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Characterisation and identification of grapevine cultivars (*Vitis vinifera* L.) from northwestern Spain using microsatellite markers and ampelometric methods

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Summary

Nine grapevine varieties from northwestern Spain (8 commonly known as types of Caíño and one as Tinta Femia) were characterised by constructing their typical 'mean leaves' and by determining their genetic profiles with respect to 6 microsatellite markers. Leaf morphologies were compared and the similarities between the cultivars were determined. Thirty three alleles were detected at the 6 microsatellite loci analysed. The different cultivars were successfully identified by both methods. In combination, the different techniques provide a more complete variety characterisation. Synonymy between these and other Spanish and Portuguese cultivars is discussed.

K e y w o r d s : grapevine, ampelometry, molecular markers, STMS, synonymies.

Introduction

Usually ampelographic studies are sufficient to differentiate between grapevine varieties and are indispensable for their characterisation. Indeed, the only official description of grapevine variety traits is by defining their ampelographic descriptors. Traditional ampelographic methods, based only on morphology and morphometry, are, however, thought to be outdated by some authors (Dettweiller 1993). In recent years, numerous techniques for characterisation have been developed that rely on molecular markers. Unfortunately, instead of complementing ampelographic data, they tended to displace them. Nowadays, microsatellite analysis is generally used for grapevine variety characterisation (Beckman and Soller 1990, Thomas and Scott 1993, Botta *et al*. 1995, Bowers *et al*. 1996, Sefc *et al.* 1998, Sánchez-Escribano *et al.* 1999, Martín *et al*. 2003).

The present work is focused on the combination of ampelometric and molecular methods for a more complete characterisation of 9 grapevine varieties from northwestern Spain. This approach provides both molecular details of the cultivars and, via reconstruction of 'mean leaves', a better understanding of leaf morphology. This allows rapid identification of each cultivar and a comparison with others.

Material and Methods

P l a n t m a t e r i a l : All grapevine varieties (Tabs 1 and 2) belonged to the collection of the Misión Biológica de Galicia (Consejo Superior de Investigaciones Científicas, CSIC), Pontevedra, Spain. Each variety was represented by 10 specimens.

L e a f s a m p l e s a n d l e a f v a r i a b l e s : In 2000, 2001 and 2002 before veraison, at least one leaf was taken from node 8 (counting from the base) of each of 10 plants per cultivar. Each year 11 leaves of each cultivar were then selected for analysis. Several authors have reported that this provides a representative sample for the description of a typical leaf of a grapevine cultivar (Galet 1956, Branas 1974, OIV 1983, Dettweiller 1991, Martínez and Grenan 1999). Quantitative variables were recorded by taking a digital photo of each of the 11 leaves per cultivar. They were examined using imaging software. Following the method of Martínez and Grenan (1999), the analySIS 3.0 program was used to measure the quantitative base variables required to construct a 'mean leaf' for each cultivar (Fig. 1). This method requires recording of the number of teeth between the major veins (Fig. 2).

The following characterising relationships were determined: Rel.1 = Lp/L; Rel.2 = L1d/L; Rel.3 = L1g/L; Rel.4 = A+B+G; Rel.5 = A'+B'+G'; Rel.6 = a+b+g; Rel.7 = a'+b'+g'; Rel.8 = (S1d+S2d)/(L1d+L2d) Rel.9 = (S1g+S2g)/L1g+L2g), where L = linear distance between the petiolar point and the central vein end, L1 = linear distance between the petiolar point and the end of the first right (L1d) and left (L1g) lateral veins, L2 = linear distance between the petiolar point and the end of the second right (L2d) and left (L2g) lateral veins, L3 = linear distance between the starting point of the first secondary vein belonging to the second lateral vein and the end of the right (L3d) and left (L3d) secondary vein, L5d = linear distance between the petiolar point and the starting point of L3d, L5g = linear distance between the petiolar point and the starting point of L3g, S1= linear distance between the petiolar point and the bottom (towards the petiolar point) of the right (S1d) and left (S1g) lateral upper sinuses, S2 = linear distance between the petiolar point and the bottom (towards the petiolar point) of the right (S1d) and left (S1g) lateral lower sinuses, A = angle between the central vein and

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Table 1

Mean values of basic length (cm) and angle (º) variables for 'mean leaf' construction and the equations calculated from the leaf variables measured

	Caíño Tinto 1	Caíño Longo	Caíño Gordo	Caíño Tinto 2	Caíño do Freixo	Caíño Bravo	Caíño Redondo	Tinta Femia	Caíño Blanco
L (cm)	11.13	13.75	9.35	10.06	11.22	11.33	11.74	10.75	10.35
S1d (cm)	6.85	8.95	6.58	6.72	8.06	7.17	7.32	6.62	7.57
L1d (cm)	9.06	11.07	8.26	8.63	10.22	9.06	10.18	8.89	8.58
S2d	5.80	6.78	5.64	5.76	6.73	6.04	5.96	5.66	5.89
L2d (cm)	6.70	7.65	6.39	6.61	7.60	6.69	7.13	6.53	6.28
L3d (cm)	4.71	4.88	4.38	4.53	4.97	4.66	4.23	4.51	4.25
L3g (cm)	4.73	4.99	4.45	4.68	4.91	4.81	4.34	4.62	4.35
L2g (cm)	6.85	7.80	6.38	6.62	7.24	6.71	7.25	6.51	6.26
S2g (cm)	5.82	6.98	5.60	5.79	6.43	5.98	6.11	5.63	5.89
L1g (cm)	9.16	11.14	8.25	8.57	9.93	9.01	10.21	8.80	8.54
S1g (cm)	6.89	9.01	6.74	6.77	7.87	7.02	7.49	6.44	7.54
L5d (cm)	0.57	0.77	0.84	0.62	0.96	0.48	1.12	0.60	0.51
L5g (cm)	0.65	0.72	0.79	0.62	1.07	0.56	1.12	0.61	0.52
Lp (cm)	15.42	15.60	12.75	14.54	15.64	14.16	14.31	15.10	10.77
A (°)	10.67	10.87	8.33	10.22	10.35	10.97	8.48	10.18	11.72
A (º)	69.93	57.32	61.59	70.15	61.14	63.35	55.22	69.42	50.43
B (º)	59.23	45.95	51.04	58.52	50.53	51.55	45.08	59.73	39.79
B (º)	58.52	52.13	55.30	56.49	50.60	51.44	54.93	62.99	44.12
G (º)	48.02	39.09	41.00	47.98	40.14	40.83	44.61	50.55	33.65
G (º)	46.42	46.12	48.94	46.59	50.50	42.88	53.36	49.48	37.83
G' (º)	40.74	35.64	37.83	40.39	40.44	37.56	45.23	40.67	35.26
G' (º)	46.52	45.51	47.14	45.07	45.90	42.59	53.48	47.21	37.54
B' (º)	40.98	34.92	35.45	40.98	35.14	34.06	44.09	40.82	34.93
B' (º)	58.01	52.71	53.06	56.09	50.76	55.78	55.20	58.94	44.79
A' (º)	45.60	35.97	38.29	47.48	39.24	40.22	41.84	48.49	33.36
A' (º)	69.18	58.03	62.91	69.81	61.68	63.84	54.55	68.20	48.05
D (º)	56.55	43.21	49.91	57.58	51.33	51.42	42.86	57.68	37.59
D' (º)	76.71	53.40	62.75	72.66	56.38	64.74	51.94	79.58	51.86
Rel.1	75.26	54.39	62.27	69.60	51.42	59.13	48.22	78.47	50.52
Rel.2	0.96	0.79	0.89	1.01	0.92	0.97	0.72	0.95	1.13
Rel.3	0.81	0.81	0.89	0.86	0.91	0.80	0.87	0.83	0.83
Rel.4	0.82	0.81	0.88	0.85	0.88	0.80	0.87	0.82	0.83
Rel.5	0.72	0.89	0.74	0.70	0.72	0.81	0.83	0.71	0.97
Rel.6	174.87	155.57	165.83	173.23	162.23	157.67	163.50	181.89	132.37
Rel.7	173.71	156.24	163.11	170.97	158.34	162.21	163.23	174.35	130.38
Rel.8	147.99	120.67	129.87	146.88	131.11	129.94	134.93	150.96	108.70
Rel.9	143.13	114.11	123.65	146.04	125.72	125.70	128.79	147.00	105.88

the first right lateral vein, A'= angle between the central vein and the first left lateral vein, a = angle between the central vein and L1d, a' = angle between the central vein and L1g, B = angle between the first and the second right lateral veins, B' = angle between the first and the second left lateral veins, b = angle between the first right lateral vein and L2d, b' = angle between the first left lateral vein and L2g, G = angle between the second right lateral vein and the first secondary vein, G' = angle between the second left lateral vein and the first secondary vein, g = angle between the second right lateral vein and L3d, g' = angle between the second left lateral vein and L3g, D = angle between L5d and the tangent of the right leaf side from the petiolar point, D' = angle between L5g and the tangent of the left leaf side from the petiolar point.

Principal components analysis (PCA) was performed on all data, taking into account the different lengths and angles recorded (Fig. 1) and the results of the above equations using SAS system v 8.1 software (SAS Institute, Cary, NC).

Microsatellite analysis: DNA was extracted from frozen young leaves and pruned wood using the MarterPure^{TM} Plant Leaf DNA Purification Kit (Epicentre Technologies, Madison, Wis.). Extracted DNA was quantified and a working solution of DNA (10 ng·ml^{-1}) was made.The

Table 2

Allele sizes in base pairs of 9 Spanish grapevine cultivars at 6 microsatellite loci; observed heterozygosity and number of different genotypes. Boldface numbers are unique alleles

Variety	Berry color[1]	VVS2		VVMD5		VVMD7		ssrVZAG47		ssrVZAG62		ssrVZAG79	
Caíño Tinto 1	N	130	132	228	234	237	237	157	161	193	193	245	245
Caíño Tinto 2	N	130	132	228	234	237	237	157	161	193	193	245	245
Tinta Femia	N	130	132	228	234	237	237	157	161	193	193	245	245
Caíño Longo	N	140	150	222	222	237	261	157	157	**185**	195	245	245
Caíño Gordo	N	140	150	222	234	**251**	**255**	**155**	165	187	199	249	249
Caíño do Freixo	N	130	**154**	232	**236**	237	**247**	165	165	193	199	249	249
Caíño Bravo	N	132	140	222	228	237	261	157	165	193	195	243	245
Caíño Redondo	N	**136**	150	232	234	237	**241**	**151**	165	187	187	243	245
Caíño Blanco	B	150	**160**	**218**	222	237	261	157	165	195	**203**	245	249
H_0(%)[2]		100		88.88		66.66		77.77		66.66		33.33	
NDG[3]		6		7		5		6		7		4	

[1] N = black; B = white.
[2] H_0 (%) = Observed heterozygosity.
[3] NDG = Number of different genotypes.

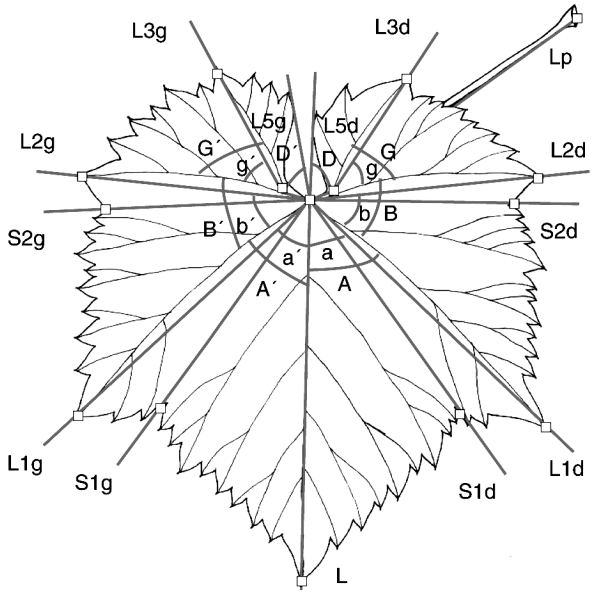

Fig. 1: Mature leaf. Basic length and angle variables measured (Martinez and Grenan 1999).

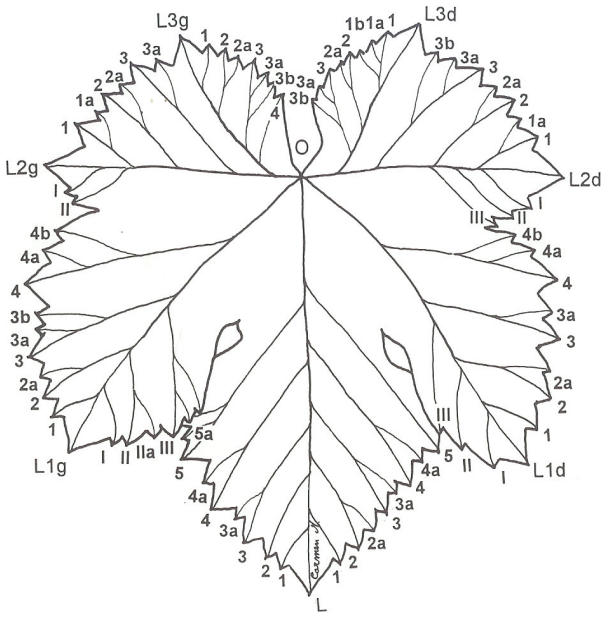

Fig. 2: Mature leaf. Number and type of teeth between the major veins (Martínez and Grenan 1999).

following STMS loci were analysed: VVS2 (Thomas and Scott 1993), VVMD5, VVMD7 (Bowers *et al.* 1996), ssrVrZAG47, ssrVrZAG62 and ssrVrZAG79 (Sefc *et al.* 1999). Due to their high discriminating power these markers were used in the European RESGEN-081 project (http://www.genres.de/vitis/). All experiments were performed in duplicates. One of the primers of each pair was labeled with a Perkin Elmer fluorophore, 6-FAM (blue), TET (green), or HEX (yellow).

Two different multiplex PCR reactions were performed according to Martín *et al.* 1993. Amplified products were separated by capillary electrophoresis using an ABI PRISM model 310 automated DNA sequencer (Perkin Elmer Applied Biosystems). The labeled fragments were detected using GENESCAN software (Perkin Elmer Applied Biosystems).

The results of the microsatellite analysis were expressed as allele sizes (number of base pairs). Allele frequencies were quantified and the observed heterozygosity calculated as the ratio between the heterozygote genotypes and the number of genotypes detected at each locus. UPGMA cluster analysis was used to obtain a dendrogram from the similarity matrix, which in turn was calculated using the simple matching coefficient. NTSYSpc (NTSYS 2000) software was used for these analyses.

Results and Discussion

Tab. 1 shows the means of the quantitative variables measured and the calculations used to reconstruct the 'mean leaf' for each cultivar (Fig. 3). PCA showed the first three components to be responsible for more than 89 % of the total inter-cultivar leaf variation. The variables with the greatest weight in PC1 were angles A, B, G, D and the sums of these angles (Rel. 4, Rel. 5, Rel. 6, Rel. 7). In PC2, the length of the main veins L1 and L2 (positive weighting) and the relationship Rel. 1 (Lp/L) (negative weighting) had the greatest weight. In PC3, the lengths of the petiole (Lp) and vein L3 (positive weighting) and the relationships Rel. 2 and Rel. 3 (negative weighting) had the greatest weight. With respect to PC1, cultivars with the widest angles (Caíño Tinto 1, Tinta Femia and Caíño Tinto 2) are grouped at the extreme right of the axis (Fig. 4). With respect to PC2 (Fig. 4), cultivars with the largest leaves (such as Caíño Longo) were located in the background of the figure; Caíño Blanco, with the smallest leaves, was located in the front of the figure. Finally, with regard to PC3, Caíño Redondo and Caíño Gordo were located in the lowest part of the figure; both have small L3 veins and small petioles and some of the highest Rel 2 and Rel 3 values.

The 6 microsatellite loci analysed were multiallelic (Tab. 2). The most common alleles were VVMD7-237 and ssrVZAG79-245 (frequency >61 %). Eleven alleles (*i.e.* one third of those discovered) were detected only once. The number of different genotypes per loci varied between 4 at locus ssrVZAG79 and 7 at VVMD5 and ssrVZAG62. The observed heterozygosity (H_o) varied between 33.33 % (ssrVZAG79) and 100 % (VVS2).

Caíño Tinto 1, Caíño Tinto 2 and Tinta Femia may be clones, they showed the same allelic profile, as reflected by the single group they formed in the clustering analysis performed with the results of the microsatellite analysis (Fig. 5). Caíño Bravo was situated close to these cultivars, coincided with them in one allele at each locus and there is possibly a parental relationship.

Caíño Longo and Caíño Blanco formed another group. These cultivars coincided fully at locus VVMD7, and in one other allele at each of the other loci. Caíño do Freixo, Caíño Gordo and Caíño Redondo had the smallest number of alleles in common with the others.

Caíño Tinto 1, Caíño Tinto 2 and Tinta Femia coincided not only in their SMTS profiles; PCA analysis also grouped them together showing identical leaf morphology independent of leaf size. The other cultivars showed different allelic

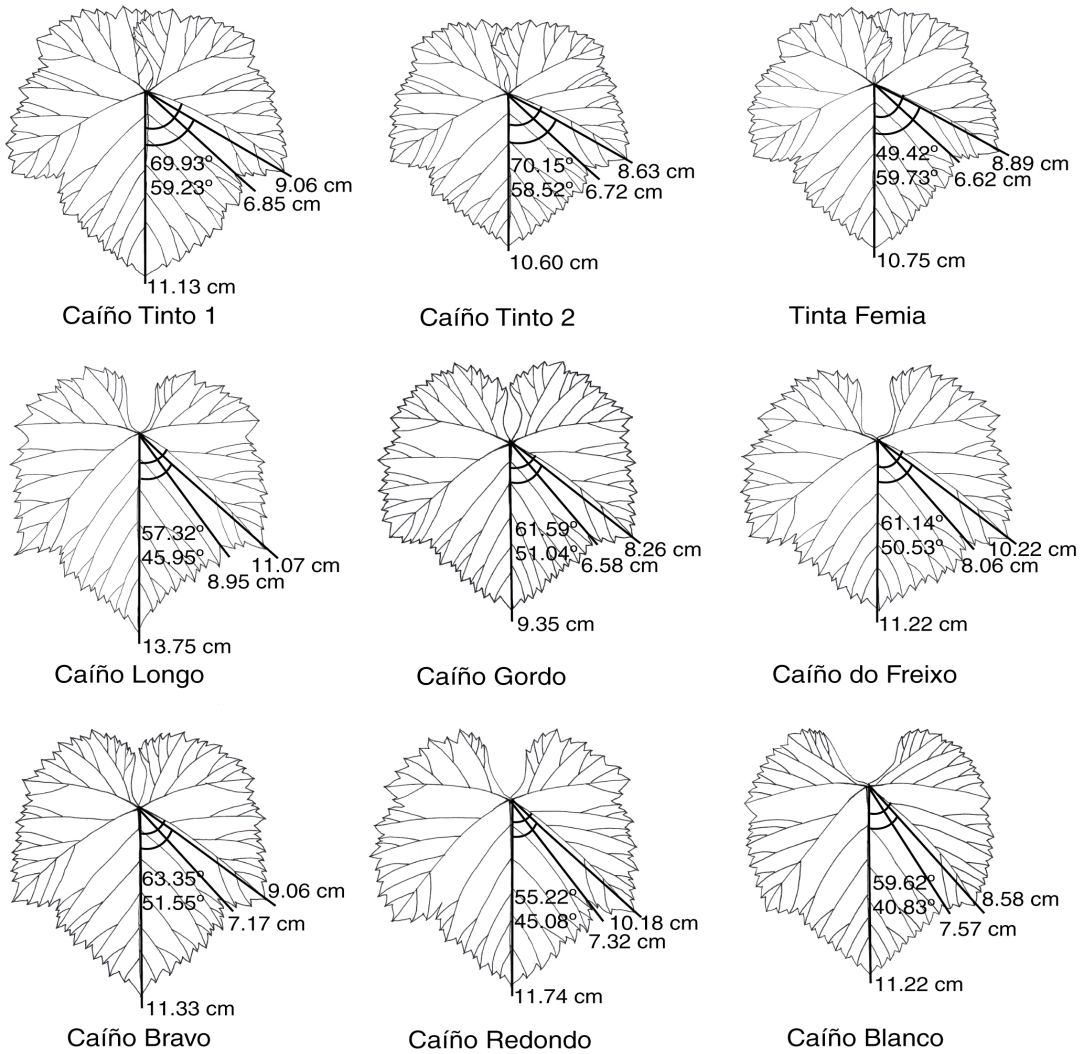

Fig. 3: Spanish grapevine cultivars: 'Constructed mean leaves'.

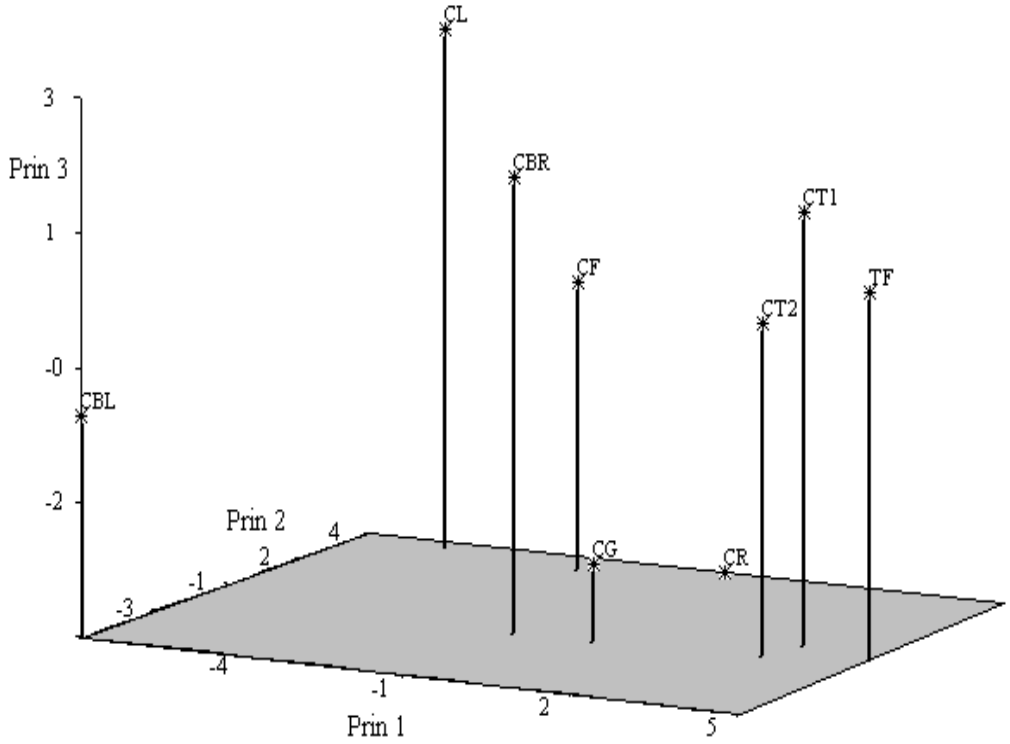

Fig. 4: Projection of 9 grapevine cultivars into the planes defined by the three first principal coordinates. CT1 = Caíño Tinto 1; CL = Caíño Longo; CG = Caíño Gordo; CT2 = Caíño Tinto 2; CF = Caíño do Freixo; CBR = Caíño Bravo; CR = Caíño Redondo; TF = Tinta Femia; CB = Caíño Blanco.

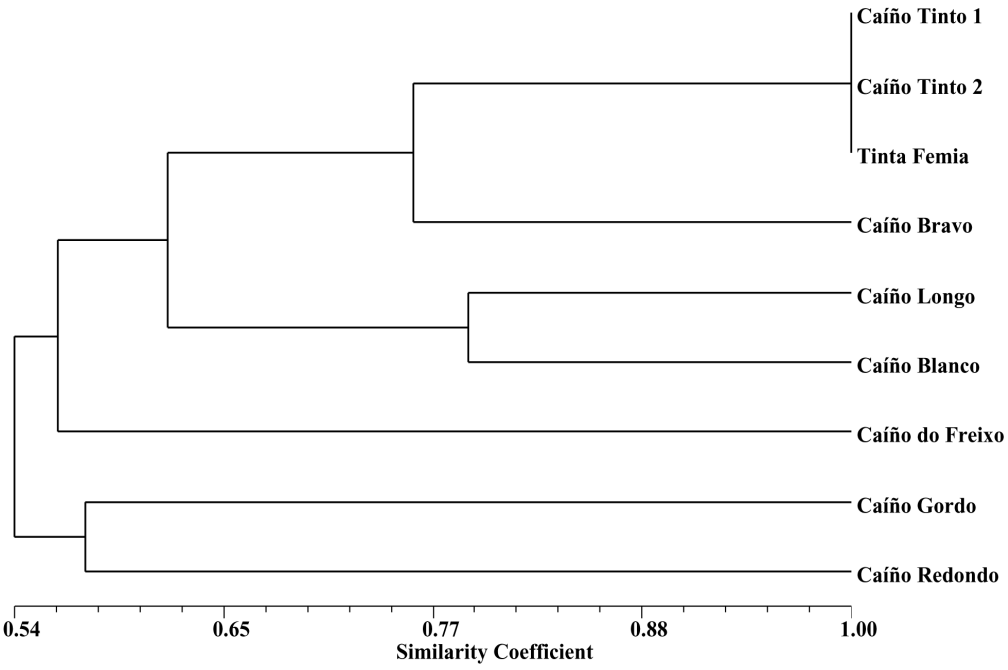

Fig. 5: Dendrogram generated by UPGMA cluster analysis using the similarity matrix obtained from the microsatellite data of 9 Spanish grapevine varieties.

combinations for the 6 microsatellite loci, and their leaves were clearly different from each other. They are therefore different varieties sharing the same name Caíño.

A comparison of the present results with descriptions published by other authors or with information from databases showed the existence of several synonymies. Caíño Tinto (Tinta Femia) and Caíño Bravo are confirmed as synonyms of the Portuguese varieties Borraçal and Amaral (Azal Tinto), respectively (Pinto *et al.* 2003). Further, Caíño Blanco had the same microsatellite profile as Cainho de Moreira from Portugal (BIOVID Proyect http://www.neiker.net/BT/). These synonymies were confirmed by comparing the present ampelographic descriptions with those reported by our group for the above Portuguese cultivars

(SANTIAGO *et al.* 2003) and with those published by other authors (CINCINATO DA COSTA 1900; VIALA and VERMOREL 1900-1910; DA MOTA and DA SILVA 1986).

The results of the present work were also compared to those of MARTÍN *et al.* (2003) who analysed the same microsatellite loci in 176 grapevine cultivars belonging to the collection of El Encín (Alcalá de Henares, Madrid). Caíño Tinto showed the same profile as the variety known as Caíño in that collection. The cultivar named Caíño Longo at El Encín appears to have been incorrectly identified or labeled; in fact it appears to be Caíño Bravo. Caíño Gordo had the same allelic combination as Albarín Negro in the El Encín collection. Caíño Redondo coincided with Espadeiro, and Caíño do Freixo with Ferrón, while Tinta Femia/Caíño Tinto was found to be totally different from the cultivar preserved under the name of Tinta Femia de Aldán at El Encín. A more complete ampelographic comparison with the El Encín varieties is necessary to confirm these possible synonymies.

The present results show that ampelographic descriptions are still necesary to characterise grapevine varieties since they complement laboratory data from molecular analyses.

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