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Highly variable AFLP and S-SAP markers for the identification of 'Malbec' and 'Syrah' clones

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Summary

The retrotransposon-based sequence-specific amplification polymorphism (S-SAP) and the amplified fragment length polymorphism (AFLP) marker systems were used to assess the clonal variation of 14 'Syrah' and 22 'Malbec' (*Vitis vinifera* L.) clones. The utility of S-SAP markers was compared to that of AFLP markers. On the basis of our results, S-SAP is more informative marker system and showed higher average number of polymorphic bands per cultivar group than AFLP. Relationships among clones were analyzed by cluster analysis using unweighted pair-groups using arithmetic averages and in both cases revealed well defined groups of clones, in which 'Malbec' clones were separated from 'Syrah' clones. High variability of some clones could also be seen within these clusters. The different levels of polymorphism for 'Malbec' and 'Syrah' obtained in this study suggest that 'Malbec' exhibits a higher mutation frequency than 'Syrah'. Our results indicate that higher proportion of polymorphic bands in S-SAP makes it a less labour-intensive and more efficient approach for developing markers for clonal identification.

K e y w o r d s : *Vitis vinifera* L., grape, AFLP, retrotransposons, S-SAP, clonal variation

Introduction

Perennial grapevine genotypes have been clonally propagated for hundreds of years, so they currently exhibit great variability, attributable to different types of mutation. Clonal selection procedures have given rise to various phenotypes, which show differences on agronomical, phenological and oenological levels. For breeding and propagation purposes, tools for distinguishing grapevine clones are highly necessary to ensure maintenance of germplasm clones, allow their identification, certification, tracking and for patenting purposes. There is a lack of knowledge of the genetic constitution and relationships of grapevine clones. Conventional clonal identification based on morphological and phenological traits have been mainly used, but these techniques have reached their limits in distinguishing several clones of a single cultivar, since they are difficult, ambiguous, time consuming, and subjective. Moreover, the results can be blurred by environmental factors, such as climate, pathogens, soil characteristics or plant nutritional status. These markers have been superseded by DNA-based methods, rendering identification possible in a cost effective, reliable and reproducible way, allowing confirmation of the clonal identity of very young plants in nurseries and foundation plantings, thus saving costs in germplasm management. In general, investigations into clonal variation within grapevine cultivars have shown that the degree of detected genetic divergence usually depends on the marker system applied and on the scope and type of plant samples used (Forneck 2005). Although SSRs have been successfully used to identify cultivars, are not powerful tools neither for the detection of clones, for a specific grapevine cultivar (Silvestroni *et al.* 1997, Imazio *et al.* 2002, Rita *et al.* 2002); nor for the detection of somaclonal variation in *V. vinifera* (Schellenbaum *et al.* 2008). AFLP (Amplified Fragment Length Polymorphism) (Vos *et al.* 1995) can screen a large portion of the genome, and are thus a promising marker type for the routine identification of grape somatic mutants and clonal variants (Cervera *et al.* 1998, Scott *et al.* 2000, Blaich *et al.* 2007).

However, additional markers are necessary to serve as individual clone specific markers to be applied in breeding, propagation and tracking processes (Blaich *et al.* 2007). Another marker system based on the presence of retrotransposons (RTs), including Sequence-Specific Amplified Polymorphism (S-SAP) (Waugh *et al.* 1997), is an alternative to and have revealed more polymorphisms than AFLPs (Waugh *et al.* 1997, Bretó *et al.* 2001). RTs are the commonest class of eukaryotic transposon elements, are ubiquitous, widely dispersed, and range from a few copies to more than 10,000 copies per haploid plant genome (Flavell *et al.* 1992, Kubis *et al.* 1998, Böhm and Zyprian 1998, Suoniemi *et al.* 1998). They can be used as a source of informative markers because of their ability to integrate into a multitude of loci in the genome and thereby generate insertional polymorphisms between individuals, thus becoming a powerful tool for genotyping and clone identification (Labra *et al.* 2004). According to Venturi *et al.* (2006), S-SAPs appeared to be the only marker system at the time with the capacity to differentiate clones in apple. RTs have repeatedly been used for studying polymorphisms among

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differents species of *Vitis,* varieties (Moisy *et al*. 2008), and clones, and have revealed promising results. Only a few RTs have been described in grapevine so far, *Tvv-1* (Pelsy and Merdinoglu, 2002), *Vine*-1 (Verriès *et al.* 2000), and *Gret1* (Kobayashi *et al.* 2004) the last two being inserted directly upstream or inside coding sequences of the *Adhr* and *Vvmyb*A1 genes, respectively. This indicates that RTs can alter gene expression, being responsible for phenotypic variation in this species (Verriès *et al.* 2000). *Vine-1* which has been identified in the 'Danuta' grape genome is 2.396 bp long and flanked by a 5-bp duplicated target site (Verriès *et al.* 2000). Its structure has two almost identical LTRs (Long Terminal Repeats, 287 bp) in the same orientation and a single open reading frame of 581 amino acids. Labra *et al.* (2004) successfully used a *Vine-1* RTs based S-SAP technique for *Vitis vinifera* L. genotyping, and it was effective in identifying polymorphism and defining genetic distances not only among cultivars, but also for clonal identification.

Since 1990, Argentinean viticulture has experienced a significant increase in profitability, and their cultivated area has consequently increased, mainly with cultivars with high oenological value, such as 'Malbec' and 'Syrah'. The first variety comes from southwest France, more precisely from the Quercy area and the vineyard of Cahors, where it is known as Cot. In Argentina, 'Malbec' has become the emblematic cultivar, since this cultivar has found the most propitious ecological features for its development, mainly in the province of Mendoza, producing exceptional red wines, which are gaining recognition at the international level. This grape variety is widely planted in Argentina today, covering 22,500 ha (INV 2005), which is two-thirds of the world surface area. 'Syrah', the French Rhone Valley red grape cultivar, now planted all over the world, occupies fourth place on the national level, covering 12,000 ha (INV 2005). This cultivar is also well adapted to the soil and climatic conditions of Argentina, where it is gaining remarkable acceptance, especially in the province of San Juan.

Clonal and sanitary selections for improving the agricultural performance of several grapevine cultivars have been performed by the National Institute of Agriculture and the Faculty of Agriculture of the National University of Cuyo, Argentina, following protocols proposed by the O.I.V. (International Organization of Vine and Wine). After twenty years of selection, 22 'Malbec' and 14 'Syrah' clones with high oenological quality and productivity have been identified. They meet the requirements for recognition as clones.

The goal of this study was to assess clonal variation in 'Malbec' and 'Syrah' cultivars that exhibit different valuable phenotypes, by using AFLP and S-SAP markers. Additionally, the effectiveness of the two techniques in distinguishing clones was evaluated.

Material and Methods

Plant Material: Thirty six *Vitis vinifera* samples, including 14 'Syrah' and 22 'Malbec' clone accessions, kindly provided by the National Institute of Agriculture, were used in the AFLP and S-SAP protocols. DNA was extracted from young expanding leaves of shoot tips of individual field-grown vines using the modified CTAB method described by Kump and Javornik (1996).

AFLP analysis: AFLP reactions were performed as described by Vos *et al.* (1995) using *Pst*I (5'-GACTGCGTACATGCAG-3') and *Mse*I (5'-GATGAGTCCTGAGTAA-3') primers without a selective base in pre-amplification and with two or three selective bases in amplification (Table).

S-SAP analysis: S-SAP reactions were performed as described by Labra *et al.* (2004) using *Eco*RI (5'-GACTGCGTACCAATTC-3') and *Mse*I (5'-GATGAGTCCTGAGTAA-3') primers in pre-amplification. Selective amplification was performed with *Vine*LTR2 primers, fluorescently labelled with Cy5, and *Mse*I+2 or *Mse*I+3 primers (Table). Amplification conditions were the same as for AFLP analysis. AFLP and S-SAP bands were resolved on 6 % polyacrylamide, 7 M urea and 1x TBE gels at 55 °C and visualized by an automated ALFexpress DNA sequencer (GE Healthcare).

Data analysis: Separation of the fragments was done at 1500 V, 60 mA and 15 W for 360 min at 55 °C. Fluorescent signals were collected every 1 s and stored in a computer. A fluorescence labelled molecular marker (Cy5 Sizer 50-500, Amersham Biosciences) comprising 10 fragments in the size range of 50 to 500 pb was used as an external size marker. Allele sizes were determined using the software Allele Locator 1.03 (Amersham Biosciences). Fragments of the same size present in all clone samples were used to correct for the smiling effect of the gels. Only strong markers were scored as binary data, with either with presence (1) or absence (0). Monomorphic markers were excluded from further diversity assessment. All calculations were performed by NTSYSpc 2.1 software (Exeter Software Co., New York). The genetic similarity among clones was calculated using simple matching genetic distance (SM = m/n), where m is the number of matches and n is the total number of variables. Cluster analysis was generated from the similarity matrix by the unweighted pair group method, using an arithmetic averages (UPGMA) algorithm, with the "FIND" option enabled to detect all possible trees. The goodness of fit of the clustering to the data matrix and comparison between S-SAP and AFLP matrices was estimated by the COPH and MXCOPM subprograms of NTSYS, which calculated the cophenetic values and performed matrix comparison analysis, respectively. Variance components within the two grapevine populations and relationships among them were calculated using analysis of molecular variance (AMOVA) with the Arlequin program (Schneider *et al.* 1997). To compare the efficiency of the two methods, the number of total and polymorphic bands and the polymorphic information content (PIC) were calculated for AFLP and S-SAP data. The PIC values were determined over all loci as:

$$\mathrm{PIC} = 1 - \sum (p_1^2 + p_0^2),$$

where p_1 and p_0 represent, respectively, the presence and absence of alleles. Reproducibility of AFLP and SSAP markers was tested by repeated amplification of two select-

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Number of amplified and polymorphic bands, percentage of polymorphism and PIC values indicated for AFLP and S-SAP markers from data of 'Malbec' and 'Syrah' clones

Primer combination	Total bands amplified	Polymorphic bands	Polymorphism (%)		PIC
			Syrah	Malbec	
AFLP					
PstI-ACA+MseI-AG	64	42	34.37	59.38	0.4004
PstI-ACA+MseI-CT	39	23	25.64	57.89	0.3821
PstI-AAC+MseI-AG	63	47	34.92	73.02	0.4728
PstI-AAC+MseI-CT	51	31	25.49	60.78	0.3115
PstI-AGA+MseI-AG	45	25	26.67	55.56	0.3503
PstI-AGA+MseI-CT	46	29	23.91	63.04	0.3508
PstI-AGA+MseI-CTG	27	16	37.04	59.26	0.3992
PstI-AGA+MseI-CAT	17	13	35.29	76.47	0.3961
PstI-ACA+MseI-CTA	59	42	61.02	69.49	0.4976
Mean	45.67	29.78	33.82	63.88	0.3956
S-SAP					
LTR2+MseI-CG	35	27	68.57	77.14	0.4952
LTR2+MseI-AG	49	35	52.63	87.72	0.3572
LTR2+MseI-ACG	57	50	32.14	75.00	0.4847
LTR2+MseI-ACC	28	20	49.18	80.33	0.3844
LTR2+MseI-AGC	61	49	22.45	71.43	0.3968
LTR2+MseI-CTT	46	41	45.65	89.13	0.4751
LTR2+MseI-CTC	20	15	30.00	75.00	0.3633
Mean	42.29	33.86	42.95	79.39	0.4224

ed clones of 'Malbec' and 'Syrah', respectively. All amplified banding profiles were compared and all bands which did not amplify above threshold two times were excluding from the data set.

Results and Discussion

A F L P a n a l y s i s: This study was performed using nine primer combinations of *Pst*I and *Mse*I primers, with two or three selective nucleotides. Altogether 36 clone accessions were included in the analysis, but four 'Malbec' clones (M1, M2, M3, M4) and one 'Syrah' clone (S63) failed to be amplified. Electrophoretic analysis of the amplified products revealed a total of 411 bands, giving 33.82 % and 63.88 % of polymorphic bands in 'Syrah' and 'Malbec' clones, respectively (Table).

The average number of fragments per primer combination was 45.7 and the average number of polymorphic bands across all samples was 29.78 (Table). All tested clones could be easily distinguished with polymorphic fragments. The obtained polymorphism, especially the group of 'Malbec' clones, is much higher than previous reports, but two clones above all, M3 and M5, are the major contribution to polymorphism. Imazio *et al*. (2002) were able to distinguish 16 out of 24 'Traminer' clones by using three primer combinations, yielding 40 polymorphic bands from 117 AFLP total amplicons. The value obtained in the present work was also higher than reported in Fanizza *et al.* (2005), who used 11 'Primitivo' clones and found only 9 polymorphisms among 3,000 AFLP bands analyzed, assessing 50 primer combinations. In addition, Moncada *et al*. (2005) and Moncada and Hinrichsen (2007), working with 46 'Cabernet Sauvignon' and 26 'Carmenere' clones, reported 5.9 % and 2 % polymorphism, respectively.

S - S A P a n a l y s i s: In this study, *Vine*LTR2 was combined with seven different *Mse*I primers, with two or three selective nucleotides. Seven primer combinations gave rise to 296 bands. Using the S-SAP approach, polymorphism was 42.95 % for 'Syrah' and 79.39 % for 'Malbec', which is higher than with the AFLP approach. The average number of fragments per primer combination was 42.3 and the average number of polymorphic bands in the two groups of clones was 33.86% (Table). In the present study, it was confirmed that S-SAP analysis revealed a higher polymorphism level than AFLP. The high proportion of polymorphic bands in S-SAP, occurring from insertional polymorphism, makes it a less labour-intensive and more efficient approach for developing markers. S-SAP, applied to 'Pinot Blanc' and 'Pinot Gris' clones, failed to reveal genetic variation (Labra *et al.* 2004). Verries *et al.* (2000) stated that polymorphism among *Vitis vinifera* cultivars observed using *Vine-1* probes suggested that transposition of this element may have contributed to *Vitis vinifera* genetic variability, but not variability among clones, which is not in agreement with our results. Wegscheider *et al.* (2009) reported a modified S-SAP method using universal primers for retrotransposons, allowing the differentiation of 4 out of 5 'Pinot Noir' clones. Retrotransposon induced mutations were responsible for clonal variation among 'Pinot Noir' clones, resulting in genetic and even phenotypic differences. S-SAP analyses, using primers based on the LTRs of Vine-1, were successful in distinguishing particular clones, such as 'Traminer' clones, but failed to

distinguish 'Pinot' clones, indicating different clonal variability in different cultivars (Imazio *et al.* 2002, Labra *et al.* 2004). Pelsy *et al.* (2003) assessed the discriminative power of S-SAP, relying on the LTRs of grapevine retrotransposons, within 12 *Vitis vinifera* varieties, confirming their efficiency in distinguishing each individual variety.

The results of reproducibility test carried out with AFLP primer combinations appeared to be highly consistent (97,2 %), while the reproducibility of the SSAP markers was lower (82%), which can be due to the high copy number of retrotransposon sequences in winegrape genome and consequently poorer PCR repetitions.

Diversity of AFLP and S-SAP bands: The degree of polymorphism detected by the AFLP approach was compared to that obtained with S-SAP analysis (Table). PIC values were higher for S-SAP (0.42) than AFLP (0.39). Similarity matrices using a simple matching coefficient were obtained for AFLP and S-SAP data, respectively. The resulting dendrograms based on UPGMA clustering are shown in the Figure. Clustering analysis in both cases revealed well defined groups of clones, in which 'Malbec' were clearly separated from 'Syrah' clones. High variability of some clones could also be seen within these clusters, especially with the S-SAP approach, with which 2 subgroups of 'Malbec' could be detected. One of them was grouped more closely to the 'Syrah' clones than to the other 'Malbec' cluster. Discrepancy between molecular marker techniques is probably due to AFLP and S-SAP explore different regions of the genome or they are reflecting different underlying divergences. The correlation coefficient (r) between the AFLP and S-SAP approach was 0.58. Clustering results were confirmed with high co-phenetic correlation for AFLP (r = 0.95) as well as for S-SAP (r = 0.88).

AMOVA analyses of AFLP and S-SAP dissimilarity matrices resulted in highly significant ($P < 0.001$) genetic variance within and between groups. The variance among clone populations accounted for 51.3 % of total variance and the variance within the clone population accounted for 48.7 %.

'Malbec' and 'Syrah' clones displayed a high level of genetic diversity ranging from 0.72 and 0.88 to 0.95, respectively (data not shown). This value was higher than with younger cultivars, such as Carmenere, which origin is not clear, showed 0.90 % genetic similarity (Moncada and Hinrichsen, 2007). A plausible explanation is that 'Malbec' and 'Syrah' are old cultivars, so have accumulated a larger number of somatic mutations. The genotype of a grape cultivar can often be hundreds or even thousands years old, but it is usually impossible to know the age of a cultivar (Vouillamoz and Grando 2006). In their study, these authors revealed that one of the most ancient western European cultivars, 'Pinot', is still in cultivation and had generated several economically important cultivars, among them, 'Syrah'. The last cultivar is the progeny of 'Dureza' and 'Mondeuse Blanche', being 'Pinot' the second and the third relative ancestor of 'Dureza' and 'Syrah', respectively. A recent research discovered the parentage of 'Malbec' which is a progeny of 'Madeleine Noir des Charentes' and 'Prunelard' and half-sibling of 'Merlot' (Boursiquot *et al.* 2009). Because high morphological diversity has been detected, all this information should be considered in clonal selection. The different levels of polymorphism for 'Malbec' and 'Syrah' obtained in this study suggest that 'Malbec' exhibits a higher mutation frequency than 'Syrah', which may be due to different processes, such as chimerism, tissue-specific and time-specific methylation, and stress-related dynamic transposition events (Benjak *et al* 2006). This polymorphism may have arisen in Argentina,

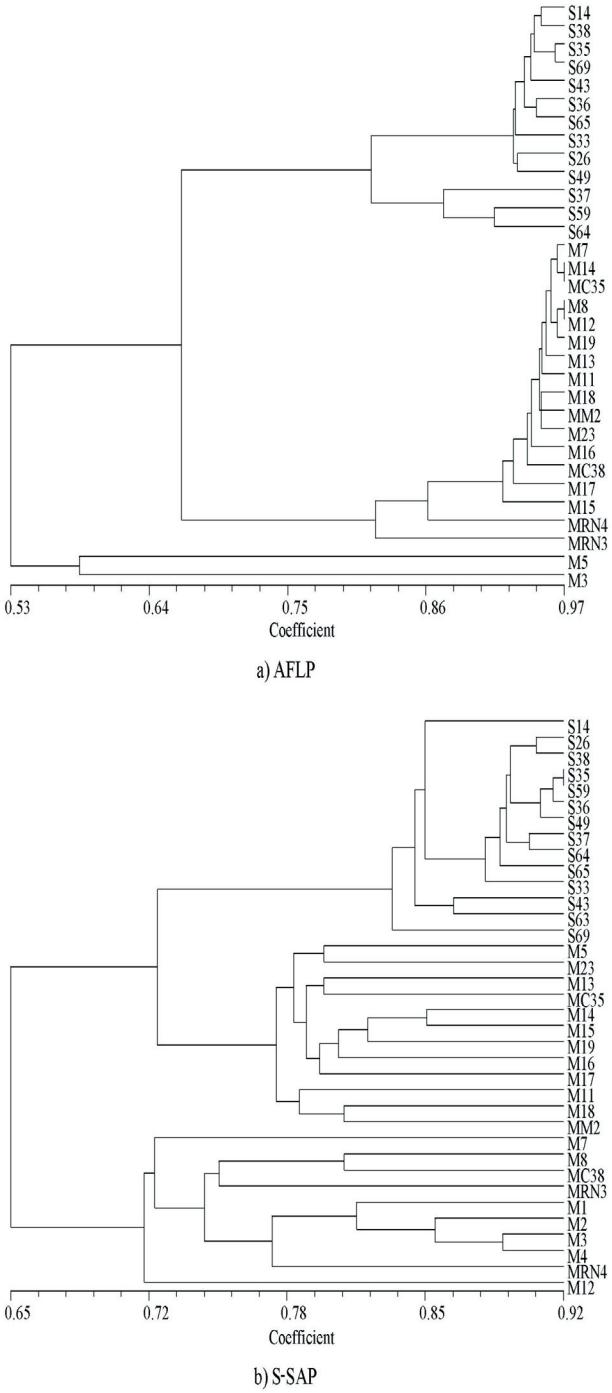

Figure: UPGMA dendrograms based on simple matching coefficient showing the genetic relationships among 'Syrah' and 'Malbec' clones obtained by: **a**) AFLP analysis and **b**) S-SAP analysis.

after many cycles of propagation and intensive cultivation by growers, taking into account that 'Malbec' and 'Syrah' varieties were introduced in this country by the middle of the XIX century (Maurín Navarro 1966). The most variable clones, M3 and M5, highly differed from the French M19, which was used as control. According to agronomical and oenological studies by Scarsi (2002), they also belong to different groups determined by qualitative and quantitative characters analyzed by principal component analysis. 'Malbec' 5 is a less productive clone, with high polyphenol content but it produces high quality wine. This clone is associated with the rest at about 54 % similarity. 'Malbec' 3 is also a low yielding clone and it is not recommended for growing due to difficulties in the accumulation of sugars during ripening and high content of vegetable notes, astringency, bitterness and high acidity.

Conclusions

Our results indicate that the identification and distinction of 'Malbec' and 'Syrah' clones could be greatly improved by using AFLP and retrotransposon-based molecular markers, such as S-SAP. These markers make clone identification feasible, which is highly desirable for breeding, propagation and tracking processes. The high genetic diversity detected probably evolved due to the high level of mutations, which may have take place in Argentina and are responsible for the variation in agronomical and wine qualities. S-SAP markers show greater ability than AFLP to detect DNA polymorphism in inter- and intra-specific variants. Our results indicate that S-SAP may be an excellent source of hyper-variable markers in grapevine. Discordance between different marker systems can be very informative for understanding genetic relationships within the study group. The detection and further sequencing of polymorphic bands, will allow their transformation into a friendlier marker system, such as SCARs. Moreover, the proposed molecular system appears to be a promising marker system for future studies in grapevine.

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