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RESEARCH ARTICLE

Evaluation of diversity among Argentine grapevine (*Vitis vinifera* L.) varieties using morphological data and AFLP markers

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Keywords: ampelography, Criollas, genetic variability, molecular markers.

Abbreviations:
AFLP: Amplified Fragment Length Polymorphism;
NTSYS:Numerical Taxonomy and Multivariate Analysis System;
O.I.V.: Office International du Vin;
PCR: Polymerase Chain Reaction;
RAPD: Random Amplified Polymorphic DNA;
RFLP: Restriction Fragment Length Polymorphism;
SSR: Simple Sequence Repeats.

Half of the Argentine grapevine growing area is cultivated with local varieties generically called

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"Criollas". These materials differ in morphology, physiology and enological aptitudes from traditional European varieties. To discriminate among them, we used morphological markers to evaluate the genetic diversity and phenetic relatedness of 9 Criollas, 6 European and 1 American varieties and compared with AFLP markers. Three AFLP primer combinations generated a total of 111 scorable fragments. Dendrograms obtained with morphologic and AFLP markers agreed in clustering the "Criollas" separately from the European and American varieties assayed, except for Muscat d' Alexandrie and Tempranillo which clustered with Criollas in the case of AFLP. Comparison of UPGMA dendrograms of morphological and AFLP markers using the Mantel test indicated a not significant correlation of r = 0.33. Nevertheless, AFLP and selected morphological characters appear as useful and complementary techniques for grapevine identification and for evaluation of genetic diversity. Among the "Criollas", AFLP similarities ranged from 76 to 98% (Dice coefficient), indicating an important source of genetic diversity that can be exploited in future breeding programs. To our knowledge, this is the first report using AFLP markers to assess genetic variability on these materials.

Argentina is one of the largest grape and wine producing countries in South America. In the last decades its viticulture and enology industries have acquired great importance from an economical point of view. Currently, 45% of the grapevine cultivated area is covered with a group of varieties generically called "Criollas", a term given to American-born individuals descendant from European parents, although the possibility that some of these varieties arrived as seeds cannot be excluded. It is likely, that these varieties were introduced in Argentina soon after the Spaniard conquerors arrived to the New World. Settlers began planting vines as early as 1556, at Santiago del Estero province (Maurín-Navarro, 1967) and later in the foothills of the Andes Mountains, in Mendoza and San Juan provinces. Currently, around 70% of the total area cultivated with "Criollas" is in Mendoza, whereas San Juan accounts for 20% of the surface (INV, 2001).

Due to their rusticity, the "Criollas" have called the attention of local plant physiologists, who have noticed significantly higher tolerance to some environmental stresses when compared with European traditional varieties (Kaiser and Cavagnaro, 2001). These varieties can grow in soils with low water availability and high salt concentration, and still maintain their characteristic high yield and vigour. Characterizing the diversity of local populations would allow a more useful application of these materials in breeding programs.

Some "Criollas" varieties such as Moscatel Amarillo, Criolla Chica, Torrontés Mendocino and Torrontés Riojano, give raise to valuable regional wines. Torrontés Riojano has been internationally recognized for originating a dry wine with a Muscat taste (Agüero et al. 2001). The rest of the "Criollas" shows relatively low enology quality, only appropriate as table wines.

Molecular markers like RFLP (Bowers and Meredith, 1996), RAPD (Gogorcena et al. 1995; Grando et al. 1995), AFLP (Sensi et al. 1996; Cervera et al. 1998) and SSR (Bowers et al. 1996; Sefc et al. 2000) have been used for genetic studies in grapevine. These studies have increased the understanding of the relatedness of cultivars within and among regions. The high level of heterozygosis that present vegetatively propagated grapevines (Reisch, 2000) allows the distinction of the most important cultivars by using almost any molecular marker technique. The recent availability of these molecular markers has facilitated research in *Vitis* genetics (Reisch, 2000). AFLP (Vos et al. 1995) is a PCR-based fingerprinting technique that is particularly useful for this purpose, since it can detect a large number of polymorphism in a single reaction. It presents a good repeatability generating primarily dominant markers which are distributed throughout the genome (Cervera et al. 1998). The goals of this work were to i- evaluate the genetic diversity of Argentine grapevine germplasm using AFLP and morphological data, ii- compare the phenetic relationships obtained by both systems of analysis and iii- compare European with Criollas varieties.

MATERIALS AND METHODS

Plant material

Nine "Criollas" varieties: Cereza, Criolla Grande, Criolla Chica, Pedro Giménez, Moscatel Rosado, Moscatel Amarillo, Torrontés Riojano, Torrontés Sanjuanino and Torrontés Mendocino, 6 European varieties: Chardonnay, Syrah, Cabernet Sauvignon, Malbec, Muscat d´Alexandrie and Tempranillo, and 1 American hybrid rootstock: SO4 (*Vitis berlandieri* x *Vitis riparia*), were assayed. All accessions were taken from the collection vineyard at the INTA Luján de Cuyo and the Agricultural College, Universidad Nacional de Cuyo, Mendoza, Argentina.

Morphological characters analysis

Fifty-three characters (Table 1), analyzed and described by Alcalde, 1989, using "Criollas" varieties, were numerically codified using a qualitative multi-status criterion (from 0 to 8, depending on the variables of each character) (Sneath and Sokal, 1973) and used to design a numbered-data matrix. The corresponding morphological characters of the European varieties, described by O.I.V. were also included in the analysis. Modal values of morphological descriptors from 15 vines per European and Criollas varieties were analyzed in 20 consecutive years.

DNA extraction

For each variety, young leaves from 5 vines, were independently collected and used for DNA isolation as

reported by Bowers et al. 1993. Three replicates of DNA extraction from the same varieties were made. DNA was quantified either by visual comparison with lambda DNA on ethidium bromide stained agarose gels or by spectrophotometry using a Pharmacia Gene Quant Spectrophotometer (Pharmacia, Biotech, Columbus, OH).

AFLP analysis

AFLP reactions were carried out following the instructions supplied with the GIBCO-BRL Life Technologies AFLP™ kit, with minor modifications. 250 ng of genomic DNA was double digested with 1.25 units of each *Eco*RI and *Mse*I, and linked to their respective adapters by using 0.25 unit of T4 DNA ligase. Digested and ligated DNA fragments were used as templates for the first amplification reaction. For the first amplification reaction primers complementary for the adapter nucleotides *Eco*RI and *Mse*I, with selective 3' nucleotide, were used. The reaction products were diluted 3-fold with TE buffer. The second amplifications were performed with a combination of *Eco*RI and *Mse*I primers that had three selective nucleotides each. Primer combination used were the following: *Eco*ACT-*Mse*CTG; *Eco*ACC-*Mse*CTG; *Eco*ACC-*Mse*CAA and *Eco*ACC-*Mse*CTC.

PCR conditions were as follow: the first amplification mixture was prepared in a total volume of 25.5 ml and amplified using 20 cycles of 30 s denaturation at 94ºC, 60 s annealing at 56ºC and 60 s extension at 72ºC. The second amplification was performed in a 20 ml final volume with 13 cycles of 94ºC for 30 s, 65ºC for 30 s with a decrease of -0.7ºC per cycle, and 72ºC for 1 min; followed by 23 cycles at annealing temperature of 56ºC. AFLP reaction products were separated in 6% (w/v) denaturing polyacrylamide in 1X TBE buffer and visualized with silver-staining, using the Promega Silver Staining kit as indicated by the manufacturer.

Morphological qualitative multi-status data were numerically transformed according to Sneath and Sokal 1973, and used to design a data matrix of pair wise similarities between genotypes, by calculating the Simple Matching Coefficient (SMC). AFLPs were scored for presence or absence in each grapevine genotype and used for calculating genetic similarities using the Dice Coefficient (DC) (Sneath and Sokal, 1973). Both matrices were used to obtain the respective phenograms using the algorithm UPGMA (Unweighted Pair Group Method with Arithmetic Averages) (Sokal and Michener, 1958) from the software NTSYS-pc (version 1.80, Rohlf, 1993).

Comparison between both methods was performed for the varieties which morphological and AFLP data were available. The correlation between the two data sets was studied by performing a Mantel test using the software NTSYS-pc.

RESULTS AND DISCUSSION

Morphology analysis

The UPGMA dendrogram obtained using morphological characters clearly separated the "Criollas", European and the American varieties (Figure 1a) (group A, B and C). Members of the European and "Criollas" groups presented more than 40 and 45% similarity, respectively. These two clusters diverged at a similarity index of 37% based on the dendrogram. Criolla Grande and Cereza showed a very high degree of similarity (0.73%) indicating that they are closely related varieties (Table 2). On the other hand T. Riojano seems to be more related to T. Sanjuanino (0.70%) than to T. Mendocino (0.43%), despite their similar names. Members of each these group of varieties have similar morphology. The three Torrontés (T. Riojano, T. Sanjuanino and T. Mendocino) are aromatic and produce a dry muscat wine appreciated as a characteristic regional wine, specially T. Riojano, whereas Criolla Grande and Cereza are not aromatic, possess a lower enological value and are good as table grapes (Alcalde, 1989). The American rootstock "SO4" and the Criolla "Cereza" were the most distantly related genotypes (SMC = 0.085) (Table 2).

AFLP analysis

The primer combinations *Eco*ACT-*Mse*CTG; *Eco*ACC-*Mse*CTG; *Eco*ACC-*Mse*CAA yielded the best amplification products. A total of 111 bands, ranging in size from 100 to 500 base pair, were identified. Of those, 81 showed a clear polymorphism, representing 73% of the total bands. Polymorphic bands were scored for presence or absence in 16 grapevine materials. Faint bands were not included in the analysis due to their low reproducibility across multiple reactions. Bands that showed the same mobility were considered as identical DNA fragments. The same AFLP patterns were repeatedly found when different plants from the same varieties were independently assayed (data not shown).

Comparison of the cophenetic values obtained from the UPGMA cluster analysis, with Dice's similarity matrix demonstrated a correlation of 0.74, indicating that data in the matrix was fairly well represented by the dendrogram. The varieties were clustered showing general agreement with their regions of origin. Four major clusters, diverging at genetic similarity coefficient (DC) of 0.82, clearly separated French, the American hybrid, "Criollas" and Spanish varieties (groups A, B, C and D) (Figure 1b). The first cluster (A) included the French varieties Malbec, Syrah, Chardonnay and Cabernet Sauvignon. SO4 was considered a separate group by itself (C). A third group (B) was conformed by all the "Criollas" varieties, except Criolla Grande. Unexpectedly, Criolla Grande clustered separately with the Spanish variety Tempranillo (group D). Nevertheless, group B was more closely related to Tempranillo (DC = 0.82) than to the French materials (DC = 0.79). The clusters originated are conformed by materials that share not only their places of origin, but also many morphological (Figure 1a) (Alcalde, 1989) and

physiological (Kaiser and Cavagnaro, 2001) characters, as well as technological aptitudes (Alcalde et al. 1997). As suggested by the AFLP analysis, there is a genetic basis for much of this variability. Although, there are varieties that share similar names like the Criolla variety Pedro Giménez and the Spanish variety Pedro Ximénez (not analyzed in this work), they do not show ampelographic similarities. The former displays hairy shoots, whole or tri-lobullated leaves, roundish berries, branchy bunches, and medium fruit set while Pedro Ximénez shows cottony shoots, penta-lobullated leaves, elliptic berries, conic bunches, and late fruit set. Comparisons of these two varieties at a molecular level would provide more conclusive data on weather they correspond to different genotypes and to determine their degree of relatedness.

Among the "Criollas", the DC between pairs of cultivars ranged from 0.76 (for T. Riojano and Criolla Grande) to 0.95 (for T. Mendocino and Moscatel Amarillo) (Table 3). Cervera et al. 1998, using AFLP and the same coefficient on a large grapevine collection from La Rioja -Spain- and the same coefficient, reported ranges between 0.70 and 1.00. Thus, Argentine grapevine genotypes represent an important source of genetic variability, that could be exploited in breeding programs.

The finding that "Criollas" comprise a group more or less separate from the most other *vinifera* varieties tested, except Muscat d'Alexandrie and Tempranillo, was somewhat surprising since grapevine culture in Argentina data since the colonial times and has not been enough time to allow a natural evolution. This fact could reflect foundation of the "Criollas" from Spanish varieties more than evolution. Nevertheless, the results obtained by Agüero et al. 2003 strongly argued for a New World origin for some of the "Criollas" from Spanish and Mediterranean varieties. The close genetic relatedness between Criolla Grande and Tempranillo, as indicated by AFLP dendrogram, could reinforce this hypothesis.

Cervera et al. 1998 proposed that, if the number of AFLP loci analyzed is sufficiently large, materials showing similarities higher than 90% could be considered as cultivars from the same variety, thus they represent very similar genotypes differing only in a few loci. In our case, T. Riojano with T. Mendocino (DC: 0.93), and T. Mendocino with Moscatel Amarillo (DC: 0.95) (Table 3) appear to share much of their genetic background, and could be considered as very closely related genotypes. The three "Torrontés" (T. Riojano, T. Mendocino and T. Sanjuanino) also share many ampelographic features (Alcalde, 1989), although T. Riojano and T. Sanjuanino are, morphologically, most closely related (Figure 1a). Agüero et al. 2001 using microsatellite markers, reported that T. Riojano and T. Sanjuanino are both progeny from the same cross (Criolla Chica x Muscat d' Alexandrie) whereas, for T. Mendocino, Criolla Chica is one putative parent but there is uncertainty about the other one.

The possibility that Criolla Chica and Muscat d' Alexandrie could be the progenitors of T. Riojano, T. Sanjuanino and Mocatel Amarillo is in agreement with the outside linkage of the former to the other "Criollas". Following this argument it is probable that other "Criollas" were also originated from the same cross.

The fact that Criolla Grande clustered outside the group B, suggests that this variety could be another putative progenitor of some Criollas; this hypothesis could be tested using microsatellites in future research.

Comparison between AFLP and morphology

To provide an objective comparison, matrices of cophenetic values, generated from AFLP and morphological data, were compared using the Mantel test. Not significant and quite low correlation between the dendrograms was obtained ($r = 0.33$, $P = 0.9741$) after doing 250 random permutation with the Mxcomp procedure from NTSYS program. We believe that correlation between them could be improved if there was more morphological markers analyzed as was previously reported by other researchers (Martínez de Toda and Sancha, 1997a; Martínez de Toda and Sancha, 1997b) or more primer combination of AFLP were used.

Working with 16 ryegrass varieties, Roldán-Ruiz et al. 2001 reported correlation values of $r = -0.06$ between AFLP and 15 morphological characters. In comparison with ryegrass, grapevine genotypes appears to be environmentally more stable, as suggested by the higher agreement between phenotypic and molecular analysis. Apparently, in ryegrass there is much environmental influence accounting for the morphological variability observed. Therefore, when compared with DNA fingerprinting techniques, morphological traits are relatively less reliable and inefficient for precise discrimination of closely related genotypes and analysis of their genetic similarities. However, morphological traits, are useful for preliminary, fast, simple, and inexpensive varietal identifications and can be used as a general approach for assessing genetic diversity among phenotipically distinguishable cultivars, although they are inefficient on account of the time and cost involved.

CONCLUDING REMARKS

Both the morphological and genetic analysis allowed to separate the "Criollas", French, Spanish and American materials, except for Muscat d' Alexandrie and Tempranillo which clustered with Criollas in the case of AFLP. The correlation between the two systems was neither significant nor very high.

Our AFLP and morphology results suggest that the "Criollas" germplasm share a common genetic background differing, in genotype and morphology, from the French, American and Spanish varieties used for comparison in this study. The high degree of polymorphism detected and the

possibility of screening a higher number of anonymous loci than morphological markers makes AFLP useful for studying genetic diversity within the "Criollas". To our knowledge, this is the first report using AFLP markers to assess genetic variability on these materials. Another type of molecular markers like microsatellite, which are highly-abundant in the grapevine genome and shows codominant nature, will certainly contribute to determine the relationships between "Criollas" and European varieties and within "Criollas" and could be used for parentage analysis in further investigation.

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APPENDIX

Tables

Table 1. Descriptive names of the 53 morphological characters examined.

Tips	**Mature leaves**	**Bunchs**
Form	Blistering of upper side blade	Size
Intensity of anthocyanic colouration	Intensity of blistering	Density
Colouration	Colouration	Shape
Density of postrate hairs	Anthocyanic colouration of upper side blade	**Berries**
Shoots	Brightness of upper side blade	Colour of skin
Aspects	Lobe number	Shape
Density of erect hairs	Density of postrate hair on lower blade side	Size
Colouration	Sideface	Bloom
Young leaves	Teeth	Thickness of skin
Aspect	Shape	Flavour firmness of flesh
Density of erect hairs	Petiole sinuses	**Pedicels**
Colour of the upper side	Shape	Length
Tendrils	Margin	Degree of separation from pedicel
Length	Veins-petiole sinuses ratio	**Woody shoots**
Inflorescence	Upper leaf sinuses	Colour
Sex (morphology)	Shape	Size
Sex (physiology)	Shape of base	**Phenology**
Insertion of first inflorescence	Petiole point colour	Time of bud burst
Mature leaves	Base vein colour	Time of full bloom
Size	Petioles	Time of berry full maturity
Length	Density of hairs	
Blade shape	Length	
	colouration	

Table 2. Genetic similarity values of grape varieties using Simple Matching coefficient with morphological markers.

	Malbec	SO4	Cabernet	Syrah	Chardon	Tempra	MoAllo	MoRos	PGimen	ToMen	ToSan	ToRioj	Cereza	CrChic	CrGra
Malbec	1.0000														
SO4	0.1714	1.0000													
Cabernet	0.5660	0.1143	1.0000												
Syrah	0.5283	0.1143	0.5849	1.0000											
Chardon	0.4906	0.1429	0.5283	0.4151	1.0000										
Tempra	0.4898	0.1935	0.4694	0.4286	0.4082	1.0000									
MoAllo	0.4000	0.1923	0.2857	0.3714	0.2857	0.4375	1.0000								
MoRos	0.4151	0.2000	0.4151	0.3019	0.2642	0.3878	0.4286	1.0000							
PGimen	0.3585	0.2857	0.3774	0.3208	0.3396	0.3878	0.5714	0.4717	1.0000						
ToMen	0.3962	0.1429	0.3774	0.2830	0.3585	0.4286	0.4857	0.4906	0.4906	1.0000					
ToSan	0.3019	0.1143	0.3208	0.3396	0.3962	0.3469	0.4000	0.5283	0.4528	0.4151	1.0000				
ToRioj	0.4340	0.1429	0.3962	0.4151	0.3774	0.4490	0.4857	0.5283	0.5283	0.4151	0.6981	1.0000			
Cereza	0.3774	0.0857	0.4717	0.4340	0.3962	0.3061	0.3429	0.3585	0.4528	0.3019	0.4340	0.3962	1.0000		
CrChic	0.4528	0.1429	0.4151	0.2830	0.3396	0.4490	0.5714	0.5094	0.4340	0.3962	0.3962	0.3962	0.3962	1.0000	
CrGran	0.3585	0.0857	0.4340	0.3962	0.3774	0.3061	0.5143	0.4528	0.5472	0.3774	0.5094	0.4906	0.7358	0.5283	1.0000

References: Chardon: Chardonnay, Tempra: Tempranillo, MoAllo: Moscatel Amarillo, MoRos: Moscatel Rosado, PGimen: Pedro Giménez, ToMen: Torrontés Mendocino, ToSan: Torrontés Sanjuanino, ToRioj: Torrontés Riojano,CrChic: Criolla Chica, CrGran: Criolla Grande.

Table 3. Genetic similarity values of grape varieties using Dice coefficient with AFLP markers.

	Malbec	SO4	Cabernet	Syrah	Chardon	Tempra	MosAle	MosAllo	Mos Ros	PGimen	ToMen	ToSan	ToRioj	Cereza	CrChic	CrGran
Malbec	1.0000															
SO4	0.7692	1.0000														
Cabern	0.8252	0.7040	1.0000													
Syrah	0.8444	0.7521	0.8000	1.0000												
Chardon	0.8120	0.7652	0.7969	0.8833	1.0000											
Tempra	0.8333	0.7778	0.8058	0.7786	0.7752	1.0000										
MosAle	0.8054	0.7634	0.7639	0.7647	0.7463	0.8276	1.0000									
MosAllo	0.8088	0.8136	0.7939	0.8293	0.8595	0.7879	0.8175	1.0000								
MosRos	0.8000	0.8376	0.7692	0.7541	0.8000	0.8397	0.8088	0.8455	1.0000							
PGimen	0.8085	0.8618	0.7941	0.7500	0.8095	0.8759	0.8169	0.8527	0.9063	1.0000						
ToMen	0.8112	0.8000	0.7971	0.8000	0.8438	0.8345	0.8611	0.9466	0.8615	0.8971	1.0000					
ToSan	0.8082	0.7813	0.7801	0.8120	0.7786	0.8310	0.8844	0.8358	0.8421	0.8777	0.8794	1.0000				
ToRioj	0.8116	0.8667	0.7970	0.8000	0.8130	0.8507	0.8633	0.9206	0.9280	0.9313	0.9323	0.8971	1.0000			
Cereza	0.7820	0.8174	0.7813	0.7667	0.7627	0.8527	0.8060	0.8595	0.8500	0.8730	0.8750	0.8397	0.9268	1.0000		
CrChic	0.7692	0.8160	0.7246	0.7077	0.7188	0.8058	0.8333	0.7939	0.8154	0.8529	0.8116	0.8369	0.8722	0.8281	1.0000	
CrGran	0.8169	0.8065	0.8175	0.8062	0.8031	0.8696	0.7692	0.7692	0.8062	0.8444	0.8029	0.7571	0.8333	0.8346	0.7737	1.000

References: Chardon: Chardonnay, Tempra: Tempranillo, MosAle: Muscat d´Alexandrie, MosAllo: Moscatel Amarillo, MosRos: Moscatel Rosado, PGimen: Pedro Giménez, ToMen: Torrontés Mendocino, ToSan: Torrontés Sanjuanino, ToRioj: Torrontés Riojano, CrChic: Criolla Chica, CrGran: Criolla Grande.

Figures

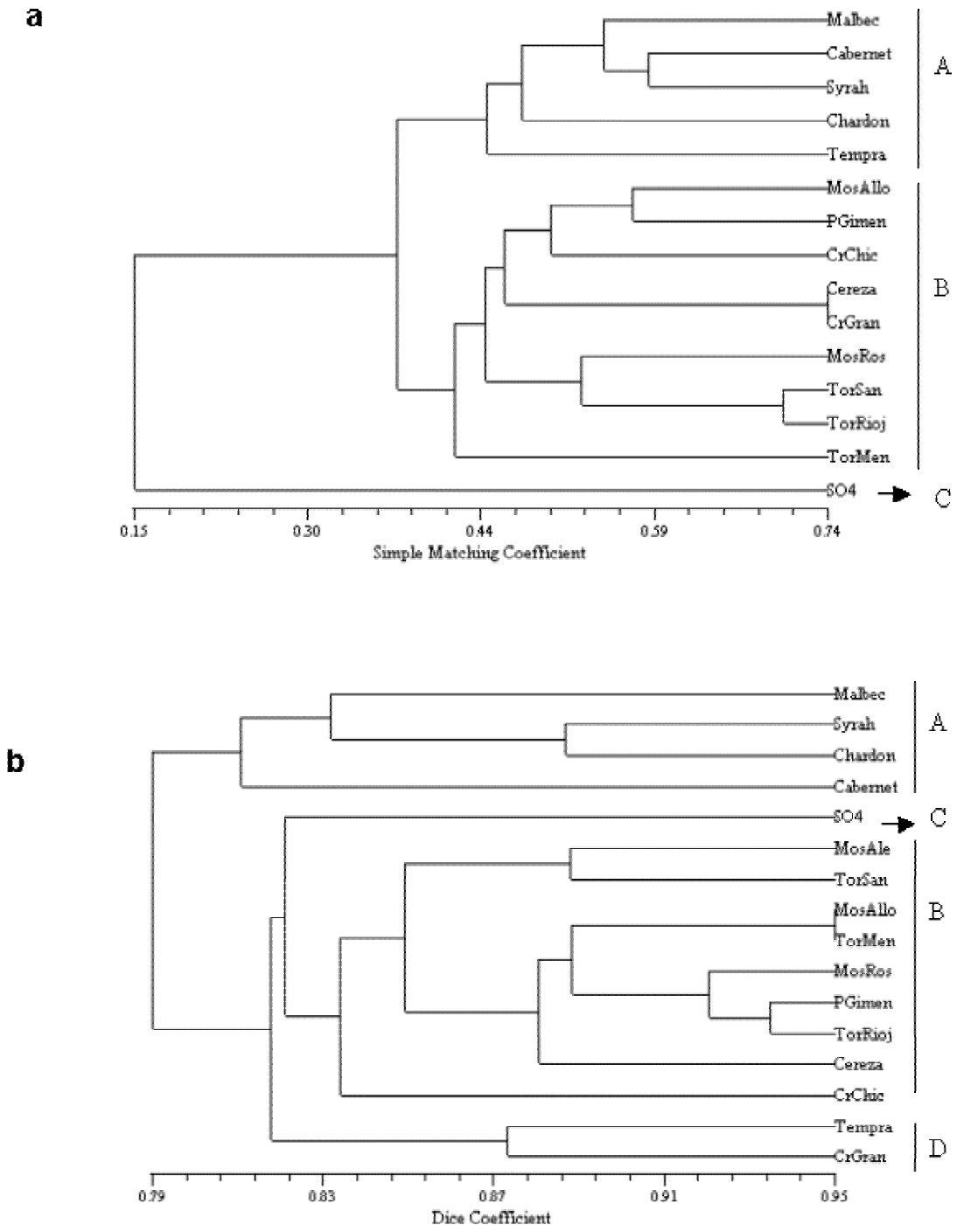

Figure 1. Dendrograms of grape varieties using morphological (a) and AFLP (b) data. Chardon: Chardonnay; Tempra: Tempranillo; CrChic: Criolla Chica; CrGran: Criolla Grande; PGimen: Pedro Giménez; MoRos: Moscatel Rosado; MoAllo: Moscatel Amarillo; ToRioj: Torrontés Riojano; ToSan: Torrontés Sanjuanino; ToMen: Torrontés Mendocino. Clusters of European, “Criollas”, American accession, and Spanish and “Criolla” varieties are indicated with letters “A”, “B”, “C” and “D”, respectively.