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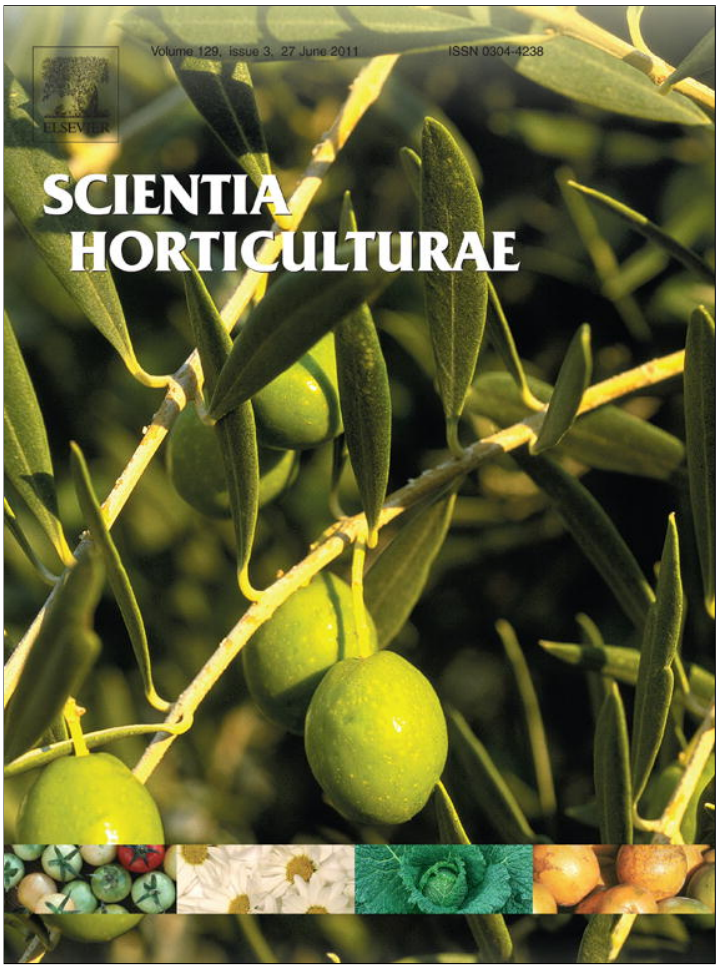

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Microsatellite characterization of grapevine (*Vitis vinifera* L.) genetic diversity in Asturias (Northern Spain)

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ABSTRACT

The genetic heritage of the Asturian grapevine (*Vitis vinifera* L.) has been declining over the past century due to the phylloxera attack and the further abandonment of this culture. In addition, efforts in recent years to restore the Asturian vineyard with the pulling-up of old vineyards and replanting with cultivars endorsed by Cangas Quality Wine regulations are contributing even more to this genetic erosion. The aim of this study was the evaluation and identification of the phytogenetic resources of the Asturian grapevine. A total of 293 accessions were collected in old vineyards and analyzed through nine microsatellite markers. Forty-two different genotypes were obtained, including six profiles with allelic variations. Only 27 cultivars were identified when compared with national and international databases; some of them had not been found in this region before. Homonymies and synonymies have also been detected. These results provide an overview of the status of current grapevine phytogenetic resources in Asturias. Despite the substantial genetic erosion that the Asturian vineyard has suffered, a higher variability than expected has been detected. The finding of new grapevine genotypes is a fact of great importance. The genetic grapevine resources are being drastically reduced all over the world, so this new genetic material has to be included in germplasm banks for its conservation and further agronomical and enological evaluation.

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1. Introduction

Grapevine (*Vitis vinifera* L.) has been cultivated for about 5000 years and its vegetative propagation has favoured widespread diffusion of many cultivars around the world, generating numerous synonymies and homonymies. This sort of propagation, together with the sole use of a few cultivars allowed by the different Designations of Origin (D.O.) has led to a substantial decrease in grapevine diversity. In addition, the phylloxera attack suffered in the 19th century along with diseases like downy and powdery mildew have also contributed to this genetic erosion (This et al., 2006). In Spain, the pulling-up of around 349,000 ha in recent years, due to a restructuration process, and vineyard replanting are causing the disappearance of old vineyards, where the greatest amount of cultivar variability is present. Furthermore, very many autochthonous cultivars are in danger of extinction, despite playing an important role in the diversification of wines (Cabello, 2004). With the aim of preserving grapevine phytogenetic resources, numerous studies on the surveying, localization, characterization and maintaining of cultivars in germplasm banks are being carried out worldwide (Aradhya et al., 2003; Halász et al., 2005; Heuertz et al., 2008; Leão et al., 2009; Maletic et al., 1999). A pool of genes of agronomical and enological potential will thus be available for grapevine genetic improvement programs (Bautista et al., 2008).

Precise identification and characterization of cultivars is required to conserve and maintain genetic resources. DNA genotyping by microsatellite markers (SSRs) is considered the best method for grapevine cultivar identification (Thomas and Scott, 1993), besides allowing the detection of synonyms and homonyms (Maletic et al., 1999; Martín et al., 2003; Yuste et al., 2006), as well as having applications in pedigree reconstruction (Bautista et al., 2008). Accurate identification of cultivars together with a phenological, sanitary (Zdunic et al., 2007), agronomical (Pommer et al., 2000) and ampelographic (Martínez and Pérez, 2000) descriptions will enable a better knowledge of the genetic heritage of grapevines.

The area in which grapevines have been cultivated in Asturias (northern Spain) has decreased from 5493 ha in the 19th century to 100 ha at the present time. In the 19th century, Asturian wine achieved a high level of quality, recognised in several national and international exhibitions (Feo, 1986). During the 20th century, however, most vineyards were abandoned owing to the expansion of coal-mining activities, the migration of the rural population to the cities and the difficulty of managing the vineyards because of the rugged terrain. After the phylloxera plague, the introduction

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of French cultivars also contributed to accentuating the erosion of the autochthonous germplasm. In the last 15 years, however, the viticulture sector has been developed once more; proof of which is the recognition of the Cangas Quality Wine appellation in 2008 (B.O.P.A., 2008). The cultivars endorsed by the regulations of this appellation are used in new vineyards, as well as in the replanting of old ones, leading to the extinction of other cultivars. The loss of grapevine phytogenetic resources in the Asturian vineyard is becoming obvious, as no stock of some cultivars (Carrascón, Agudiello, Rondal Negro, Mallén, Pardusco Prieto, Picudo, Rondales, Conrasión and Bondal), mentioned in bibliography as existing before the phylloxera attack (García, 1914; Naredo, 1914), have been reported in recent studies of ampelographic and genetic characterization (Martínez and Pérez, 1999, 2000; Martínez et al., 2002; Moreno-Sanz et al., 2008; Santiago et al., 2005). The aim of the present study is to analyse Asturian grapevine diversity through microsatellite markers (SSRs).

2. Materials and methods

2.1. Plant material

A survey was conducted between 2005 and 2007 in search of grapevine cultivars in old vineyards in Asturias older than 50 years. Sampling was carried out with the intention of covering all the observed phenotypic variability. Fresh young leaves were collected in the field, frozen and preserved at −80 °C. A total of 293 accessions were analyzed. Cabernet Sauvignon and Chardonnay cv. (supplied by the Subestación Enolóxica of Ribadumia, Xunta de Galicia, Spain) were included as reference material.

2.2. DNA extraction and quantification

The DNeasy® Plant Mini Kit (Quiagen, Hilden, Germany) was used for DNA extraction from 65 mg of leaf weight of each sample. Quantification of the extracted DNA was performed by electrophoresis in 1% agarose gels in 1X TAE buffer (0.04 M Tris–acetate, 0.001 M EDTA, pH 8.0). Gels were stained for 20 min in 2 $\mu g\,ml^{-1}$ ethidium bromide in milliQ water and visualised on an ultraviolet transilluminator. DNA was quantified using the Gene Tools software (Syngene, Cambridge, UK) by comparison with known concentrations of λ phage DNA (Bioron, Ludwigshafen, Germany). A working solution of 5 ng μl^{-1} DNA was prepared for each sample.

2.3. Microsatellite analysis

Two multiplex PCR were carried out:

- **PCR1**, containing the set of six SSRs proposed by the European project GENRES081 (http://www.eu-vitis.de/index.php), namely VVS2 (Thomas and Scott, 1993), VVMD5 and VVMD7 (Bowers et al., 1996), VVMD27 (Bowers et al., 1999), VrZAG62 and VrZAG79 (Sefc et al., 1999). The amplification mixture was prepared according to the protocol described by García-Beneytez et al. (2002) with the following modifications: 0.06 μM VVS2 and 0.1 μM VVMD7 and VrZAG62.
- **PCR2**, containing VVMD28 (Bowers et al., 1999), VrZAG67 and VrZAG112 (Sefc et al., 1999); with 2 mM $MgCl_2$, 250 μM of each dNTP, 0.075 U/μl of Tth polymerase (Biotools), 0.15 μM VVMD28, VrZAG67 and VrZAG112 and 25 ng of the template DNA.

Both PCR were performed in 12 μl reaction volume. A primer of each pair was labelled with a fluorochrome (6-FAM, NED, PET or VIC) so that the size range of fragments amplified by primers labelled with the same fluorochrome did not overlap.

Amplification reactions were carried out in a GeneAmp® PCR System 9700 thermal cycler (Applied Biosystems, Langen, Germany) following the protocol described by Martín et al. (2003), modifying the annealing temperature to 54 °C for PCR2. A final cycle of 90 min at 65 °C was added in both amplification reactions to favour the formation of +A alleles (Matsumoto et al., 2004).

The PCR reaction was checked in 3% agarose gels and PCR products were analyzed on an ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems) as described in Moreno-Sanz et al. (2008). The internal standard used to assign sizes to the DNA fragments was GENESCAN500LIZ. Fluorescently labelled fragments were sized using GeneMapper® Software v.4.0 (Applied Biosystems), rounding off the decimal places to reach integer allele numbers.

2.4. Grapevine microsatellite databases consulted

SSR profiles were compared with the following national and international databases for cultivar identification (classified according to the SSR markers analyzed in common):

GENRES081 SSR markers: El Encín Germplasm Bank, Madrid (BGV, Dr. Cabello, personal communication); Swiss *Vitis* Microsatellite Database (SVMD, http://www1.unine.ch/svmd/); *Vitis* database of the Polytechnic University of Madrid (ETSIA-UPM, http://www.sivvem.monbyte.com/sivvem.asp), *Vitis* international Variety Catalogue (VIVC, http://www.vivc.de/index.php) and the Estación de Viticultura y Enología de Galicia database (EVEGA, Dr. Díaz, personal communication).

GENRES081 SSR markers and VVMD28: Grapevine microsatellite database of Centro di ricerca per la viticoltura of Conegliano (CRA – VIT), Italy (Dr. Crespan, personal communication).

GENRES081 SSR markers and VVMD28, ZAG67 and ZAG112: Greek *Vitis* Database (GVD, http://gvd.biology.uoc.gr/gvd/contents/databases/index.htm).

VVS2, VVMD5, VVMD7, VVMD27 and VVMD28 SSR markers: Grape SSR Fingerprinting from NCGR-University of Davis (NCGR, http://www.ars.usda.gov/Main/docs.htm?docid=13743).

VVS2, VrZAG47, VrZAG62, VrZAG79 markers: Bulgarian Grape nSSR Database (BgGD, http://bulgenom.abi.bg/Grape%20nSSR%20Database.htm).

2.5. Data analysis

The number of alleles, allele frequencies, expected (H_e) and observed (H_o) heterozigosity, probability of identity (PI) and probability of null alleles (r) were calculated using IDENTITY v.4 software (Wagner and Sefc, 1999). The polymorphic information content (PIC) was calculated according to Botstein et al. (1980).To assess the structure of the genetic diversity, two approaches were used: graphic clustering from similarity data and principal coordinates analysis (PCoA). Both analysis were performed using NTSYS-PC v.2.2 software (Rohlf, 2005). Cluster analysis was performed from a presence/absence data matrix obtaining a dendrogram based on Jaccard's similarity coefficient by means of the Unweighted Pair-Group Method with Arithmetic Averages (UPGMA); cophenetic correlation coefficient was also calculated. Bootstrap analysis was performed with 1000 replicates with WinBoot software (Yap and Nelson, 1996). In the case of the PCoA, the two principal coordinates obtained were used to visualise the dispersion of the genotypes in a graphic.

3. Results and discussion

The lack of knowledge regarding a substantial part of grapevine biodiversity in Asturias led us to carry out surveys to find, catalogue and identify the genetic heritage of this species. Conservation

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of these phytogenetic resources is a priority, because some cultivars existing before the phylloxera plague have not been recorded till now. At the same time, the replanting of old vineyards is causing the loss of other remaining minority cultivars. From 2005 to 2007, a total of 293 individual vines with different morphological characteristics or different names were recorded.

The name assigned to each accession was that given by the vine growers, but in those cases in which the name was unknown to them, the genotypes with no match found with the databases were identified by a code ("GEN" followed by a number). Most of the Asturian vine growers are able to identify the most widespread cultivars in this region, such as Albarín Tinto, Albillo (Chasselas Doré), Cabernet Sauvignon, Carrasquín, Garnacha Tintorera (Alicante Bouschet), Mencía, Moscatel, Mouratón and Verdejo Tinto. However, when asked for the names of other different cultivars found, these were completely unknown to them.

3.1. Microsatellite analysis

All nine analyzed loci were polymorphic. A total of 98 alleles were detected, ranging from 9 for ZAG67 and ZAG112 to 13 for VVS2 and VVMD28 (Table 1). The highest allelic frequencies were found for ZAG62-187 (38.6%) and ZAG112-236 (37.5%). ZAG62-187 was found to be one of the most frequent alleles in a study of a broad Spanish germplasm collection performed with the six loci proposed by the EU Project GENRES081 (Martín et al., 2003). 23.5% of the alleles showed the minimum allelic frequency possible (1.1%) (Table 2).

The number of unique genotypes per locus varied from 15 for ZAG62 and ZAG112 to 24 for ZAG79 (Table 1). The most frequent genotypes were 187:193 (25%) and 126:140 (25%) from markers ZAG62 and ZAG67, respectively. More than 40% of each locus genotypes were unique.

H_o and H_e values ranged from 75% (VVMD27 and ZAG112) to 93.2% (VVMD5 and VVMD28) and from 75.9% (ZAG67) to 88% (VVMD28), respectively. The H_o for each SSR marker was higher than H_e, except for markers VVMD27 and ZAG112, which also showed a positive probability of null alleles. The probability of null alleles in ZAG112 was close to zero (0.6%), while for VVMD27 it presented a value of 5.4%. The average H_o for the whole SSR set was 85.1% (Table 1).

The PI value estimates the probability that two unrelated (randomly sampled) individuals will have an identical genotype for each single SSR marker analyzed, or for a whole set of SSR markers. This probability becomes very small if many highly polymorphic loci are considered. The PIC value assesses the usefulness of each microsatellite marker for reliable distinction. Accordingly, the most informative marker for the present study was VVMD28, with a PI of 0.026 and a PIC of 0.868; while the least informative markers were ZAG67 and ZAG112, with a PI of 0.094 and 0.092 and a PIC of 0.723 and 0.726, respectively. The overall probability that two individuals drawn at random from a given population share identical genotypes at the nine typed loci is about four chances in a billion ($PI = 3.922 \times 10^{-12}$) (Table 1).

3.2. Cultivar identification

Analysis using this set of nine SSR markers allowed the detection of 42 different genotypes (Table 3). After comparison with the consulted grapevine SSR databases and the bibliography (De Sebastián et al., 2005; Díaz et al., 2010; Gago et al., 2009; González-Andrés et al., 2007; Ibáñez et al., 2003, 2009; Martín et al., 2003, 2006; Santana et al., 2010; Sefc et al., 2000; Vargas et al., 2009; Vilanova et al., 2009; Vouillamoz et al., 2006; Zinelabidine et al., 2010), only 29 of them were identified (Table 3). Some accessions showed the same microsatellite profile for all the analyzed SSR markers: Chasselas Doré/Chasselas Rosé and Moscatel Blanco de grano menudo/Moscatel Rojo. The unique ampelographic difference observed in both cases was the berry colour, which has been previously reported for different cultivars (Martín et al., 2003).

When compared with the databases, a variation of 1 bp was observed in some cases for an allele, but was considered the same profile if this variation also occurred with some of the reference cultivars used to make the databases conversion.

An accession of Mencia cv. and another of Albarín Tinto cv. showed three alleles for one locus. Mencía accession presented the alleles 141:146:148 for the locus VVS2 and Albarín Tinto accession the alleles 176:184:186 for the locus VVMD27. The fact of finding accessions with three alleles for a locus could be explained by the phenomenon of chimerism. Chimeras have been previously reported in grapevine (Bertsch et al., 2005; Franks et al., 2002; Martínez et al., 2006; Zulini et al., 2005). Vouillamoz et al. (2006) found three alleles in several loci, especially at VVS2.

De José Blanco cv. and the accessions corresponding to the unknown profiles GEN12, GEN13 and GEN14 were all collected under the name of "Productora", the way vine growers call hybrids. De José Blanco cv. has four unique alleles, each one in a different marker. This was previously reported in another study (Martín et al., 2003) which argued that it might be due to a probable hybrid origin of this cultivar in keeping with its ampelographic characters. De José Blanco and Chasselas presented one allele of each marker in common. The unknown profile GEN15 also showed four markers with a unique allele each one; so it could also have a hybrid origin. The GEN15 microsatellite profile matched with the profile of an unknown cultivar from the CRA – VIT database which was collected in Georgia. The researchers who analyzed this cultivar also suppose, in line with their molecular data, that it might be a hybrid (Dr. Crespan, personal communication). GEN16 was identified by some vine growers as Jaen and by others as a type of Moscatel. Martínez and Pérez (1999) suggested this cultivar could be a hybrid produced by a series of crosses between *Vitis labrusca* and Moscatel cv. made after phylloxera plague; this could be possible because of the slight taste of both muscat and foxé of its berries. We think GEN16 could be Jaen-Moscatel cv., reported by Naredo (1914). However, according to said author, Jaen-Moscatel cv. was cultivated in Asturias before the phylloxera crisis. The profile of GEN12 matched with an unknown genotype of an accession localized in Castilla y León that presented a high percentage of alleles in common with other accession catalogued as a hybrid (Santana et al., 2010). GEN05 and GEN17 genotypes matched with the profiles of Bouqseb and Begugnol cv., respectively, for the SSR markers analyzed in common with the databases consulted (Table 3), but we do not consider these profiles completely identified due to the lack of complementary information about them (ampelographic descriptions, photographs).

A cultivar with rosé berries named by a vine grower as Verdello matched with the Cardinal cv. SSR profile reported by Ibáñez et al. (2009) for the eight SSR markers we analyzed in common (VVS2, VVMD5, VVMD7, VVMD27, VVMD28, ZAG62, ZAG67 and ZAG112), but differed in one allele for markers VVMD5 and VVMD7 with respect to the Cardinal cv. profile reported by the ETSIA-UPM *Vitis* SSR database and in 2 bp with the alleles of the VrZAG79 locus of Cardinal profile of the VIVC database (Table 3).

Petit Bouschet profile matched with the profile of Negrón de Aldán (Petit Bouschet) of the Spanish collection BGV (Martín et al., 2003). Ibáñez et al. (2003) obtained two different profiles when analyzing two accessions of Negrón de Aldán. The genotype of one of them matched with ours for the nine SSRs analyzed, although for the locus VrZAG79 our profile was heterozygous, while the one of that study was homozygous for the smaller allele. According to the VIVC database, Negrón de Aldán and Petit Bouschet are two different cultivars. In this latter database our profile matched

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Table 1
Number of observed genotypes per locus (OG), number of observed alleles (NA), expected (H_e) and observed (H_o) heterozigosity, probability of null alleles (r), probability of identity (PI) and polymorphic information content (PIC) of the nine SSR markers for the cultivars discriminated in this study (including allelic variations).

Locus	OG	NA	H_e	H_o	r	PI	PIC
VVS2	18	13	0.804	0.864	−0.033	0.062	0.780
VVMD5	22	11	0.842	0.932	−0.049	0.042	0.825
VVMD7	19	12	0.811	0.886	−0.042	0.058	0.789
VVMD27	21	10	0.850	0.750	0.054	0.039	0.833
VVMD28	23	13	0.880	0.932	−0.028	0.026	0.868
ZAG62	15	10	0.765	0.864	−0.056	0.086	0.734
ZAG67	17	9	0.759	0.818	−0.034	0.094	0.723
ZAG79	24	11	0.862	0.864	−0.001	0.033	0.847
ZAG112	15	9	0.760	0.750	0.006	0.092	0.726
Mean	–	10.889	0.815	0.851	–	–	–
Cumulative		98	–	–	–	3.922×10^{-12}	–

Table 2
Allele sizes (AS) in bp and allele frequencies (AF) for the nine SSR markers in the cultivars discriminated in this study (including allelic variations).

SSR loci																
VVS2		VVMD5		VVMD7		VVMD27		VVMD28		ZAG62		ZAG67		ZAG79		ZAG112	
AS	AF	AS	AF	AS	AF	AS	AF	AS	AF	AS	AF	AS	AF	AS	AF	AS	AF
119	0.011	220	0.023	234	0.034	173	0.057	217	0.034	179	0.011	126	0.341	238	0.011	232	0.205
121	0.011	224	0.182	236	0.011	176	0.114	228	0.045	180	0.011	132	0.148	240	0.045	236	0.375
129	0.284	226	0.136	238	0.011	178	0.159	230	0.011	185	0.170	134	0.011	244	0.091	240	0.068
131	0.034	230	0.068	240	0.330	180	0.068	234	0.182	187	0.386	140	0.307	246	0.114	242	0.205
133	0.045	233	0.068	244	0.136	182	0.170	236	0.170	191	0.011	144	0.011	248	0.170	244	0.102
135	0.068	235	0.114	248	0.068	183	0.034	244	0.136	193	0.205	150	0.057	250	0.034	246	0.011
139	0.102	237	0.273	250	0.170	186	0.239	246	0.045	195	0.057	152	0.057	252	0.239	248	0.011
141	0.091	239	0.091	252	0.023	188	0.011	248	0.136	199	0.023	154	0.045	256	0.068	250	0.011
144	0.023	245	0.023	254	0.023	191	0.125	252	0.011	201	0.023	156	0.023	257	0.136	252	0.011
146	0.011	250	0.011	256	0.011	198	0.023	254	0.023	203	0.102		–	259	0.057	–	–
148	0.295	252	0.011	258	0.159		–	258	0.080	–	–	–	–	261	0.034	–	–
150	0.011	–	–	262	0.023	–	–	260	0.045	–	–	–	–	–	–	–	–
155	0.011	–	–	–	–	–	–	267	0.080	–	–	–	–	–	–	–	–

with that for Petit Bouschet, so finally we identified it under this name. Petit Bouschet profile also matched with the one of the white cultivar Auxerrois of the SVMD database for the six SSR markers analyzed in common. Chardonnay shared the genotype of five loci with Petit Bouschet (Table 3). Auxerrois and Chardonnay cvs. belong to the progeny of the cross between Pinot × Heunisch Weiss, being Pinot a common ancestor with Petit Bouschet (VIVC, http://www.vivc.de/index.php).

The GEN18 profile matched with Aramon cv. profiles reported by Ibáñez et al. (2009) and the Davis NCGR database; although for the latter database a difference of 2 bp was observed for one allele of locus VVMD28. On the other hand, the GEN18 profile also matched with the Amor-nao-me-deixes cv. profile reported by the ETSIA-UPM database. In addition, GEN18 presented a similarity level of 0.50 with Petit Bouschet (Fig. 1), which comes from a cross of Aramon × Teinturier du Cher (Galet, 2000). According to the ampelographic description of Aramon cv. carried out by this author, our accession seems to be this cultivar. No descriptions of Amor-nao-me-deixes were found for comparison, although, according to data not published, it is suspected that Amor-nao-me-deixes is actually Aramon cv. (Dr. Ortiz, personal communication).

Carrasquín genotype matched with the profile of Carrasco cv. reported by Gago et al. (2009), although Suárez (1879) considered them as different cultivars in the basis of ampelographic data.

Cluster analysis of the data (Fig. 1) allowed us to group cultivars that are supposedly autochthonous to Asturias and the neighbouring regions of north-western Spain (Albarín Blanco, Verdejo Tinto, Espadeiro, Albarín Tinto, Carrasquín and Mencía), together with others not yet identified, and two international cultivars (cluster 1 in Fig. 1).

Cluster 2 shows the teinturier cultivars Garnacha Tintorera (Alicante Bouschet), Morrastel Bouschet and Petit Bouschet, although they are more related with other not teinturier cultivars than between them.

Variation among the two principal coordinates accounted for 10.3% and 8.3%, respectively. Cultivars considered autochthonous of Northern Spain, grouped in cluster 1 in the dendrogram, became also grouped on the PCo analysis (Fig. 2).

Some of the cultivars reported in this study had already been detected in previous studies (Martínez and Pérez, 1999; Martínez et al., 2002), but others are reported for the first time. Some of them are probably autochthonous and relatives of the most widespread cultivars, such as GEN01 and GEN06, very close genetically to Albarín Blanco and Verdejo Tinto cv., or GEN02 close to Albarín Tinto and Carrasquín cv. (Fig. 1), but a greater number of loci must be analyzed to confirm these hypotheses. These unknown cultivars are in danger of extinction; no more than three accessions have been found for each one.

3.3. Synonymies and homonymies

This study revealed the denominations Pedro Jiménez and Blanca Extra to be homonymies of Palomino; Mencín as a synonymy of Mencía, and Negrona as a synonymy of Mouratón (Table 4). Blanca Extra is the usual name for Palomino cv. in Asturias (Martínez et al., 1999), but we consider it a homonymy because Blanca Extra is a synonymy of the Picapoll cv. (Vitis International Variety Catalogue, http://www.vivc.bafz.de/index.php). On the other hand, the synonymy between Albarín Tinto and Albarín Francés reported in a previous study for the set of six SSR markers proposed by GENRES081 (Moreno-Sanz et al., 2008) was confirmed with the addition of the three new SSR markers analyzed (VVMD28, ZAG67 and ZAG112). Moreover, a new synonymy for Albarín Tinto cv. was found: Albarinón. Albarín Tinto is a cultivar that is more sus-

Table 3
Genetic profiles (allele sizes in bp) of the different cultivars (including allelic variations found) obtained for the nine SSR loci analyzed in this study. Letters in the "DB" column refer to the database where the matches were found: a = NCGR; b = ETSIA-UPM; c = González-Andrés et al. (2007); d = Ibáñez et al. (2003); e = Ibáñez et al. (2009); f = Martín et al. (2003); g = Martín et al. (2006); h = Sefc et al. (2000); i = SVMD; j = Vilanova et al. (2009); k = Santana et al. (2010); l = Díaz et al. (2010); m = GVD; n = Vargas et al. (2009); o = Gago et al. (2009); p = Vouillamoz et al. (2006); q = Zinelabidine et al. (2010); r = CRA-VIT; s = EVEGA; t = VIVC; u = BgGD.

Cultivar[a]	DB	SSR loci																	
		VVS2		VVMD5		VVMD7		VVMD27		VVMD28		ZAG62		ZAG67		ZAG79		ZAG112	
Chardonnay[REF]		133	139	233	237	240	244	178	186	217	228	187	195	140	154	244	246	242	242
Albarín Blanco	b,g,j,k,t	129	148	220	237	240	258	178	186	234	246	185	193	132	140	246	248	236	242
Albarín Tinto	b,c,f,g,h,l,t	139	148	224	237	254	258	176	186	236	248	187	199	126	126	252	252	236	242
Cabernet Sauvignon[REF]	a,c,f,h,i,j	135	148	230	239	240	240	173	186	234	236	187	193	126	140	248	248	232	236
Cardinal	e,m,n	131	131	224	235	250	250	176	182	244	267	185	185	126	140	252	256	236	244
Carrasquín	b,f,g,o,t	139	148	224	237	240	258	176	186	236	248	187	193	126	150	252	252	236	236
Chasselas Doré	c,e,f,i,t	129	139	226	235	240	248	182	186	217	267	193	203	126	154	252	259	242	244
Chasselas Rosé	c,e,f,i,t	129	139	226	235	240	248	182	186	217	267	193	203	126	154	252	259	242	244
De José Blanco[b]	b,f,t	121	129	226	226	248	256	182	182	217	244	180	203	126	144	250	250[c]	242	242
Dona Blanca	b,c,f,g,k	133	148	220	233	240	250	178	178	228	248	185	203	126	140	248	248	232	242
Espadeiro	b,f,g,l	135	148	235	237	240	244	173	186	234	244	187	187	126	154	246	248	236	244
Furmint	b,t	129	150	224	239	240	250	176	191	228	248	187	203	140	150	238	250	242	244
Garnacha Tintorera	a,b,c,h,k,t	129	141	224	237	240	244	178	191	244	260	187	187	132	140	244	257	232	240
Garnacha Tintorera[b]		129	141	224	237	240	244	178	178[c]	244	260	187	187	132	140	244	257	232	240
Godello	b,c,d,f,g,h,k,t	148	155	224	237	240	244	182	186	234	258	185	187	126	132	252	252	232	242
Italia[b]	b,e,f,m,p,q,t	129	146	230	237	244	248	176	176[c]	234	244	191	203	140	156	256	257	232	250
Lairén	b,f,k,t	129	141	226	237	240	248	182	191	234	258	185	193	126	152	240	257	232	242
Lairén[b]		129	141	226	237	240	248	182	182[c]	234	258	185	193	126	152	240	257	232	242
Morenillo II/Mandón	b,c,f,g,t	139	148	224	239	240	240	180	191	244	258	185	187	132	140	257	259	232	236
Morenillo II/Mandón[b]		139	148	224	239	240	240	180	180[c]	244	258	185	187	132	140	257	259	232	236
Mencía	b,c,d,f,g,j,k,t	141	148	224	235	250	258	178	186	236	236	187	193	126	132	248	252	236	242
Morrastel Bouschet	a,b,c,f,g,k,t	135	148	224	233	240	244	178	180	236	244	187	187	140	152	244	259	232	236
Moscatel Blanco de grano menudo	b,c,f,g,k,m,n,t	129	129	226	235	234	250	176	191	246	267	185	195	126	140	252	256	236	236
Moscatel Rojo	f	129	129	226	235	234	250	176	191	246	267	185	195	126	140	252	256	236	236
Moscatel Rojo[b]		129	144[c]	226	235	234	250	176	191	246	267	185	195	126	140	252	256	236	236
Mouratón	b,c,f,g,k,l,t	133	148	233	237	250	258	178	186	248	248	187	203	126	126	248	252	232	242
Petit Bouschet	b,c,f,g,k,t	129	148	233	237	240	244	178	186	236	260	187	195	126	140	244	246	236	240
Palomino	a,b,c,f,k	129	141	226	239	240	250	182	191	236	248	187	193	132	152	252	257	232	236
Roseti	a,b,e,m,n,q	129	131	224	230	240	250	182	182	234	258	185	187	132	156	244	252	244	244
Savagnin Blanc	b,g,t	148	148	230	237	244	258	186	186	234	236	187	193	126	132	246	252	236	242
Sumoll	b,c,f,k,t	129	141	224	239	244	250	176	178	234	236	185	187	140	152	248	261	232	236
Verdejo Tinto	b,c,f,h,k,l,t	139	148	237	237	240	258	173	186	234	248	187	187	126	140	246	248	236	242
GEN01		148	148	237	239	258	258	180	186	234	254	187	193	126	140	246	248	236	242
GEN02		148	148	226	237	240	258	186	186	236	248	187	193	134	150	240	252	236	244
GEN05	q	135	148	224	237	250	254	182	191	234	234	187	203	126	126	252	257	236	236
GEN06		129	148	237	239	240	258	180	186	234	254	185	193	126	140	246	248	236	236
GEN08		135	148	226	237	240	258	173	186	234	248	187	193	132	154	240	248	236	236
GEN10	u	139	148	230	237	240	258	178	186	236	248	187	193	126	132	252	261	242	242
GEN12	k	129	141	237	250	244	262	183	191	236	244	187	193	140	140	244	256	240	248
GEN13		129	133	224	252	244	262	183	183	236	244	187	201	150	150	257	261	232	246
GEN14		129	144	224	245	250	252	178	198	248	258	193	203	140	140	244	246	232	252
GEN15	r	129	148	230	245	238	252	188	198	228	252	179	199	126	126	246	252	232	240
GEN16	s	119	133	235	237	236	250	182	182	230	267	187	201	140	140	248	257	232	236
GEN17	r	135	148	235	237	240	258	173	186	236	244	187	193	132	140	246	248	236	236
GEN18	a,b,e,k,t	129	139	233	233	240	244	178	191	228	260	187	195	126	140	244	257	240	244

REF, reference cultivar.

[a] When the profile could not be identified, the code given to the unknown genotype was reported, instead of the cultivar name.

[b] Accession profile that showed an allelic variation with respect to the cultivar genotype.

[c] Allelic variation with respect to the cultivar genotype for this marker in the databases examined.

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Table 4
Identification based on the SSR databases consulted, generalized denominations, particular names, synonymies and homonymies for the cultivars named by the vine growers.

Cultivar ID	Generalized names in Asturias	Particular names[a]	Synonymies	Homonymies
Albarín Blanco	Albarín Blanco		Blanca del País; Blanco Verdín	Savagnin Blanc; Godello
Albarín Tinto	Albarín Tinto		Albarín Francés; Albarinón; Albarín Negrín; Tinto Serodo	
GEN18 (Aramon)		Verdello		
Cabernet Sauvignon	Cabernet Sauvignon			
Cardinal		Verdello		
Carrasquín	Carrasquín			
Chasselas Doré	Albillo	Jerez racimo pequeño		
Chasselas Rosé		Perdigueira		
Dona Blanca		Blanca Castellana; Rondal		
Garnacha Tintorera	Garnacha Tintorera			
Godello	Godello	Verdello Blanco		
Italia		Catalana		
Mencía	Mencía	Tinta del País	Mencín	
Morrastel Bouschet		Tinta Alicante	–	–
Moscatel Blanco de grano menudo	Moscatel Blanco de grano menudo			
Moscatel Rojo	Moscatel Rojo			
Mouratón	Mouratón	Mencía	Negrona	
Palomino	Blanca Extra			Blanca Extra; Pedro Jiménez
Savagnin Blanc	Albarín Blanco			Albarín Blanco
Verdejo Tinto	Verdejo Tinto	Tinta del País	Verdello Tinto	

[a] Not considered synonymies nor homonymies because they are not a widespread denomination for the cultivar.

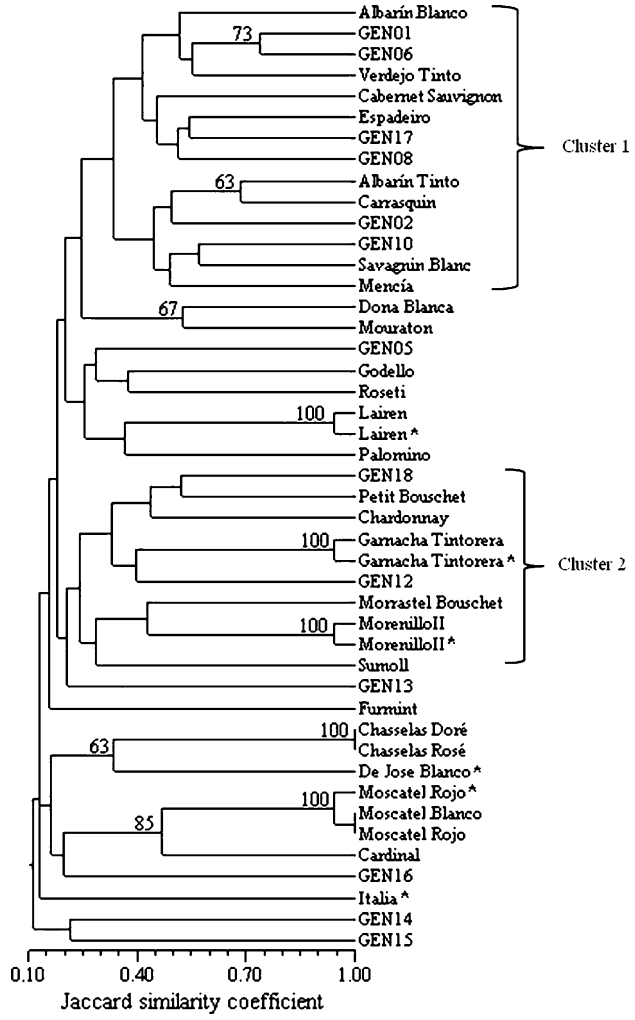

Fig. 1. Dendrogram generated by applying the UPGMA method, using Jaccard's coefficient matrix, of the cultivars discriminated in this study (*: allelic variations). The cophenetic correlation coefficient was 0.863. Bootstrap values greater than 50% are indicated at the corresponding branches.

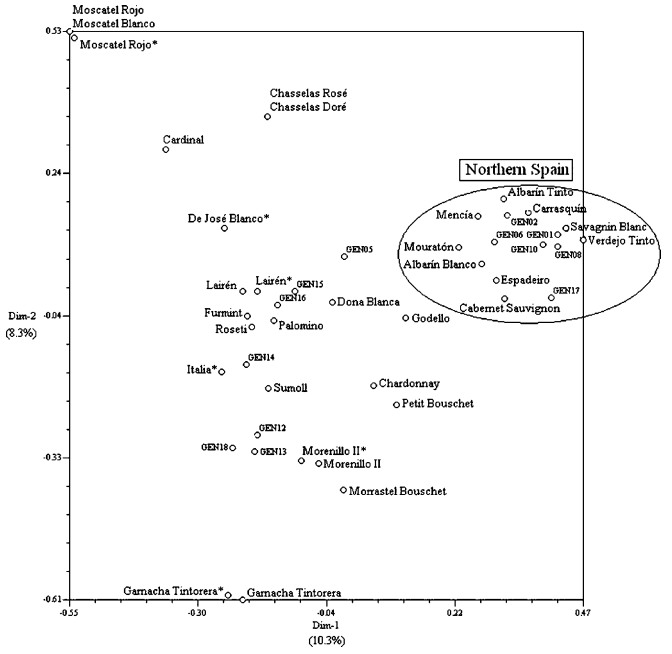

Fig. 2. Two-dimensional plot obtained from principal coordinate analysis of the cultivars discriminated in this study, as well as the reference cultivars. (*: allelic variations).

ceptible than others to exhibiting a certain degree of millerandage when conditions favour this phenomenon, but the name of Albarinón is used by vine growers for stocks where millerandage is always highly expressed. The phenotypical variation observed in Albarín Tinto cv. is worth noting, which indicates an old origin. Some stocks exhibit whole leaves, others trilobed leaves with or without a tooth on the upper lateral sinus, with different blistering of upper side of blade, and with different shapes of bunches.

Some of the genotypes identified were represented by less than three accessions and only one single vine grower gave them a name. When this given name did not correspond to the cultivar name obtained by comparison with the SSR databases, it was not considered a synonymy due to not being representative (Table 4). This is the case of Dona Blanca cv., which was called Blanca Castellana by

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one vine grower alone, not being a widespread designation for this cultivar. Although another vine grower identified this white cultivar as Rondal, according to the bibliographic references (Naredo, 1914), Rondal is a red cultivar, not white. For the two accessions of Chasselas Rosé cv. analyzed, one was collected under the name of Perdigueira and the other as unknown (Table 4).

3.4. Allelic variations

As regards cultivar genotype, some accessions showed variation in a single allele (Fig. 1 and Table 3), which may be a clonal variation of the cultivar. This is the case of De José Blanco, Garnacha Tintorera, Italia, Lairén, Mandón and Moscatel Rojo cultivars. Except for Moscatel Rojo, the variation is due in all cases to the lack of the major allele; the heterozygous original genotype of the cultivar thus becomes homozygous for those accessions. This occurs in De José Blanco for the ZAG79 marker; in the six accessions tested we obtained a homozygous genotype, 250:250, while in the examined database (Martín et al., 2003) the cultivar genotype was 250:252. It is worth noting that a very small peak of 252 bp could be appreciated for all these accessions. However, its very low intensity, which did not reach the minimum threshold, led us to not take it into consideration. It is worth noting in the case of Garnacha Tintorera, Italia, Lairén and Mandón cvs. that, regardless of the variety, the allelic variation always occurs in the same SSR marker (VVMD27), always consisting of the absence of the allele VVMD27-191. The only accession of Italia cv. analyzed showed this variation in its profile, as well as one accession out of four in Garnacha Tintorera cv., one out of three in Lairén cv. and one out of two in Mandón cv. In the accessions that showed this allele, the peak intensity was less than half that of the minor allele. One accession out of five of Moscatel Rojo presented a single allele variation for the VVS2 marker, where, instead of having the cultivar genotype 129:129, it showed a heterozygous one, 129:144.

Analyses were repeated in different years for the accessions that showed allelic variation and these are currently being studied ampelographically. According to preliminary results (data not published), they seem to match with their respective cultivar descriptions.

4. Conclusions

The Resolution OIV/VITI 424/2010 of the International Organisation of Vine and Wine (OIV) considers of urgent need to protect the grapevine world heritage, especially wild and cultivated plant material with high risk of disappearance. The OIV promotes the collection, identification and conservation of this kind of plant material and encourages research activity within this scope. In Asturias, despite the reduced vineyard surface and the heavy genetic erosion suffered during years, 42 different genotypes have been detected through the molecular analysis of 293 accessions, 13 of them not being identified in the databases consulted. These unknown cultivars are in danger of extinction and some of them seem to be closely related to autochthonous plant material. Cultivars not recorded in Asturias before have been identified as well. Homonymies and new synonymies have also been detected, as well as allelic variations of some cultivars. These results provide an overview of the status of current grapevine phytogenetic resources in Asturias, finding higher variability than expected. It is still not too late to recover and conserve the remaining diversity after the genetic erosion process that the Asturian vineyard has undergone and make it available in germplasm banks.

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