vines. All the negative plants were analysed three times over a minimum of two years for the presence of viruses; dormant canes were tested during winter for all the viruses; in the vegetative period, young leaf was sampled in spring for the presence of the GFLV, GFkV, and ArMV, and adult leaf in autumn for GLRaV (Table 2).

Identification of cultivars

Sixty-three clones were analysed through six loci microsatellites (VVS2, VVMD5, VVMD7, VVMD27, VrZAG62, VrZAG79), proposed by the GENRES 081 Project (http://www.genres.de/vitis) for grapevine cultivar identification. DNA extraction and amplification was carried out as in Moreno-Sanz et al. (2008). PCR products were analysed by means of an automated ABI PRISM® 3100 DNA sequencer (Applied Biosystems, Foster City, USA) and the fluorescence labelled fragments were sized using the Genemapper Software v.4.0 (Applied Biosystems).

Table 2. Number of vines tested by ELISA and part of the plant analysed

Year	Organ tested	Virus and vines of each cultivar analysed					
		GLRaV1	GLRaV2	GLRaV3	GFLV	GFkV	ArMV
2005	AL/YL	AT (27), CR (24), VT (25), M (21)					–
2006	DC	AT (28), CR (26), VT (19), M (27)					–
	AL/YL	AT (18), CR (17), VT (14), M (17)					–
2007	AL/YL	AT (18), CR (17), VT (12), M (17)					
2008	AL/YL	AT (18), CR (17), VT (12), M (17)					

DC – dormant canes (sampled for all the viruses), YL – young leaf (sampled for GFLV, GFkV, ArMV), AL – adult leaf (sampled for GLRaV); GLRaV – *Grapevine leafroll-associated virus*, GFLV – *Grapevine fanleaf virus*, GFkV – *Grapevine fleck virus*, ArMV – *Arabis mosaic virus*; AT – Albarín tinto, CR – Carrasquín, VT – Verdejo tinto, M – Mencía

Table 3. Analysis of variance applied to agronomic and enological data from each cultivar

Cultivar	Factor	Probable alcohol content (%, v/v)	Yield/vine (kg)	Titratable acidity (g/l tartaric acid)
Albarín tinto	year	***	***	***
	vineyard	***	**	***
	year × vineyard	***	***	ns
Carrasquín	year	***	**	***
	vineyard	***	***	***
	year × vineyard	ns	ns	ns
Verdejo tinto	year	***	***	**
	vineyard	***	ns	***
	year × vineyard	ns	ns	**
Mencía	year	ns	**	***
	vineyard	**	**	ns
	year × vineyard	*	ns	*

ns – no significant differences; *, **, *** – significant at the 0.05, 0.01, 0.001 levels of probability, respectively

Statistical analysis

Average and standard deviation values were calculated for each cultivar, year, and vineyard for the following parameters: probable alcohol content, yield, and titratable acidity. Data were subjected to an Analysis of Variance using the SPSS v 11.5.1. (SPSS Inc., Chicago, USA) statistical package to study whether there were any differences depending on the year and the vineyard for each parameter.

RESULTS AND DISCUSSION

Plant material

The plantation of autochthonous cultivars has spread in recent years with the aim of offering the consumer typical wines from each region. Moreover, these cultivars are adapted to local climatic conditions and can present genetic characteristics of tolerance and resistance to biotic and abiotic stresses (Borgo et al. 2005). Their clonal selection is therefore being undertaken to obtain healthy plant material of good quality with a guarantee of varietal identity.

Viticulture goes a long way back in time in Asturias, but practically disappeared in the 20th century. In recent years, this culture has undergone a process of recovery, with new plantations and the obtaining of the region-specific wine appellation "Cangas Quality Wine" in 2008. The most widespread quality red cultivars are Mencía, Verdejo tinto, Albarín tinto, and Carrasquín.

Mencía is also cultivated in the neighbouring areas of Castilla-León and Galicia and clones have recently been certified in these regions. It provides fruity, delicate wines which are dark cherry in colour. Verdejo tinto is a synonym of the Merenzao cultivar from Galicia (Moreno-Sanz et al. 2008); it produces rosé wines with a low anthocyanins concentration. There exist four clones of this cultivar that have recently been certified in Spain, three of which have not yet been commercialized. Albarín tinto is a synonym of the Caíño gordo cultivar from Galicia, and Tinta Francesa and Alfrocheiro Preto from Portugal (Zerolo, Cabello 2006). Carrasquín is only present in Asturias. There are no certified clones of Albarín tinto or Carrasquín cultivars in Spain; these vines produce fresh, acidic wines. With the exception of Mencía, the other cultivars are in danger of extinction, occupying an extension of less than 100 ha in Spain.

The agronomic and enological observations carried out over three years showed differences in probable alcohol content, yield and titratable acidity between vineyards and years, although some significant interactions between year and vineyard were observed for the Albarín tinto, Verdejo tinto, and Mencía cultivars (Table 3). In fact, the good climatic conditions in 2006, with higher temperatures than 2004 and 2005, produced a better maturation and the concomitant increase in the probable alcohol content and decrease in titratable acidity. The

Table 4. Average (aver) and standard deviation (SD) values for all the studied clones and for the preselected clones for the parameters: probable alcohol content, yield per vine, and titratable acidity

Cultivar	Year		Probable alcohol content (%, v/v)		Yield per vine (kg)		Titratable acidity (g/l tartaric acid)	
			all clones	preselected clones	all clones	preselected clones	all clones	preselected clones
Albarín tinto (17)	2004	aver	11.07	11.75	1.54	1.64	10.70	11.39
		SD	1.57	0.98	0.65	0.66	1.20	0.76
	2005	aver	11.42	12.37	1.55	1.68	9.72	9.03
		SD	2.13	1.69	1.08	0.96	1.50	0.57
	2006	aver	12.82	12.52	0.79	1.56	7.15	7.30
		SD	1.28	1.07	0.65	0.80	1.15	1.16
Carrasquín (16)	2004	aver	10.94	11.34	2.14	2.56	10.13	9.83
		SD	1.33	1.22	0.83	0.90	0.98	1.36
	2005	aver	11.76	12.05	1.92	2.16	11.26	10.88
		SD	1.74	1.28	1.11	0.87	1.66	1.35
	2006	aver	13.49	13.71	0.91	0.96	7.85	7.92
		SD	0.84	0.90	0.67	0.55	1.15	1.11
Verdejo tinto (12)	2004	aver	10.89	12.35	1.36	1.14	7.72	8.25
		SD	1.65	0.70	0.64	0.57	1.77	2.06
	2005	aver	11.77	12.22	1.22	1.71	6.56	6.33
		SD	1.71	1.24	0.59	0.60	1.37	1.26
	2006	aver	13.63	14.04	0.35	0.35	5.27	5.17
		SD	1.39	1.14	0.21	0.24	0.72	0.73
Mencía (17)	2004	aver	11.09	11.29	1.87	2.01	7.86	8.04
		SD	1.20	1.33	0.73	0.86	0.71	0.56
	2005	aver	11.45	12.19	1.76	1.96	6.53	6.13
		SD	1.56	0.94	0.77	0.53	0.86	0.73
	2006	aver	11.96	12.22	1.25	1.16	5.31	5.04
		SD	1.20	1.40	0.87	0.89	1.03	0.72

In brackets – number of final preselected clones

Mencía cultivar suffered this effect of the year less than the other cultivars for the probable alcohol content parameter (Table 4).

The differences found between vineyards can be explained, on the one hand, by the mesoclimate of each vineyard (different altitudes and orientation, since they are on mountains slopes) and, on the other, as a result of different rootstocks, canopy management or fertilization (Table 1) (Smart 1985; Howell et al. 1987; McCarthy 1992; Jackson, Lombard 1993; Spayd et al. 1994; Main et al. 2002). In our case, the rootstock in all the vineyards is probably the same, because the I.N.D.O. (1982) reported that the 94.87% of the asturian vineyard is grafted on Rupestris de Lot.

Intracultivar variability was found within the same vineyard. As an example, Table 5 shows the maximum and minimum values, relative standard deviation, mean, and standard deviation for the Albarín tinto cultivar in one of the vineyards studied. Substantial differences can be observed, mainly in the yield per vine parameter, with a relative standard deviation of 59.18% in 2005. Variability in the same vineyard may be explained by the existence of genetic variability or by the sanitary state of the vines. In fact, important attacks by downy mildew seriously affected the final yield of some of the vines studied, producing considerable differences between vines for this parameter. Genetic variability is necessary to carry out clonal selection and is more abundant in old vineyards (Bessis 2007). It allows the selection of clones with good agronomic and enological performances so as to be able to test

Table 5. Average (aver), standard deviation (SD), relative standard deviation (rSD), maximum (max), and minimum (min) values obtained for all the vines of the cultivar Albarín tinto at one of the vineyards studied

Year		Probable alcohol content (%, v/v)	Yield/vine (kg)	Titratable acidity (g/l tartaric acid)
	aver	11.80	1.38	11.28
	SD	0.71	0.36	0.70
2004	rSD	6.02	26.09	6.21
	max	12.83	2.00	12.38
	min	11.00	0.70	10.13
	aver	12.96	1.47	8.96
	SD	1.07	0.87	0.86
2005	rSD	8.26	59.18	9.60
	max	14.35	2.90	10.50
	min	11.13	0.10	8.03
	aver	12.36	1.85	6.95
	SD	0.95	0.43	0.27
2006	rSD	7.69	23.24	3.88
	max	13.45	2.65	7.35
	min	10.59	1.30	6.60

them under homogeneous conditions and select the best ones for the production of quality grapes.

Average values for each vineyard, year, and cultivar were calculated for the measured parameters. Individual values obtained for each vine were compared with the average of its vineyard for each year. For each cultivar, vines with above average values of probable alcohol content and yield for its vineyard for at least two years were selected for testing by ELISA. Some vines with very good values for only one of these parameters were also tested.

ELISA test

A total of 150 individual vines were examined by ELISA (Table 6). 39.3% of plants proved positive for viruses; the most infected cultivar was the

Table 6. ELISA test for the GFLV, GFkV, GLRaV-1, GLRaV-2, GLRaV-3, and ArMV viruses

	Albarín tinto	Carrasquín	Verdejo tinto	Mencía	Total of plants
Total vines tested by ELISA	43	37	35	35	150
+ GFLV	0	1	2	0	3 (2.0%)
+ GFkV	0	7	5	2	14 (9.3%)
+ GLRaV-1	11	1	14	1	27 (18.0%)
+ GLRaV-2	7	3	6	6	22 (14.7%)
+ GLRaV-3	2	0	1	3	6 (4.0%)
+ ArMV	0	0	0	0	0 (0.0%)
Infected plants: total number (%)	18 (41.9)	12 (32.4)	18 (51.4)	11 (31.4)	59 (39.3)

GFLV – *Grapevine fanleaf virus*, GFkV – *Grapevine fleck virus*, GLRaV – *Grapevine leafroll-associated virus*, ArMV – *Arabis mosaic virus*;

Table 7. Microsatellite profiles of the Albarín tinto, Carrasquín, Verdejo tinto, and Mencía cultivars

Loci	Albarín tinto	Carrasquín	Verdejo tinto	Mencía
VVS2	139:148	139:148	139:148	141:148
VVMD5	224:237	224:237	237:237	224:235
VVMD7	254:258	240:258	240:258	250:258
VVMD27	176:186	176:186	173:186	178:186
VrZAG62	187:199	187:193	187:187	187:193
VrZAG79	252:252	252:252	246:248	248:252

Verdejo tinto, with 51.4% positive results. The most frequent virus was GLRaV-1 (18.0%), followed by GLRaV-2 (14.7%). Some of the plants proved positive for more than one virus. For instance, one of the Mencía plants tested proved positive for GLRaV-2 and GLRaV-3; one of the Albarín tinto for GLRaV-1 and GLRaV-2, and another for GLRaV-2 and GLRaV-3. The Verdejo tinto cultivar presented a high number of plants infected with more than one virus. All the plants analysed proved negative for ArMV.

Viral infections can reduce yield, fruit quality, longevity, rooting ability, and successful grafting, with the corresponding economic cost (Walter, Martelli 1996; Akbaş et al. 2009; Lee, Martin 2009; Uyemoto et al. 2009). In our study, we included not only those viruses whose testing is mandatory, but also GLRaV-2, following the recommendation of the ICGV (International Council for the Study of Virus and Virus-like Diseases of the Grapevine, http://www.icvg.ch/data/recomm.pdf). This virus is not included in sanitary selection protocols because it has always been regarded as one of minor importance. However, the association of several molecular variants of this virus with a graft incompatibility condition was reported (Greif et al. 1995; Borgo et al. 2006; Fiore et al. 2008). Komar et al. (2007) documented that the elimination of GLRaV-2 in Chardonnay plants had a greater beneficial effect than the elimination of other viruses on their growth, yield, and fruit maturity. This virus is frequently tested in clonal selections in Spain because of its incidence (Cretazzo et al. 2010). Padilla et al. (2007) found the most frequent viruses in plant material from clonal preselections in Spain to be GFkV, GLRaV-3, and GLRaV-2; all the material they tested was negative for ArMV. In a study on the incidence of GLRaV in old vineyards from the

Table 8. Sanitary status and origin of the preselected clones

Vineyard	Clones preselected	Sanitary status	Observations
1	0	–	no clone preselected; vineyard abandonned by owner in 2006
2	0	–	no clone preselected; symptoms of trunk diseases in the vineyard
3	AT (8), M (4)	good	generalised *Empoasca vitis* attack on the vineyard in 2003
4	CR (1), VT (4), M (3)	*Botrytis* in VT in 2003	
5	AT (2), CR (6), VT (3), M (4)	good	
6	AT (1), CR (9), VT (1), M (1)	good	generalised light *Botrytis* attack on the vineyard in 2004
7	0	–	viruses in the clones analysed
8	AT (3), VT (4), M (3)	*Colomerus vitis* on VT in 2005	
9	AT (3), M (2)	good	
10	0	–	viruses in the clones analysed
11	0	–	viruses in the clones analysed

[1]AT – Albarín tinto; CR – Carrasquín; VT – Verdejo tinto; M – Mencía; in brackets – number of final preselected clones

Ribeira Sacra, near to Asturias, García-Berrios et al. (2008) found the most frequent viruses to be GLRaV-1 (47% of total symptomatic plants) and GLRaV-2 (39%). We also found these two viruses to be the most frequent of all the viruses analysed.

Identification of cultivars

After the analysis of viruses and the study of the agronomic and enological data, 63 clones finally considered for the selection process were analysed by microsatellite analysis to confirm their identity. Until recently, identification of grapevine cultivars was based on ampelographic descriptions. However, these require a long time to be completed and differentiation between related cultivars is sometimes difficult. At present, analysis of microsatellite markers is the best method to accomplish fast and accurate identification and is being used worldwide for this purpose (Maletic et al. 1999; Ibáñez et al. 2003; Heuertz et al. 2008). The results (Table 7) were compared with national and international databases. A clone designated as Carrasquín neither corresponds to this cultivar nor to any other existing in all the databases consulted (Germplasm bank at El Encín, Madrid; *Vitis* database of the School of Agricultural Engineers of the Polytechnic University of Madrid (ETSIA-UPM): http://www.sivvem.monbyte.com/sivvem.asp; Swiss *Vitis* Microsatellite Database (SVMD): http://www1.unine.ch/svmd/; Greek *Vitis* Database: http://gvd.biology.uoc.gr/gvd/contents/databases/index.htm; Grape SSR Fingerprinting from NCGR University of Davis: http://www.ars.usda.gov./Main/docs.htm?docid=13743; Ibáñez et al. 2003; Martín et al. 2003, 2006) and so was removed from the selection process.

After the study of the agronomic, enological, ELISA, and microsatellite data, a final number of 62 clones (Table 8), corresponding to the cultivars Albarín tinto (17 clones), Carrasquín (16), Verdejo tinto (12), and Mencía (17), were selected to continue the clonal selection process. Three replicates of 10 vines each were grafted on 110R and planted in a plot under homogeneous conditions in 2007 for further comparative studies. The clonal selection of these cultivars will allow to obtain high-quality plant material, thus improving wines, and to preserve the grapevine natural resources of this region. In addition Albarín tinto, grown in other regions of Spain and in Portugal, and Carrasquín produce wines with a high acid content. This feature is of great interest because the climatic change is causing a decrease of the acidity of wines, necessary for a good quality and a better conservation of this product.

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