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SSR-based assessment of genetic diversity in South American *Vitis vinifera* varieties

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Abstract

Six SSR loci, previously developed for grapevine, were analyzed to evaluate the genetic variability and cultivar relatedness in a collection of 25 autochthonous *Vitis vinifera* varieties from Perú and Argentina.

The number of alleles per locus ranged from 6 to 13, while the number of microsatellites genotypes varied between 9 and 16. The expected heterozygosity varied between 0.71 and 0.89 and the polymorphism information content ranged from 0.70 to 0.88 indicating that the SSRs were highly informative. It was possible to identify 76 different genotypes, with all accessions showing-at least one-specific combination of alleles. Triallelic loci were observed with some SSR. Sequence analysis revealed that variation in the number of repeats and insertion/deletions (InDels) accounted for the polymorphisms observed. Clustering analysis resulted in four separate groups of varieties sharing at least 75% of the markers. A few cases of synonymies were found within the Peruvian accessions. Varieties were clustered following a general pattern of shared morphological and enological traits, rather than geographical origin.

Keywords: *Vitis vinifera* L.; Grape; Microsatellite; Genetic diversity; Synonyms

1. Introduction

Cultivation of grapevine in America is, historically, a recent event. Although the origins and dispersal of American viticulture is quite unclear -and currently debated—it is believed that *Vitis vinifera* was first introduced by the early Spanish conquerors in the fifteenth century. Initial attempts to cultivate this specie in the Antilles islands and other tropical regions of Central America were unsuccessful due to unfavorable weather conditions [1]. It is apparent that only after 1519, when Spaniards settled in high altitude regions of Mexico, grapevines were successfully grown [2]. Later in the sixteenth century, Perú became an important secondary center for grapevine cultivation and spread to other regions. Whether plants were introduced directly from Spain or from Mexico is unclear. It is speculated that Spaniards would have introduced varieties from the Canary Islands since many trips to South America followed this route, serving the islands as a convenient stop for re-stocking food supplies [3]. Others hypothesize that grapevines were introduced to Perú, most likely in the form of raisin seeds, directly from Spain [4]. These originally-sexually propagated plants grown in a different environment for almost five centuries could explain the little morphological similarities, observed today, between South American and most Spanish cultivars [4]. As a distinctive group, these varieties are locally called ‘‘Criollas’’ [5].

Criollas from Perú were introduced in the North of Chile by Spanish conquerors Diego de Almagro or Pedro de Valdivia [6], and then to Argentina from La Serena, Chile in 1556 [1], although other introductions directly from Perú, cannot be ruled out.

Traditional European varieties for wine-making are nowadays economically important in many viticulture regions of South America. Despite of this, the Criollas still represent a

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high proportion of the grapevine-cultivated surface, especially in Perú and Argentina, and are of importance in local economies. Criollas varieties are used for different purposes: table grapes, raisins, production of unique regional wines (e.g. Torrontés Riojano and Pisco) and for the juice industry. Also of interest is the fact that some Criollas are significantly more tolerant to environmental stresses – namely, drought and salt – than the traditional European varieties [7,8].

It is important therefore to organize, describe and characterize these valuable germplasms in terms of their oenological and agronomical aptitudes, morphology and genetic diversity. The latter is a challenging goal since few records exist regarding the origins of the Criollas grown in different regions. Moreover, variations of the plant phenotypes due to accumulation of mutations throughout 500 years of cultivation, aggravated by the intense traffic of plants among countries, could have given raise to new denominations – synonyms – for identical genotypes. In other cases, varieties sharing a few distinctive morphological traits were called the same—homonyms [9].

A reliable method for Criollas variety characterization would aid the organization and management of germplasm collections by propagating and maintaining only the non-redundant accessions. Morphological descriptions of Argentinean [9,10] and Peruvian Criollas are not complete and lack the comparative analysis of both sets of varieties. Since morphological traits are highly influenced by environmental factors the data obtained from varieties grown in Perú and Argentina are not reliably comparable.

Molecular markers have proved to be a powerful tool for fingerprinting, assessing genetic variation and studying relatedness among cultivars of many species. This is due, in part, because they are not influenced by the environment. Simple sequence repeats (SSR) in particular have become the marker of preference for many grapevine genetic studies including genotyping of varieties and clones [11–14], genetic mapping [15–20], pedrigree reconstruction [21–24] and characterizing the genetic diversity within germplasm collections [25–28].

In this work, we use SSR markers as a tool for characterizing the genetic variation and cultivar relatedness in a collection comprising the most important Criollas varieties cultivated in Perú and Argentina.

2. Materials and methods

2.1. Plant material

Sixteen Peruvian and nine Argentinean Criollas were included in this study. The formers were collected from viticulture regions of Perú, whereas the latter were obtained from the grapevine germplasm collection of E.E.A. Luján de Cuyo INTA, Mendoza, Argentina. Names, sources and distinctive features of the varieties are shown in Table 1.

2.2. DNA extraction and PCR amplification

For each variety young leaves from four individual vines were independently collected. Genomic DNA was extracted according to the procedure described by Bowers et al. [29]. Two replicates of DNA extraction from each variety were done.

Table 1
Denomination, provenance, berry color and use of 25 Criollas varieties

Variety	Provenance	Berry color	Use[a]
Negra Corriente Tacna	Tacna Valley, Perú	Red-black	P, W
Negra Corriente Majes	Majes Valley, Perú	Red-black	P, W
Negra Corriente ICA	ICA Valley, Perú	Red-black	P, W
Negra Cantarita	Majes Valley, Perú	Red-black	P, W
Italia Moquegua	Moquegua Valley, Perú	Green yellow	P, T, R
Italia Majes	Majes Valley, Perú	Green yellow	P, W, R
Italia Tacna	Tacna Valley, Perú	Green yellow	P, T, R
Quebranta	ICA Valley, Perú	Red	P, W, T
Burdeos Vitor	Vitor Valley, Perú	Red-black	W
Burdeos Tacna	Tacna Valley, Perú	Red-black	W
Moscatel Perú	Majes Valley, Perú	Red-brown	P, W
Uvina	Lima, Perú	Red-black	P, W
Borgoña	Majes Valley, Perú	Red-black	W
Mollar ICA	Mollar Valley, Perú	Red	P
Mollar Majes	Majes Valley, Perú	Red	P
Rosada Vitor	Vitor Valley, Perú	Pink	P
Criolla Chica	Luján, Mendoza, Argentina	Red	W, J
Criolla Grande	Luján, Mendoza, Argentina	Red	W, J
Cereza	Luján, Mendoza, Argentina	Red	W, J
Torrontés Riojano	Luján, Mendoza, Argentina	Yellow	W, J
Torrontés Mendocino	Luján, Mendoza, Argentina	Yellow	W, J
Torrontés Sanjuanino	Luján, Mendoza, Argentina	Yellow	W, J, T
Moscatel Amarillo	Luján, Mendoza, Argentina	Yellow	W, J
Moscatel Rosado	Luján, Mendoza, Argentina	Pink	W, T, R
Pedro Giménez	Luján, Mendoza, Argentina	Yellow	W, J

[a] Pisco (P), Wine grape (W), Table Grape (T), Raisin (R), Grape juice (J).

DNA was quantified either by visual comparison with lambda DNA molecular marker on ethidium bromide stained agarose gels or by spectrophotometry using a Pharmacia Gene Quant Spectrophotometer (Pharmacia, Biotech, Columbus, OH).

PCR reactions were carried out in a final volume of 20 μl, containing 50 ng of genomic DNA, 1.5 units of *Taq* polymerase (Invitrogen), 1 × *Taq* polymerase buffer (Invitrogen), 2 mM $MgCl_2$ (Invitrogen), 0.2 mM of each dNTP (Invitrogen) and 200 μM of each primer. Amplification reactions were carried out on a PTC-100™ (MJ Research Inc.) thermal cycler using the following cycling profile: 94 °C for 2 min followed by 40 cycles at 92 °C for 30 s, 52–56 °C for 1 min, and 72 °C for 2 min, and a final extension step at 72 °C for 7 min.

Six grapevine SSR markers fully characterized in previous studies were used: VVMD5; VVMD7 [30], VVMD31; VVMD32 [31], VrZAG62 and VrZAG79 [32]. These set of microsatellites are highly polymorphic and have being considered the most suitable ones for assessing variation among European grapevine collections (European project GENRES #081) (see http://www.genres.de/vitis/).

Aliquots of the amplification products were checked in 2% agarose gel electrophoresis and visualized with ethidium bromide. The remaining PCR products were separated on 6% (w/v) polyacrylamide gels, visualized by silver staining as described by the Promega Silver Staining kit and documented by scanning. Fragment lengths were estimated by comparison with a 100 bp DNA Ladder (Promega) and SSR alleles for cv. Malbec of known fragment sizes, ran in adjacent lanes in the same gel [33].

2.3. Cloning and sequencing of SSR regions

Alleles from different loci exhibiting complex patterns were sequenced. For this, microsatellites bands were excised from the polyacrylamide gels, eluted in water and re-amplified using the corresponding SSR primer combinations. PCR products were resolved by agarose gel electrophoresis, stained with ethidium bromide, excised from the agarose gel and purified using the QIAquick Gel Extraction Kit (QIAGEN, Valencia, CA, USA). These DNA fragments were cloned into pGEM-T Easy vector system (Promega) and recombinant vectors were transformed into JM109 competent cells (Promega). Plasmids were extracted using the Wizard Plus Miniprep DNA Purification system (Promega) and used as template for the sequencing reactions using the vector universal T7 or SP6 primers. The PCR conditions for sequencing reactions were as recommended by Applied Biosystems and used a fluorescent dye terminator. DNA was sequenced with an ABI Prism 377XL Automated DNA sequencer (Applied Biosystem Foster City, CA). The regions corresponding to the cloning vector were deleted using the Chromas Version 2.3 software (Technelysium) for editing sequences. The sequences from each microsatellite locus were aligned separately using the BioEdit program [34].

2.4. Data analysis

Various genetic parameters for 26 varieties over six SSR loci were calculated. For each locus, genotypes showing one and two bands were scored as homozygous and heterozygous, respectively. Alleles and genotypes frequencies were calculated. Observed heterozygosity (H_o) was calculated as the ratio between the number of heterozygous individuals and the total number of genotypes per locus. Expected heterozygosity (H_e) was estimated according to the formula $H_e = 1 - \sum p_i^2$, where p_i is the frequency of the ith allele for the studied locus [35]. The polymorphism information content (PIC), was calculated as $1 - \sum p_i^2 - \sum \sum 2 p_i^2 p_j^2$, where p_i equals the frequency of the ith allele and p_j the frequency of the $(i + 1)$th allele [36]. The effective number of alleles (n_e) was obtained according to [37], $(n_e = \sum p_i^2)^{-1}$, and the probability of identity (PI), was calculated using the following formula $\sum p_i^4 - \sum \sum (2 p_i p_j)^2$ [38]. H_e, H_o and PIC were calculated using the software IDENTITY 1.0 program for the analysis of microsatellite data (http://www.boku.ac.at/zag/forsch/MANUAL.rtf). The discrimination power (D) is an estimation of the probability that two randomly sampled accessions could be distinguished by their SSR profiles [39,40]. This parameter was calculated as $D = 1 - C$, where C is the probability of coincidence or, in other words, the probability that two varieties match by chance at one locus ($C = \sum p_i^2$, where p_i being the frequency of different genotypes for a given locus). The Total Probability Identity for all loci combined was calculated.

Similarity matrices, generated according to the Simple Matching coefficient [41] were used to perform cluster analyses by the Unweighted Pair Group Method with Arithmetic Averages (UPGMA) [42] using the software NTSYS-pc [43]. Dendrograms representing the estimated similarities among the Criollas varieties were constructed with the TREE program of NTSYS-pc. Cophenetic values were calculated as a mean for measuring the correlation between the varieties pair-wise similarities and the dendrogram.

3. Results

Twenty-five Peruvian and Argentinean varieties were genotyped at six different microsatellite loci. All the primer pairs used gave satisfactory amplification products and were multiallelic. Fragment lengths for SSR alleles and varieties are shown in Table 2. The total number of different alleles per locus ranged from 6 (in VVMD5) to 13 (in VrZAG79), with a total of 58 alleles considering all loci. In average per locus, 9.67 alleles (n) and 5.95 effective alleles (n_e), were found (Table 3). For all individual loci, allele frequencies were not homogeneous. The three most frequent alleles accounted for 44% (VrZAG62) (185, 187 and 198 bp) to 88% (VVMD5) (228, 232 and 240 bp) of the variation found (Table 4). Overall, the most frequent alleles were VVMD5—228 bp and VVMD7—329 bp (Table 4).

The observed genotypes at each locus and their frequencies are shown in Table 5. Samples in which only a single allele per locus was detected were considered homozygous instead of heterozygous with a null allele. The number of different microsatellite genotypes ranged from 9 (in VVMD5) to 16 (in VrZAG79) with an average of 12.6 and a total of 76. In general, the distribution of frequencies for genotypes was more uniform

Table 2
Allele size, in base pairs, of six microsatellites loci from 25 grape varieties

Variety	VVMD 31	VrZAG 62	VVMD 5	VrZAG 79	VVMD 7	VVMD 32
Negra Corriente Tacna	210:210	197:198	228:240	242:250	239:249	260:260
Negra Corriente Majes	210:210	197:198	228:240	242:250	239:249	260:260
Negra Corriente ICA	210:210	197:198	228:240	242:250	239:249	260:260
Negra Cantarita	210:213	187:200	228:240	248:252	239:248	260:260
Italia Monquegua	213:218	185:203	228:232	250:258	249:249	265:277
Italia Majes	205:213:218	185:203	228:232:238	240:250:258	239:249	265:277
Italia Tacna	213:218	185:203	228:232	250:258	249:249	265:277
Quebranta	210:212	189:197	228:240	243:250	239:249	260:277
Criolla Chica	210:213	185:189:198	228:240	252:258	239:249	260:277
Criolla Grande	213:215	185:203	232:232	250:259	234:249	265:265
Cereza	212:218	198:206	228:232	248:258	207:232:249	262:277
Burdeos	210:212	187:202	228:238	246:261	239:263	241:253
Burdeos Tacna	210:212	187:202	232:240	257:259	NA	260:277
Moscatel Perú	212:212	NA	232:232	240:250	239:248	260:260
Uvina	206:206	185:200	228:244	246:250	239:239	253:253
Borgoña	206:206	185:200	228:244	246:250	239:239	253:253
Mollar ICA	212:220	188:199	222:228:240	246:261	239:239	253:281
Mollar Majes	212:220	188:198:203	228:240	246:257	251:251	259:281
Torrontés Sanjuanino	210:210	198:199	228:240	246:257	239:251	259:259
Torrontés Riojano	212:215	187:198	228:232	257:259	251:251	260:270
Torrontés Mendocino	212:215	187:198	228:232	246:257	251:251	241:281
Moscatel Amarillo	212:222	189:205	228:232	246:257	239:251	241:281
Moscatel Rosado	212:222	189:205	228:232	246:259	239:251	281:281
Pedro Gimenez	213:215	187:198	232:240	246:257	251:251	260:281
Malbec	210:212	189:203	228:238	245:259	239:263	241:253
Rosado Vitor	212:217	197:205	232:238	246:260	239:251	253:260

NA: no amplification was obtained.

than the observed for alleles (Tables 4 and 5). For VVMD5, however, two genotypes (BC and BE) accounted for more than 60% of the variation (Table 5).

For each locus, the number of unique genotypes varied from 3 (VVMD5) to 10 (VrZAG79) (indicated as genotypes with frequency = 0.038, Table 5). Rosada Vitor and Cereza presented unique microsatellite genotypes for five and four loci, respectively.

All the accessions showed at least one variety-specific combination of alleles (genotype) that allowed accurate fingerprinting of the varieties. Thus, by using six primer combinations it was possible to discriminate among the 25 Criollas tested. This is also supported by the extremely low value obtained for the Total Probability Identity (9.66×10^{-7}), which indicates the probability of miss-identifying a variety of this sample set when using all six SSR markers.

The expected heterozygosity varied within a narrow range between 0.71 (VVMD5) and 0.89 (VrZAG62) with an average of 0.81 (Table 3) over the six loci. The observed heterozygosity was 1.0 for VrZAG62 and VrZAG79 and 0.92 for VVMD5, indicating a high level of genetic diversity within the Criollas for these loci. The mean observed heterozygosity was slightly higher (0.818) than the expected (0.81) by a random union of gametes.

Calculated PIC values ranged from 0.70 to 0.88 and classified the six loci as highly informative markers (PIC > 0.7) (Table 3). Their values were always equal or lower than the corresponding expected heterozygosity values. Also, n_e was positively correlated with PIC. Four of the six microsatellites were very informative, with a probability of identification equal or below 0.10 (VVMD5, VVMD32, VrZAG62 and VrZAG79).

Table 3
Expected (H_e) and observed heterozyygosity (H_o), number of alleles (n), number of effective alleles (n_e), polymorphism information content (PIC), probability of identity (PI), probability of coincidence (C) and discrimination power (D) of six SSR loci used in 25 Criollas varieties

Primer	H_e	H_o	n	n_e	PIC	PI	C	$D = 1 - C$
VVMD 31	0.818	0.731	10	5.496	0.785	0.100	0.101	0.899
VrZAG 62	0.891	1.000	12	9.191	0.882	0.041	0.086	0.914
VVMD 5	0.712	0.923	6	3.467	0.705	0.228	0.216	0.784
VrZAG 79	0.867	1.000	13	7.511	0.867	0.057	0.089	0.911
VVMD 7	0.730	0.640	8	3.698	0.724	0.199	0.146	0.854
VVMD 32	0.826	0.615	9	6.385	0.843	0.090	0.095	0.905
Average	0.807	0.818	9.67	5.958	0.801	0.119		

Table 4
Fragment length (in base pairs) and allele frequencies (*F*) for 25 varieties and six microsatellites loci

VVMD 31		VrZAG 62		VVMD 5		VrZAG 79		VVMD 7		VVMD 32	
G	*F*	G	*F*	G	*F*	G	*F*	G	*F*	G	*F*
AEH*	0.038	AD	0.040	ABE	0.038	AGJ	0.038	ABF	0.040	AB	0.077
BB	0.077	AH	0.080	BC	0.308	AG	0.038	CF	0.040	AI	0.077
CC	0.154	AJ	0.160	BCD	0.038	BG	0.115	DD	0.120	BB	0.077
CD	0.154	BF	0.120	BD	0.077	CG	0.038	DE	0.080	BD	0.038
CE	0.077	BH	0.040	BE	0.308	DK	0.038	DF	0.240	BI	0.038
DF	0.077	BI	0.080	BF	0.077	EG	0.077	DG	0.120	CI	0.038
DG	0.038	CFJ	0.040	CC	0.077	EI	0.192	DH	0.080	CC	0.038
DH	0.038	CG	0.040	CD	0.038	EK	0.038	FF	0.080	DD	0.192
DI	0.115	CK	0.080	CE	0.077	EM	0.077	GG	0.200	DG	0.038
DJ	0.077	DE	0.040			EL	0.038			DH	0.115
EF	0.077	DJ	0.040			FH	0.038			DI	0.038
EH	0.077	EF	0.120			FJ	0.038			EH	0.038
		EK	0.040			GJ	0.077			FF	0.038
		FG	0.040			GK	0.038			FH	0.115
		FL	0.040			HJ	0.038			II	0.038
						IK	0.077				

* Individual letters are arbitrary codes given to the SSR alleles, as presented in Table 4.

3.1. Tri-allelic loci

Five primer pairs generated more than two alleles in five varieties (Italia Majes, Criolla Chica, Cereza, Mollar ICA and Mollar Majes) (Table 2). To confirm the occurrence of a third allele, PCR products for these loci were sequenced and compared. Fig. 1 shows the sequence alignment of Italia Majes alleles for locus VrZAG79. The highly conserved sequences flanking the microsatellite motifs, observed for the three SSR bands, indicate that they are allelic. Variation of the number of microsatellite repeats and Insertion/Deletions (InDels) account for the fragments size polymorphisms observed in polyacrylamide gels. For example, the three-allelic locus VrZAG79 of Italia Majes, showed 8, 12 and 16 repeats of a GA motif, whereas the shortest one also lacks four bases immediately after the 5′ end of the microsatellite (Fig. 1).

A few single nucleotide polymorphisms (SNPs) were observed, mostly within the microsatellite repeats, in some loci. The third allele of Italia Majes/VVMD5 (238 bp) showed four SNPs disrupting a perfect $(CT)_{17}$ repeat (data not shown).

3.2. Genetic relatedness

Pair wise genetic similarities, calculated using the Simple Matching coefficient, demonstrated similarities from 0.64 to 1.00 among the grape varieties analyzed (data not presented). The UPGMA dendrogram revealed four groups by clustering varieties with more than 75% similarity (Fig. 2). Groups A, B, C and D clustered 7, 6, 5 and 8 varieties, respectively. Putative synonymous varieties were found in three clusters. The morphologically similar varieties Negra Corriente Tacna, Negra Corriente Majes and Negra Corriente ICA, cultivated in different Peruvian valleys, shared 100% of the markers, suggesting that they are the same variety. The same appears to be the case for Italia Moquegua and Italia Tacna (group B) and Uvina and Borgoña (group C).

Table 5
Genotypes (G) and genotypes frequencies (*F*) for six SSR loci tested in 25 grape varieties from Perú and Argentina

Letter code*	VVMD 31		VrZAG 62		VVMD 5		VrZAG 79		VVMD 7		VVMD 32	
	Length	*F*	Length	*F*	Length	*F*	Length	*F*	Length	*F*	Length	*F*
A	205	0.019	185	0.140	222	0.019	240	0.038	207	0.020	241	0.077
B	206	0.077	187	0.120	228	0.404	242	0.058	232	0.020	253	0.157
C	210	0.269	188	0.040	232	0.288	243	0.019	234	0.020	259	0.058
D	212	0.269	189	0.100	238	0.058	245	0.019	239	0.400	260	0.308
E	213	0.135	197	0.100	240	0.192	246	0.212	248	0.040	262	0.019
F	215	0.077	198	0.180	244	0.038	248	0.038	249	0.220	265	0.096
G	217	0.019	199	0.040			250	0.212	251	0.240	270	0.019
H	218	0.058	200	0.060			252	0.038	263	0.040	277	0.135
I	220	0.038	202	0.040			257	0.135			281	0.135
J	222	0.038	203	0.100			258	0.077				
K			205	0.060			259	0.096				
L			206	0.020			260	0.019				
M							261	0.038				

* Letters are arbitrary codes used to designate different alleles for six SSR loci.

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allele 1 (240 bp)  AGATTGTGGAGGAGGGAACAAACCGAAAATAGGGGAGAGAAGAGGAGACAATATCTCGCTTATAGTAGTAGTAGGTTGCAGAGCAAGAAGGATGGTGAGGAAAACAATAACATTAGCCG
allele 2 (250 bp)  AGATTGTGGAGGAGGGAACAAACCGAAAATAGGGGAGAGAAGAGGAGACAATATCTCGCTTATAGTAGTAGTAGGTTGCAGAGCAAGAAGGATGGTGAGGAAAACAATAACATTAGCCG
allele 3 (258 bp)  AGATTGTGGAGGAGGGAACAAACCGAAAATAGGGGAGAGAAGAGGAGACAATATCTCGCTTATAGTAGTAGTAGGTTGCAGGGCAAGAAGGATGGTGAGGAAAACAATAACATTAGCCG
                   ********************************************************************************* *************************************

allele 1           AAGCCATCTCTGTTCTCAAGCAGAATGG--------------------GAGAGAGAGAGAGAGATAAAGGTGGTGAGGTGCTTGTGTTTCTTGATTTAATAGTGACCTTTGGAAGGGAG
allele 2           AAGCCATCTCTGTTCTCAAGCAGAATGGAAGTGAGAGAGA--------GAGAGAGAGAGAGAGATACAGGTGGTGAGGTGCTTGTGTTTCTTGATTTAATAGTGACCTTTGGAAGGGAG
allele 3           AAGCCATCTCTGTTCTCAAGCAGAATGGAAGTGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGATAAAGGTGGTGAGGTGCTTGTGTTTCTTGATTTAATAGTGACCTTTGGAAGGGAG
                   ****************************                    ****************** ****************************************************

allele 1           TTTGAAAATGGGGGCA
allele 2           TTTGAAAATGGGGGCA
allele 3           TTTGAAAATGGGGGCA
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Fig. 1. Sequence alignment of Italia Majes alleles for locus VrZAG79. The microsatellite repeats are presented in bold letters.

Comparison of cophenetic values, obtained from de UPGMA cluster analysis, with Simple Matching's similarity matrix demonstrated a correlation of 0.81 indicating that data in the similarity matrix was well represented by the dendrogram.

4. Discussion

In this work, we report, for the first time, on the application of SSR markers for assessing genetic diversity in Peruvian and Argentinean grape varieties. The level of polymorphism found for these materials at six SSR loci is comparable to that reported for other *V. vinifera* germplasms assessed with microsatellites. Working with Spanish varieties, 9–13 and 4–16 alleles per locus were reported by Martín et al. [14] and Ibañez et al. [27], respectively. Fatahi et al. [13] obtained similar results (4–16 alleles per locus) with an average of 11.4, in an Iranian grapevine collection comprising 62 varieties. The larger set of samples used in the latter study could account for the higher polymorphism observed. However, Bowers et al. [30] working with 77 European wine and table grapes and four SSR loci, reported lower polymorphism (5–11 alleles per locus with a mean of 7.5) than the obtained in the present work.

Others polymorphism indexes calculated (Table 3) are in accordance with a diploid highly heterozygous species. The percentage of heterozygotes (mean for all loci) found in our study (81.8%) was lower than the one reported by Bowers et al. [30] (85.5%) but higher than those obtained by Fatahi et al. (76%) [13], Ibañez et al. (70.7%) [27] and Sefc et al. (75.4%) [44]. Comparable lower heterozygocity results were obtained by Lópes et al. [45], Lefort and Roubelakis-Angelakis [46] and Maletic et al. [47], working with Portuguese, Greek and Croatian grapevine collections, respectively.

The SSR markers used had been developed previously and tested on several grape germplasms [13,14,27,30,44,45,48,49]. Although many authors [14,44,45] reported that the highest information content was provided by VVMD5 locus, our results indicate the contrary. Tested on Peruvian and Argentinean varieties, this locus showed the lowest number of alleles/locus, PIC value and discrimination power (*D*) (Table 3). Ibañez et al. [27], found 16 alleles with locus VVS5, but undermined the usefulness of this locus due to the presence of null alleles, the existence of varieties that do not amplify any fragment and the low number of heterozygotes observed. In our case, the most informative primer was VrZAG62, with 12 alleles, showing the highest PIC and *D* values. According to Martín et al. [14], the PIC and effective number of alleles are estimators of usefulness of SSRs for cultivar distinction [49,50].

4.1. Tri-allelic loci

The occurrence of a third allele in some variety-SSR combinations was unexpected for heterozygous loci in a diploid species. The sequencing of the three microsatellite alleles at three loci confirmed their simultaneous presence in DNA from leaf tissues. Triallelic SSR loci have been previously found in grapevine [51–53] and other crops [54,55]. Hocquigny et al. [53] demonstrated that the origin of a third allele in SSR loci of

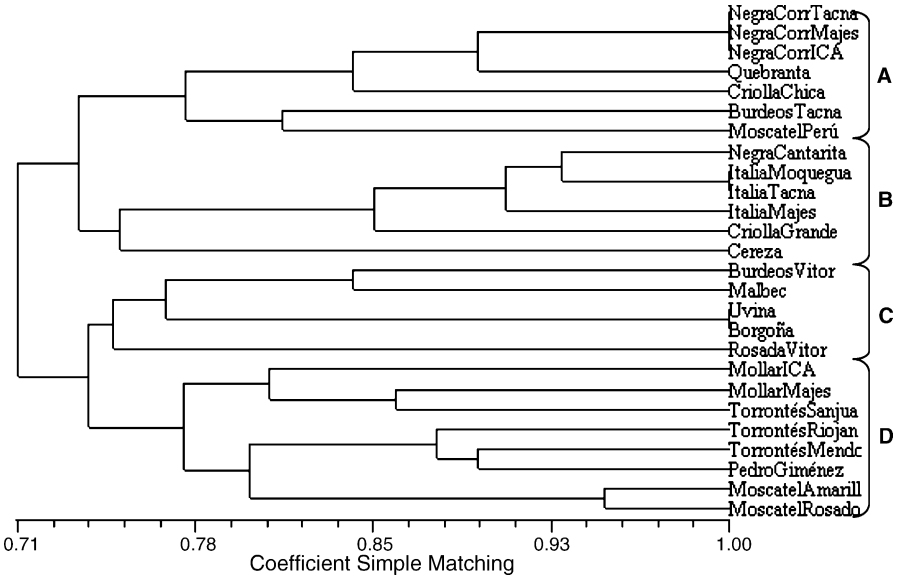

Fig. 2. Dendrogram of 25 Peruvian and Argentinean grape varieties constructed by Unweighted Pair Group Method with Arithmetic Averaging cluster analysis based on Simple Matching's similarity coefficient of shared SSR polymorphisms. Capital letters indicate groups discriminated at 75% similarity.

'Pinot gris' cultivar was the presence of a periclinal chimera meristem structure, in which genetically different cells layers, namely L1 and L2, coexist. Somatic mutations can accumulate in the L1 cell layer and thus form a stable genetic mosaicism in the meristem. Since leaf derives from the two cell layers L1 and L2, DNA extracted from this tissue will carry both sets of alleles. So far, the size variation of the alleles reported for tri-allelic SSR loci was strictly related to the differences in the number of repeats, but not to any other type of mutation. This is coherent with the observation that DNA polymerase slippage during replication seems to play a major role in the generation of mutations in microsatellites [56]. The finding that one of the three alleles at locus VrZAG79 of Italia Majes varies in the number of repeats and also lacks four bases immediately after the 5′ end of the microsatellite (Fig. 1) suggest that other types of mutations must have occurred. *Allele1* (240 pb) could have aroused from *allele2* (250 pb) (the closest reference allele) by suffering a deletion carrying part of the GA repeats and the 5′-contiguous four base pairs. Alternatively, polymerase slippage (accounting for variations in the number of repeats) and the 4 bp deletion could have occurred, independently. The third allele: 205 pb (motiv: $(CA)_8(TA)_6$) showed by locus VVMD31 seemed to be derived from *allele1*: 218 pb (SSR motiv: $(CA)_{11}(TA)_8$) by two independent slippage events, giving shorter repeat motifs. However, this is less likely since it implies two or more independent mutation events.

4.2. Genetic relatedness

Our results demonstrate that SSR analysis effectively and efficiently provided quantitative estimates of genetic similarities related to the distribution of variability among 25 Peruvian and Argentinean grape varieties. This is supported – considering the Argentine subset of samples – by Martinez et al. [5], who reported similar results using AFLP markers in a collection of Argentinean grape cultivars. A phenogram representing varieties relatedness, constructed based on the analysis of six SSR loci, showed four major clusters separated at 75% similarity. No clear separation according to their geographical origins was observed since, except for group C, the rest of the clusters included varieties from both countries; but rather followed a general pattern of shared morphological and oenological traits.

Group A clustered Criolla Chica and five varieties from Perú. All the 'Negra corriente' varieties displayed identical genotypes, and should be regarded as belonging to the same variety. Quebranta and Criolla Chica are closely related to the 'Negra corriente' group, sharing 90 and 84% of the alleles, respectively. It is believed that Negra Corriente was one of the first grapevines introduced in Perú, and that Quebranta would have derived from it (Zúñiga, personal communication). The obtained dendrogram supports this hypothesis. Both varieties are morphologically similar, show remarkable tolerance to phylloxera and other diseases and they are used for making a characteristic Peruvian beverage called ''Pisco''. Hidalgo [4] reported that Criolla Chica and Quebranta are synonyms. Our results demonstrate that, although related (84% similar), they are not the same variety. They displayed different genotypes for three (50%) SSR loci (Table 2).

Cluster B included the 'Italia' group of cultivars grown in the Peruvian valleys of Tacna, Majes and Moquegua. It has been speculated that Italia is the synonym of Muscat of Alexandria, because they show similar ampelographic features. Italia varieties are the most appreciated grapes used for Pisco. Italia Moquegua and Italia Tacna showed the same genotype for all SSR loci, and were very closely related to Italia Majes (93% similarity). In the same cluster, the Argentinean Criolla Grande and Cereza are also related. The two varieties together account for more than 50% of the surface cultivated with Criollas in Argentina. They have similar ampelography, posse high vigor

and yield and are tolerant to drought and salinity [8]. In agreement, Martínez et al. [5] observed for these two varieties 73 and 83% genetic similarity, using morphological and AFLP data, respectively. Based on its morphology, the inclusion of Negra Cantarita in this cluster was unexpected and could not be solved.

Group C comprised the European variety Malbec and a group of varieties of which very little is known regarding their origins. It is speculated that Burdeos could be a late introduction from France (Zúñiga, personal communication). The SSR profile of Uvina was identical to that of Borgoña in all six microsatellite analyzed. Although they show high morphological similarity at shoot, leaf and bunch level, Uvina presents red colored flesh berry, while Borgoña does not. The lack of differences between these two varieties detected by SSR could probably be because the possible somatic mutations responsible for the flesh berry color is located outside the microsatellite regions analyzed. If Burdeos was introduced from France, as hypothesized, one can speculate that it is possible that Uvina and Borgoña have the same origin, and thus, be more related to Malbec and Burdeos than to the Criollas. A broader study, including several French and Criollas varieties, would be needed to test this hypothesis.

Group D included the Peruvian cultivars 'Mollar ICA' and 'Mollar Majes' and the most aromatic varieties grown in Argentina (Torrontés Sanjuanino, Torrontés Mendocino, Torrontés Riojano, Pedro Giménez, Moscatel Amarillo, and Moscatel Rosado). The 'Torrontés' group is appreciated for giving rise to regional dry wines with a Muscat taste, especially Torrontés Riojano [23]. The rest of them show relatively low oenological quality, only appropriate for table wines. Based on the berry similarities it was expected that Moscatel Perú (in cluster A) would have grouped together with Moscatel Amarillo and Moscatel Rosado. Our results indicate that 'Moscatel' cultivars from Perú and Argentina are not closely related. Thus, the Muscat flavor trait in 'Moscatel Perú' could have aroused independently and thus reflects convergence rather a common origin. Mollar ICA and Mollar Majes are slightly aromatic and share few ampelographic traits with the rest of the varieties from this cluster.

In this work, we have characterized for the first time the genetic variability and established varieties relatedness for the most complete collection of autochthonous Peruvian and Argentinean Criollas accessions using SSR data.

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