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Twenty microsatellites (SSRs) reveal two main origins of variability in grapevine cultivars from Northwestern Spain

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Summary

The grapevine germplasm bank in the "Estación de Viticultura y Enología de Galicia, Xunta de Galicia", holds fifteen grapevine cultivars with a total of 98 accessions: 'Brancellao', 'Albarello', 'Caíño Astureses', 'Caíño Bravo', 'Caíño Blanco', 'Caíño Gordo', 'Albarín Negro', 'Caíño Longo', 'Caíño Redondo', 'Castañal', 'Mencía', 'Merenzao', 'Mouratón', 'Sousón', and 'Verdello'. Cultivars 'Syrah' and 'Pinot Noir' were included as references. Two different lineages were detected, one originating in 'Caíño Astureses' and the other in 'Merenzao', synonymy of the French cultivar 'Trousseau'. Cultivars from Northwestern Spain derived from both of these cultivars by hybridization and selected genotypes that had adapted to local climatic conditions and became fixed by cuttings, explaining the domestication process of these grapevine cultivars. Both lineages differed in allelic frequencies and were distributed differently in Northwestern Spain, the first lineage in the west and the second, related with the French cultivar 'Trousseau', in the east. 'Caíño Astureses' was the most frequent genotype related by hybridization, indicating the importance that this cultivar had in the origin of grapevines in Galicia. In addition a total of 13 different genotypes were identified. The identity of 'Brancellao' and 'Albarello' was confirmed by SSR-markers. Other two synonyms were 'Caíño Astureses' and 'Caíño Bravo', and 'Caíño Gordo' and 'Albarín Negro'. 'Caíño Redondo' showed two different genotypes, one related to 'Caíño Astureses' and the other to 'Merenzao'. Two cultivars included in the collection from EVEGA were not reported previously, 'Verdello' and 'Caíño Longo'.

K e y w o r d s : *Vitis vinifera*, lineage, genetic relationship, hybridisation, microsatellite markers.

Introduction

During the 90s and updated more recently, a grapevine germplasm bank was established in the "Estación de Viticultura y Enología de Galicia, Xunta de Galicia". Most of these grapevines have been collected in Galicia (Northwestern Spain) and are supposed to be traditionally grown in this region by way of a lengthy period of selection process carried out by growers. Designations of Origin (D.O.) regulations have referred to some of these cultivars as being preferred or authorized for wine production, such as 'Brancellao', 'Sousón', 'Caíño Tinto', 'Merenzao', 'Mencía', and 'Mouratón'. All of them could be found in Galicia before phylloxera spread [*Daktulosphaira vitifoliae* (Fitch)] (Santos-Solla 1992) and as they are considered to produce distinct wines, this has encouraged several studies in order to characterize and evaluate their genetic diversity.

The 'Brancellao' cultivar was cultivated all over Galicia before phylloxera (Fig. 1) and is currently recognized as preferred or authorized in 4 out of 5 D.O.: Rías Baixas, Ribeiro, Valdeorras and Ribeira Sacra. 'Albarello' was reported in Western Galicia (Martínez *et al.* 2006) and has been considered as synonymous to 'Brancellao' (Casares 1843, Crespo 1897, Chomé *et al.* 2003), but also as different when morphology is taken into account (Martínez *et al.* 2006). Other denominations for 'Brancellao' are 'Portuguese Brancelho', 'Sousón' or 'Negrâo from Vinhao', and 'Merenzao of Bastardinho' (Truel 1983).

'Sousón' is cited as being cultivated only in Galicia (Freijanes and Alonso 1997, Chomé *et al.* 2003). 'Brancellao' and 'Sousón' produce special chromatic characteristics for ageing in barrels (Río Segade *et al.* 2008).

'Mouratón' and 'Merenzao' are mainly cultivated in the D.O. of Monterrei, Valdeorras and Ribeira Sacra (Freijanes and Alonso 1997). 'Mouratón' is also cultivated in various neighbouring regions, such as Asturias and Castilla-La Mancha (Chomé *et al.* 2003). 'Merenzao' is a synonymy of the 'Trousseau' variety cultivated in the Jura region (France) and 'Bastardo' grown in the Douro Valley (Portugal).

The origin of the most important cultivar at present in Northwestern Spain, 'Mencía', is still unknown. Peñín (1997) considers that it originated from Bordeaux (France) and entered Spain westerly along the St. James Pilgrimage Route, to be profusely planted in regions of Castilla-León (Bierzo) and Galicia (Valdeorras and Ribeiro).

The 'Caíño' cultivar group is considered to be the oldest variety in Galicia (Northwestern Spain) and since 1957 the most recommended as main cultivar for new groves in the Ribeiro D.O. region, due to its potential for producing quality wines (Boe 1976). Different types of 'Caíño' were first reported between 1909 and 1911 by García de los Salmones (1914), but it was then difficult to differenti-

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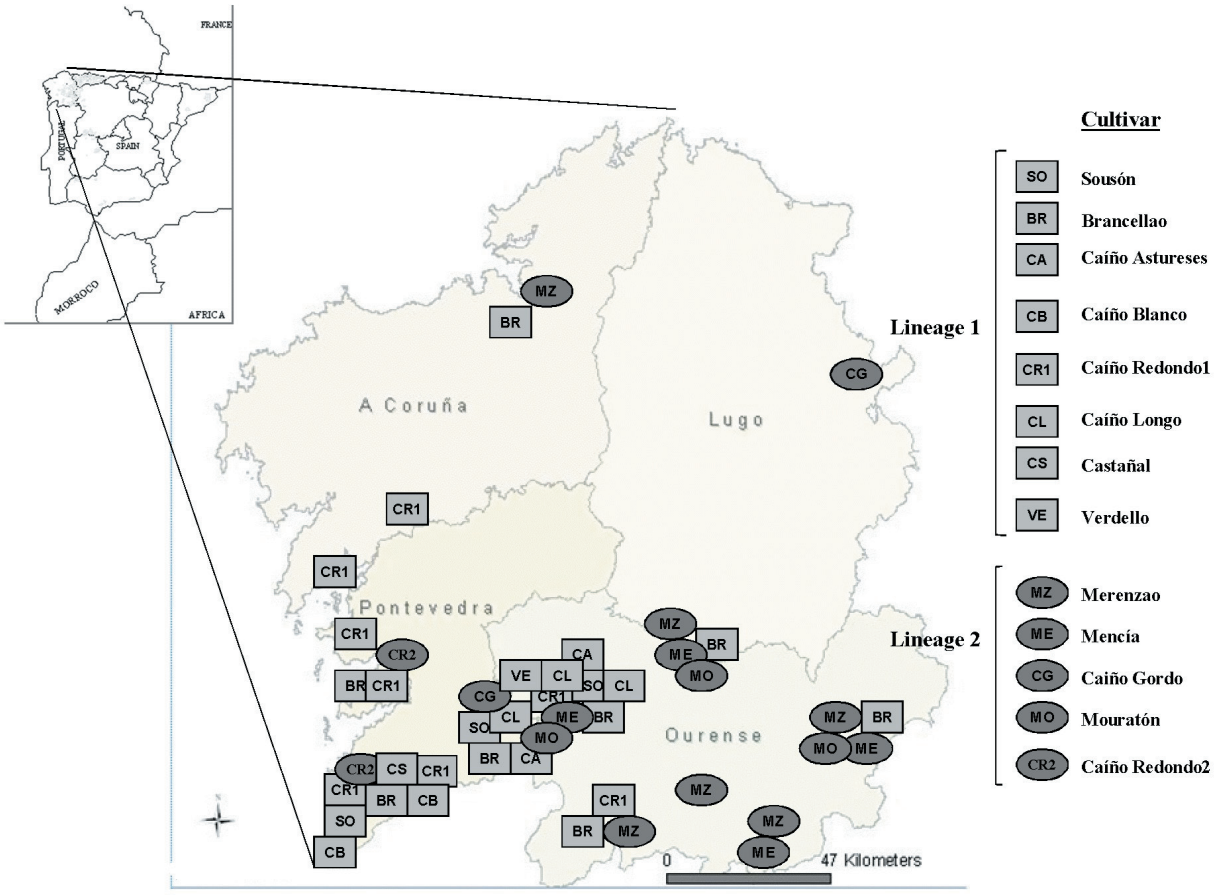

Fig. 1. Distribution of grapevine cultivars in Northwestern Spain (modified from Santos-Solla 1992).

ate between varieties (Consellería de Agricultura 1986). Freijanes and Alonso (1997) identified 'Caíño Bravo' and 'Caíño Gordo' by morphology. In addition, Santiago *et al.* (2005 a) described 'Caíño Blanco', 'Caíño do Freixo', 'Caíño Redondo', 'Caíño Tinto' (known as 'Borraçal' in Portugal) and 'Caíño Longo'. 'Caíño' from Galicia is recorded in the *Registro de Variedades Comerciales* (Chomé *et al.* 2003) as 'Caíño Tinto', which is the authorized type for planting, with 'Caíño Bravo' or 'Cachón' as synonyms (Boe 2005). Ampelography was used in the past to successfully differentiate between cultivars and to determine similarities (Truel and Boursiquot 1986; Boursiquot *et al.* 1987). Later on, molecular markers improved the identification of a great number of grapevines (Bowers *et al.* 1996, Martín *et al.* 2003). These methodologies have been used to find synonymies such as 'Caíño Tinto' and 'Tinta Femia', 'Albarín Blanco' and 'Blanco País'; and 'Blanco Verdín', 'Blanco Legítimo' and 'Raposo' (Santiago *et al.* 2005 a, 2005 b).

Three processes produced cultivar diversification in cultivated grapevines (This *et al.* 2006): sexual reproduction, vegetative propagation and somatic mutations. New genotypes produced by sexual reproduction, either by crossing or self-fertilization have been described with molecular markers as the origin of cultivars from South America.

In this study, we present the genetic characterization of the fifteen grapevine Galician cultivars from the germplasm bank "Estación de Viticultura y Enología de Galicia (EVEGA), Xunta de Galicia", aiming to determine the relationships between them.

Material and Methods

This study includes fifteen local grapevine cultivars from Northwestern Spain collected in the germplasm bank of the EVEGA, with a total of 98 accessions (Fig. 1): 11 accessions of 'Brancellao', 3 of 'Albarello', 6 of 'Caíño Astureses', 2 of 'Caíño Bravo', 1 of 'Caíño Blanco', 5 of 'Caíño Gordo', 1 of 'Albarín Negro', 4 of 'Caíño Longo', 13 of 'Caíño Redondo', 2 of 'Castañal', 16 of 'Mencía', 5 of 'Merenzao', 7 of 'Mouratón', 21 of 'Sousón', and 1 of 'Verdello'. Two main cultivars included in the collection at EVEGA, were evaluated at the same time as references for allele sizes, 1 accession from 'Syrah' and 1 accession from 'Pinot Noir'.

Each accession was grafted onto rootstock (196-17C), in the "Estación de Viticultura y Enología de Galicia, Xunta de Galicia" located in Leiro-Ourense (Spain). Grapevines were planted at 1.2 x 1.8 m, conducted by trellis and formed in espalier, with an east-west orientation. Each grapevine was pruned in a single cordon with four buds.

Molecular methods: DNA was prepared from leaves using the DNA DNeasy® Plant kit from the Quiagen Group. After quantification, final DNA concentration was adjusted to 5 $ng \cdot \mu L^{-1}$. Twenty polymorphic SSRs were considered for this study, some of them considered

the most appropriate to evaluate the grapevines (European project GENRES081, http://www.genres.de/vitis). VVS2, VVS1, VVS4, VVS5 and VVS29 (Thomas and Scott 1993); VVMD5, VVMD7 and VVMD28 (Bowers *et al.* 1996); ssrVrZAG47, ssrVrZAG62 and ssrVrZAG79 (Sefc *et al.* 1998); ssrVrZAG29**,** ssrVrZAG67, ssrVrZAG112, ssrVrZAG83 and ssrVrZAG21 (Sefc et al. 1999); and VVMD34, VVMD17, VVMD31 and VVMD27 (Bowers *et al.* 1999). VVS2, VVS29 and ssrVrZAG 79 were marked with flurochrome NED; VVS5, VVMD5, ssrVrZAG47 and ssrVrZAG67 with 6FAM; VVMD7, ssrVrZAG29, ssrVrZAG62 and ssrVrZAG112 with VIC; and VVMD28 and ssrVrZAG83 with HEX. PCR were conducted according to the methodology defined by Martín *et al.* (2003).

Statistical analyses: Populations (www.cnrs-gif.fr/pge) software was used to estimate allelic. Hierarchical analysis of the molecular variance (AMOVA) was calculated to partition the genetic diversity using Arlequin 3.1. To compute microsatellite genetic data, we applied factorial correspondence analysis (FCA) performed with Genetix4 (Belkhir *et al.* 1996–98).

Results and Discussion

Genetic relationships by SSRs: All twenty recommended microsatellites for *Vitis* identification were polymorphic in this study and identified 13 different genotypes. Identified genotypes and the references 'Syrah' and 'Pinot Noir' are shown in Tab. 1.

We found a new synonymy that had not been previously reported, 'Brancellao' and 'Albarello'; and two more were confirmed, 'Caíño Astureses' and 'Caíño Bravo', and 'Caíño Gordo' and 'Albarín Negro'. A homonym was found for 'Caíño Redondo', with two different genotypes, named 'Caíño Redondo1' and 'Caíño Redondo2'.

'Verdello' and 'Caíño Longo' genotypes from EVEGA collection were not reported previously. The other 11 cultivars were partially described previously for six microsatellites, VVS2, VVMD5, VVMD7, ssrVrZAG47, ssrVrZAG62 and ssrVrZAG79; 'Brancellao' from EVEGA corresponded to the one reported by Martín *et al.* (2006) with 'Brancelho' as synonym; 'Sousón' from EVEGA was reported by Martín *et al.* (2006), named also 'Sousao' and 'Vinhao'; 'Caíño Astureses' from EVEGA was reported by Martín *et al.* (2006) and Santiago *et al.* (2005 a) as 'Caíño Bravo'; 'Caíño Redondo1' was coincident with 'Caíño Tinto' reported by Santiago *et al.* (2005 a) and Martín *et al*. (2006); 'Caíño Blanco', 'Castañal', and 'Merenzao' were identical to the one described by Martín *et al.* (2006); 'Caíño Redondo2' was reported by Santiago *et al.* (2005 a); 'Mouratón' was described by Martín *et al.* (2006), named also 'Juan García'; 'Caíño Gordo' was reported by Santiago *et al.* (2005 a) and it was described as 'Albarín Negro', 'Bruñal' and 'Alfrocheiro Preto' by Martín *et al.* (2006) and 'Mencía' by Martín *et al.* (2006).

Two different lineages could be detected, the first having its origin in 'Caíño Astureses' and the second in 'Merenzao' (Tab. 1). Cultivars related by hybridisation share alleles for each of all 20 loci as it is shown in Tab. 1. In this study, a minimum of eight SSRs were enough to reveal the two lineages: VVS2, VVMD5, VVMD7, ssrVrZAG47, ssrVrZAG62, ssrVrZAG79, VVS29, VVS5. In all, 13 grapevine cultivars from Northwestern Spain, eight from 'Caíño Astureses' and five from 'Merenzao', could have been derived by hybridization. Hybridization was reported as the origin of cultivars from South America (This *et al.* 2006). This situation was also reflected when a multivariate analysis (FCA) was performed (Fig. 2), cultivars from lineage 1 mainly clustered in the positive part of the axe 1, and cultivars from lineage 2 in the negative part. AMOVA performed showed that genetic differentiation between lineages was 11.20 % (Tab. 2).

Six different 'Caíños' were evaluated in this study, 'Caíño Astureses', 'Caíño Redondo1', 'Caíño Redondo2', 'Caíño Blanco', 'Caíño Longo' and 'Caíño Gordo'. Three of them were related by hybridization with 'Caíno Astureses' in the first lineage (Tab. 1 and Fig. 3), 'Caíño Redondo1', 'Caíño Longo' and 'Caíño Blanco'. 'Caíño Astureses' was also the origin of 'Sousón' and 'Castañal'.

Two more 'Caíños' were also related by hybridization to the second lineage. 'Caíño Redondo2' was related to 'Merenzao' and 'Caíño Gordo' to 'Mouratón' and 'Mencía' in lineage 2. Propagation of seedlings with an unknown

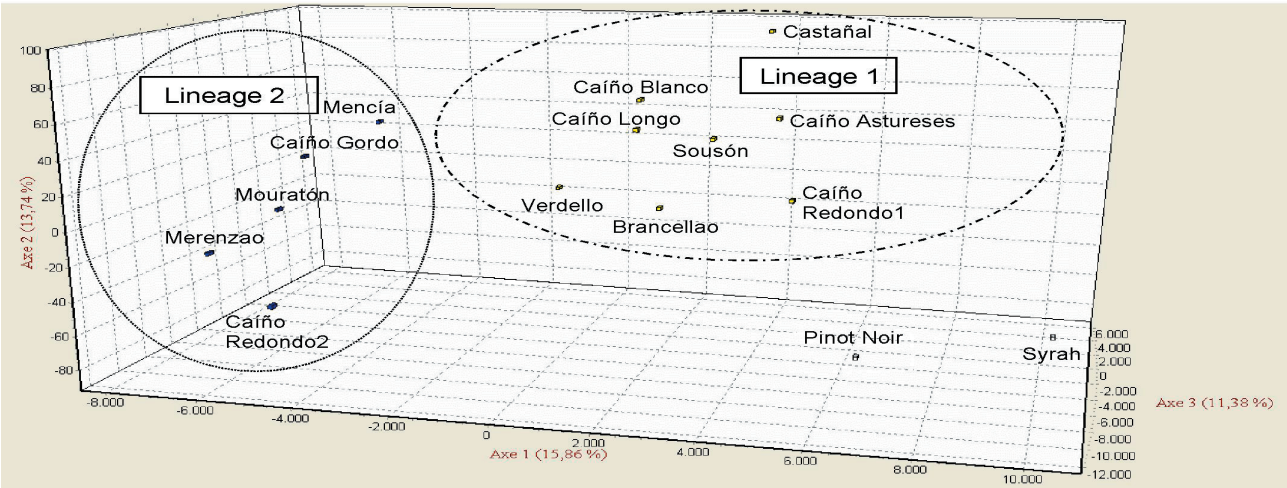

Fig. 2. Factorial correspondence analysis based on 20 SSRs of 13 Galician cultivars, 'Syrah' and 'Pinot Noir'.

Table 1

Microsatellite characterization of grapevine cultivars included in the Germplasm Bank of the EVEGA, Galicia. Shared alleles between genotypes related by hybridization were underlined. Genotypes are indicated in base-pairs

	Cultivar	Lineages	Cultivars with the same number share an allele in each loci	VVS2		VVMD5		VVMD7		ssrVrZAG 47		ssrVrZAG62		SsrVZAG79		VVS29		VVS5		ssrVrZAG112		SsrVrZAG67		SsrVrZAG29	
1	Verdello	1	6	130	150	218	228	237	255	157	165	185	193	245	249	169	169	100	121	238	238	123	137	114	114
2	Brancellao, Albarello	1	5,6	130	150	218	222	237	237	161	165	187	193	249	257	169	169	85	121	238	238	137	137	110	114
3	Sousón	1	1,5	130	132	218	222	237	261	165	165	187	195	243	249	169	179	100	121	234	238	137	151	110	114
4	Caíño Astureses	1	1,2,3,4	132	140	222	228	237	261	157	165	193	195	243	245	169	179	121	121	232	234	123	151	110	114
5	Caíño Redondo1	1	2	130	132	228	234	237	237	157	161	193	193	245	245	169	179	85	121	232	240	137	151	110	114
6	Caíño Longo	1	3	140	140	222	232	241	261	157	165	187	195	243	245	169	169	85	121	228	234	123	149	110	114
7	Caíño Blanco	1	3	140	150	218	222	237	261	157	165	195	203	245	249	169	179	121	121	232	234	123	151	114	114
8	Castañal	1	4	132	154	222	222	261	261	157	165	193	195	245	257	169	179	121	150	232	232	151	151	112	114
9	Merenzao	2	7,8	140	150	234	234	237	255	151	165	187	187	243	245	169	169	98	150	232	238	123	137	110	110
10	Caíño Redondo2	2	8	136	150	232	234	237	241	151	165	187	187	243	245	169	179	98	150	--	--	123	151	110	110
11	Mouratón	2	7,9	134	150	230	234	247	255	157	165	187	203	245	249	169	169	150	150	228	238	123	123	110	110
12	Caíño Gordo	2	9, 10	140	150	222	234	251	255	155	165	187	199	249	249	169	169	94	150	232	238	123	123	110	110
13	Mencía	2	10	142	150	222	232	247	255	157	165	187	193	245	249	169	169	94	150	232	238	123	129	110	110
14	Syrah		Reference	130	130	222	228	237	237	165	167	187	193	243	249	169	177	121	121	228	240	123	147	110	114
15	Pinot Noir		Reference	134	150	224	234	237	241	161	165	187	193	237	243	169	177	121	150	238	240	123	151	110	114

Tab. 1, continued

	Origen sample	Lineages	Cultivars with the same number share an allele in each loci	VVMD28		SsrVrZAG 83		VVMD34		VVS4		ssVrZAG21		VVS1		VVMD17		VVMD31		VVMD27	
1	Verdello	1	6	234	234	194	200	237	245	165	173	199	201	161	179	217	219	202	214	179	187
2	Brancellao, Albarello	1	5,6	234	258	200	200	237	237	165	165	189	199	161	179	217	219	202	210	183	187
3	Sousón	1	1,5	236	258	188	200	237	237	165	165	199	201	179	181	209	219	210	210	187	187
4	Caíño Astureses	1	1,2,3,4	236	266	188	200	237	237	165	165	199	203	181	189	209	221	210	214	179	187
5	Caíño Redondo1	1	2	236	258	188	200	237	237	165	165	199	201	179	189	209	217	204	214	179	183
6	Caíño Longo	1	3	236	266	188	200	237	237	163	165	199	203	179	189	217	221	204	210	179	187
7	Caíño Blanco	1	3	234	236	188	188	237	245	165	165	193	203	179	181	217	221	210	210	179	187
8	Castañal	1	4	236	264	188	194	237	237	165	173	189	203	181	189	209	219	210	210	179	187
9	Merenzao	2	7,8	234	248	190	200	237	245	165	173	199	203	179	189	217	219	202	208	173	187
10	Caíño Redondo2	2	8	234	244	200	200	237	237	165	173	193	199	179	189	209	217	208	210	173	187
11	Mouratón	2	7,9	248	248	188	190	237	245	165	165	199	199	179	179	217	217	202	210	179	187
12	Caíño Gordo	2	9, 10	236	248	188	194	237	245	165	165	199	201	179	189	217	219	202	210	177	187
13	Mencía	2	10	236	236	194	194	237	245	165	165	201	203	--	--	219	219	202	210	179	187
14	Syrah		Reference	218	228	194	200	237	237	165	171	189	205	179	179	209	221	210	214	187	189
15	Pinot Noir		Reference	218	236	--	--	237	237	165	171	199	205	181	189	209	217	214	214	183	187

Table 2

Analysis of molecular variance (AMOVA) based on the 20 SSR loci

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation	P-value
Among lineages	1	14.496	0.716	11.20	< 0.001
Within populations	24	136.150	5.681	88.80	
Total	25	150.846	6.397		

Note: d.f., degree of freedom.

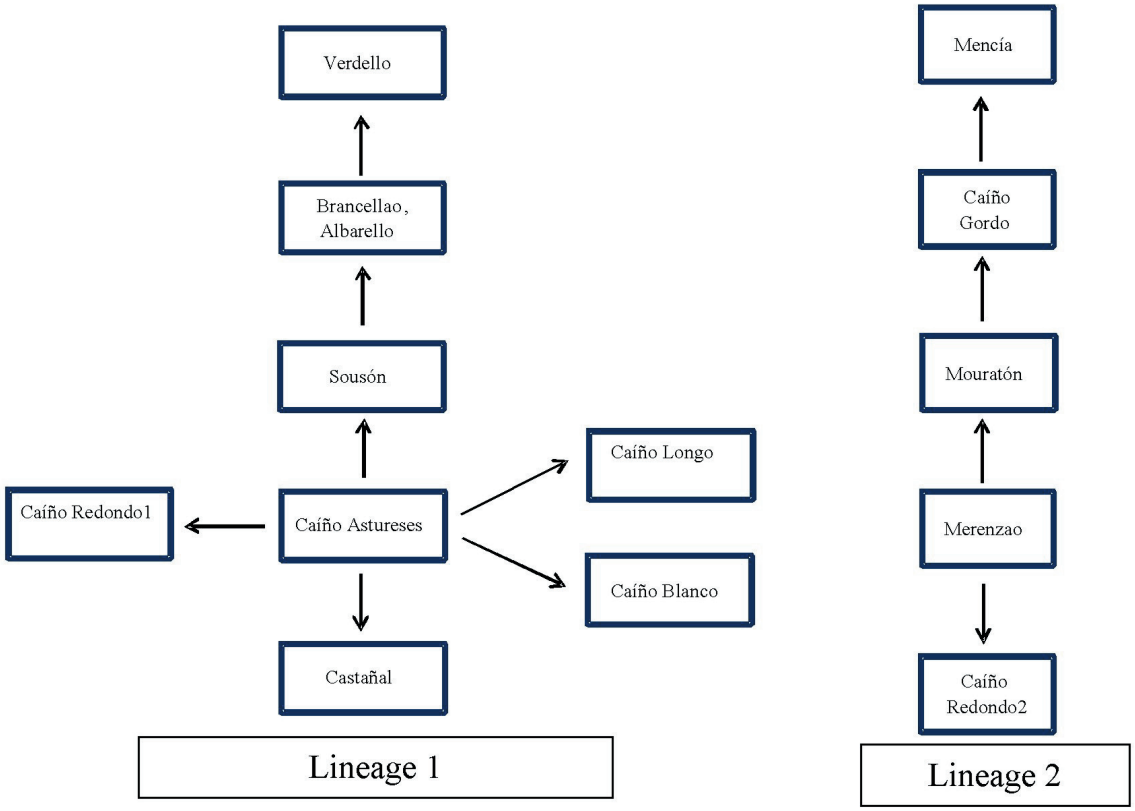

Fig. 3. Hybridisation relationships between Galician grapevine cultivars found with 20 SSRs, with 'Caiño Astureses' as origin of the first lineage and 'Merenzao' of the second one.

pollenizer explains that 'Mencía' was related by hybridization to 'Caíño Gordo', 'Caíño Gordo' to 'Mouratón', 'Mouratón' to 'Merenzao' and, finally, 'Caíño Redondo' to 'Merenzao', which would be certainly the origin of this second lineage.

The two lineages differed in allele frequencies (Tab. 1). For VVS2, allele 140 was more frequent in the 'Caíño Astureses' lineage and 150 in the 'Merenzao' lineage; for VVMD5, alleles 222 and 234; for VVMD7, alleles 237 and 255; for ssrVrZAG79, alleles 245 and 249; for VVS5, alleles 121 and 150; for ssrVrZAG29, alleles 114 and 110; for VVMD31, alleles 210 and 202; for VVMD28, alleles 236 and 236-248; and for ssrVrZAG83, alleles 188-200 and 188-194, respectively. For ssrVrZAG62, allele 193 was more frequent in the 'Caíño Astureses' lineage versus 187 in the 'Merenzao' lineage; and for ssrVrZAG67, allele 151 versus 123. For ssrVrZAG112, allele 238 was most frequent in 'Merenzao' lineage and alleles 232 and 238 were more frequent in 'Caíño Astureses' lineage. For VVMD34, 'Merenzao' lineage showed a higher frequency of allele 245 in heterozygosis. For VVS1, allele 179 was only common to 'Merenzao' lineage. 'Caiño Astureses' showed the specific allele 221 for VVMD17. For ssrVrZAG21, allele 203 was only relevant for lineage1 and allele 201 for lineage 2. Finally, for VVS4 allele 163 was specific for lineage1.

Genetic and geographic differentiation in Northwestern Spain: The current distribution of grapevine cultivars in Northwestern Iberian Peninsula can be explained roughly by the existence of two origins of diversification (Fig. 1).

'Caíño Redondo1', 'Caíño Longo2', 'Caíño Astureses', 'Verdello' and 'Sousón' are cultivated mainly in the transition area between Western Galicia and the Atlantic coast. 'Sousón' is also cultivated in Northern Portugal where it is known as 'Vinhao'. 'Albarello' is considered as originating in Galicia, as this study has confirmed, from the D.O. Valdeorras and Ribeira Sacra regions and does not overlap the

area where 'Caíño Astureses' and 'Sousón' are cultivated. This study, however, demonstrated that 'Brancellao' and 'Albarello' are synonymous. Also, 'Brancellao's close relationship with 'Caíño Astureses', 'Verdello' and 'Sousón' could be explained by its wide area of cultivation though out the entire region of Galicia.

'Mouratón' and 'Merenzao' are mainly cultivated in the eastern area of Galicia and border areas of Castilla-León. The same can be said for 'Mencía', currently a principal cultivar in the region. 'Merenzao' is a synonymy of the 'Trousseau' variety cultivated in Jura (France). 'Mencía' could have derived from 'Merenzao' after three generations of hybridization and selection and directly from 'Caíño Gordo' with which it shares alleles in each locus. These genetic relationships can explain the domestication process in these important lineages found in Northwestern Spain, where hybridization with still unknown parents and further selection identified the best genotypes, which had become climatically adapted and fixed by cuttings.

Conclusions

Microsatellites have shown the genetic relationships among some Galician cultivars and revealed a new synonymy and two homonyms that had never been previously reported. In this paper, we have demonstrated that 'Brancellao' and 'Albarello' are synonymies confirmed by SSRs. 'Caíño Redondo' from EVEGA showed two different genotypes. Two cultivars included in the collection from EVEGA were not reported previously, 'Verdello' and 'Caíño Longo'. The other 11 Galician cultivars were coincident for six SSRs with previous studies and in this work we have reported seven additional SSRs not studied before for those cultivars.

Microsatellites have also been efficient in finding two main origins of variability: the first lineage involved 'Caíño Astureses' (first lineage origin), 'Sousón', 'Brancellao' (synonym 'Albarello'), 'Caíño Redondo1', 'Caíño Longo', 'Caíño Blanco', 'Castañal' and 'Verdello'; and the second one: 'Merenzao' (second lineage origin), 'Caíño Redondo2', 'Mouratón', Caíño Gordo', and 'Mencía'. Thus, the actual distribution of grapevine cultivars in the Northwestern Iberian Peninsula can basically be explained by the existence of two genetic diversification centres, together with a French origin for cultivars related with 'Merenzao' by hybridization, as 'Mencía', which French origin was reported by Peñín (1997).

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