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Limited genetic diversity among clones of red wine cultivar 'Carmenère' as revealed by microsatellite and AFLP markers

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Summary

'Carmenère' is a fine red wine cultivar (*Vitis vinifera* L.) that has spread, unrecorded from France to other countries. It probably arrived in Chile before the *Phylloxera* crisis in Europe where it remained confused with Merlot and other red wine cultivars until the mid 1990s. In this study, genetic diversity among 26 accessions from Chile, France and Italy was analysed using microsatellite (SSR) and AFLP markers. Using 20 SSR markers, a "standard genotype" was established and three different haplotypes were found, presumably arising by a mutation at the VVMD7 and VMC5g7 loci. In the case of AFLP, using 11 primer combinations five groups were identified, with one main cluster of 22 accessions not differentiated. Combining both techniques it was possible to identify five out of the 26 accessions analysed. Together, these results suggest that 'Carmenère' exhibits a lower genetic diversity in comparison with other French red wine cultivars. This is a factor to consider when managing a clonal selection assay. Possible causes are discussed.

K e y w o r d s : clonal selection, *Vitis*, microsatellites, SSR, AFLP, genetic diversity.

Introduction

Winegrape cultivar identification has traditionally been based on ampelography but in the last decade this has been powerfully supplemented by a number of molecular tools and, in particular by microsatellite (or Simple Sequence Repeat, SSR) marker development. These markers have been used in grapes and also in many other species for a range of applications including genetic diversity studies (Aradhya *et al*. 2003), pedigree analyses (Bowers and Meredith 1997, Bowers *et al.* 1999 a), genetic mapping and QTL identification (Doligez *et al.* 2002, Adam-Blondon *et al.* 2004) and cultivar determination (Meredith *et al.* 1999, Hinrichsen *et al.* 2001).

In spite of the large volume of work at the cultivar level, molecular identification of clones remains a challenge for many varieties. However, the importance of clones in modern viticulture makes their identification crucial for the nursery and also the wine industries. One of the most important aims of germplasm collections is to maintain the maximum genetic diversity with the minimum number of accessions, in order to make the best possible use of the limited resources allocated to their preservation (Dangl *et al.* 2001). Studies approaching this issue with clones have been addressed, in particular in the 'Pinot' group where the chimeric nature of the grapevine was first described (Franks *et al.* 2002, Hocquigny *et al.* 2004). Later on, a large set of microsatellites has been applied to detect differences between clones of a limited set of cultivars such as 'Chardonnay' (Riaz *et al.* 2002), 'Tannat' (González-Techera *et al.* 2004) and 'Cabernet Sauvignon' (Moncada *et al*. 2006), or closely related cultivars considered clones of polyclonal origin (Silvestroni *et al.* 1997). On the other hand, AFLP as well as other dominant-type markers have been tested to reveal intra-cultivar variability (Cervera *et al.* 1998, Sensi *et al*. 1996, Imazio *et al.* 2002), with a diversity of results.

'Carmenère', a red wine cultivar from South-West France has been classified together with 'Cabernet Sauvignon', 'Merlot', 'Petit Verdot' and 'Hère' in the 'Carmenet' elite group, one of the 13 *sortotype* or eco-geografical groups distinguished in France according to morphological traits (Bisson, 1995). 'Carmenère's' origin is not clear and its parentage remains unknown. This cultivar was widely cultivated in the Médoc region due to its exceptional wine quality, including a particular bouquet but it was almost lost after the *Phylloxera* crisis and also because of low yields caused by coulure (Viala and Vermorel 1901-1910, Galet 2000).

This cultivar was probably distributed from the Bordeaux region to other countries before *Phylloxera*, but information about its spread was very poor until recently. In Italy it is sometimes mixed and confused with 'Cabernet franc' (Galet 2000, Pszczolkowski 2000) and it was recently re-discovered in Chile, where it used to be planted together with 'Merlot' (Hinrichsen *et al.* 2001). At present there are 6,849 ha planted in Chile (SAG 2005), most if not all coming from the same material introduced by the end of XIX century. Its relation with the few hectares remaining in France is unknown.

In this study, we analysed the genetic diversity of 'Carmenère' accessions using molecular approaches (SSR and AFLP markers). In the long term, we are interested in deter-

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mining the molecular basis of clonal variability, under the hypothesis that the accumulation of somatic mutations responsible for intra-cultivar diversity can be revealed by the use of a combination of anonymous and sequence-tagged molecular markers. In this way, we show that this cultivar presents a very limited genetic diversity, much lower than other red wine cultivars previously studied at this level.

Materials and Methods

Twenty-five 'Carmenère' accessions collected from Chilean vineyards, and recently established as clonal trials were kindly provided by the University of Talca and the Catholic University of Chile as there were no other registered clones available at the time. In addition, one accession was obtained from vineyards in Northern Italy (Tab. 1) and nine accessions (named 1205, 1276, 1308, 1345, 1369, 1379, 1470, 1478 and 1482) were obtained from the INRA-Bordeaux collection (France). All these were used in microsatellite studies. None of the materials selected for the study had any evident morphological, physiological, or production differences. Note that although all these materials are not formal clones, but accessions, both these terms will be used without distinction based on the possibility that they could have accumulated somatic mutations and because of their different geographical origins.

Table 1

The Carmenère material studied. All clones were used for microsatellite and for AFLP analysis except the French clones which were used only for microsatellite analysis

Country	Original name	Analysis name	Source**
Chile	H1P1	Cm1	CTVV
Chile	H1P7	Cm7	CTVV
Chile	H1P9	Cm9	CTVV
Chile	H1P10	Cm10	CTVV
Chile	H1P12	Cm12	CTVV
Chile	H1P13	Cm13	CTVV
Chile	H1P18	Cm18	CTVV
Chile	H1P19	Cm19	CTVV
Chile	H1P20	Cm20	CTVV
Chile	H1P21	Cm21	CTVV
Chile	H1P22	Cm22	CTVV
Chile	H1P23	Cm23	CTVV
Chile	H2P5	Cm27	CTVV
Chile	H2P6	Cm28	CTVV
Chile	H2P8	Cm29	CTVV
Chile	H2P11	Cm32	CTVV
Chile	H2P13	Cm34	CTVV
Chile	H2P15	Cm35	CTVV
Chile	H2P16	Cm36	CTVV
Chile	H2P17	Cm37	CTVV
Chile	H2P19	Cm39	CTVV
Chile	H2P21	Cm41	CTVV
Chile	H2P22	Cm42	CTVV
Chile	080215	CmPUC34	PUC
Chile	080114	CmPUC26	PUC
Italy	-	CmIt	IASMA-Trento
France	1205	-	INRA-Bordeaux
France	1276	-	INRA-Bordeaux
France	1308	-	INRA-Bordeaux
France	1345	-	INRA-Bordeaux
France	1369	-	INRA-Bordeaux
France	1379	-	INRA-Bordeaux
France	1470	-	INRA-Bordeaux
France	1478	-	INRA-Bordeaux
France	1482	-	INRA-Bordeaux

**CTVV, Centro Tecnológico de la Vid y el Vino, University of Talca, Chile; PUC, Catholic University of Chile; IASMA, Istituto Agrario San Michele all'Adige, Trento, Italy; INRA, Institut National de la Recherche Agronomique, Bordeaux, France.

DNA isolation: DNA was extracted from young, expanding leaves according to the method described by Lodhi *et al.* (1994). DNA quality was evaluated in a 0.8 % agarose gel stained with ethidium bromide and concentration was measured with a fluorimeter DyNA Quant 200 (Hoefer Pharmacia Biotech.) using Hoechst 33258 dye.

Microsatellite (SSR) analysis: The analysis was based on 20 microsatellite markers, VVS2 (Thomas and Scott 1993); VVMD5, VVMD6, VVMD7, VVMD24, VVMD25, VVMD27, VVMD28, VVMD31, VVMD32, VVMD34 (Bowers *et al.* 1996, Ibid 1999 b); and VMC2a3, VMC2c7, VMC2f12, VMC3a9, VMC3e12, VMC5g7, VMC5g8, VMC8g9, VMC9a2-1 (Vitis Microsatellite Consortium, managed by Agrogene, Moissy Crayamel, France). PCR reactions were performed using a mixture (16 µl final volume) containing 4 ng of genomic DNA, 0.3 µM of each primer, 250 µM of each dNTP, 1.6 µl 10x PCR buffer (NaCl 100 mM, Tris-HCl 200 mM, pH 8.3), 1.25 mM of $MgCl_2$ and 0.4 µl of Taq DNA polymerase in a PTC-100 thermal cycler (MJ Research, Mass, USA). Amplification conditions were adjusted to an initial denaturation of 5 min at 95 °C, followed by 40 cycles of 45 s at 95 °C, 45 s at 56 °C and 90 s at 72 °C, and a final extension of 7 min at 72 °C before stopping the reaction.

AFLP analysis: The AFLP protocol was that of Vos *et al*. (1995) but modified as described by Narváez *et al.* (2000). AFLP products were generated by double digesting 300 ng of genomic DNA with 2.5 U of both *Eco*RI and *Mse*I (New England Biolabs) restriction enzymes. Adapters were ligated to the digestion products with 100 U T4 DNA ligase (New England Biolabs) overnight at 16 ºC. A 1/10 dilution in buffer TE of the digestion products was made for doing PCR+1 with primers *Eco*RI-N (5'-GACTGCGTACCAATTC-A-3') and *Mse*I-N (5'-GATGAGTCCTGAGTAA-G-3'). Pre-amplifications were done in final volumes of 50 µl, each containing 5 ng DNA, 10 pmoles of each primer, 0.5 U Taq DNA polymerase, 0.8 mM dNTPs, 5 µl 10x PCR buffer and $MgCl_2$ 2.0 mM. The amplification program was adjusted to an initial denaturation of 2 min at 94 ºC, followed by 35 cycles of 30 s at 94 °C, 30 s at 55 ºC and 1 min at 72 ºC, finishing with a 2 min extension at 72 ºC. Selective amplifications, PCR+3, were achieved under the same reaction conditions but using 5 pmoles of each *Eco*RI-ANN and *Mse*I-GNN primers, in a final volume of 20 µl. The amplification profile was 94 ºC for 3 min, followed by 30 sec at 65 ºC and 1 min at 72 ºC in the first cycle. In the following cycles the annealing tem-

perature was reduced by 0.7 ºC per cycle, to 56 ºC. Then, 25 more cycles were repeated under these same conditions (annealing at 56 ºC), ending with 2 min at 72 ºC for the final extension.

Amplicons separation and staining: For both SSR and AFLP, DNA fragments were resolved on denaturing 6% polyacrylamide gel electrophoresis and were revealed by silver staining (Narváez *et al.* 2001). Microsatellite alleles were named from their sizes in base pairs (bp), according to allele sizes established for other cultivars. In the case of microsatellites, at least two independent reactions were performed to confirm polymorphisms.

Data analysis: Microsatellite and AFLP fingerprints were evaluated by visual inspection. Presence or absence of amplified fragments was scored with 1 or 0, respectively, constructing a binary matrix. Distance matrices were calculated using the DICE (1945) coefficient implemented by the SimQual procedure of the NTSYSpc V. 2.0 program (ROHLF, 1997). Genetic divergence was visualised using an unweighted pair group method average (UPGMA) clustering (Sneath and Sokal, 1973).

Results and Discussion

'Carmenère' identity confirmed by microsatellite markers: All materials collected were confirmed as 'Carmenère' by a preliminary analysis with markers VVMD5, VVMD6, VVMD7 and VVMD28, which are able to discriminate between the most common varieties planted in Chile (Narváez *et al.* 2001). Every sample displayed the expected allelic pattern for these four loci. An expanded analysis to 20 loci allowed the establishment of a "standard genotype" for 'Carmenère' (Tab. 2) that is better than that published previously (Hinrichsen *et al.*, 2001). This standard genotype was based on the experimental data obtained, since original alleles for each locus were unknown owing to its lack of pedigree. All loci were revealed as heterozygotic, except for VVMD32, VMD34 and VMC3e12 that showed one allele each. It was not possible to determine if these were homozygous or if they harboured a null allele. Locus VMC2c7 amplified four alleles (132:146:163:170), that could correspond to a multilocus marker.

Table 2

Standard microsatellite-based genotype of Carmenère

Locus	Genotype
VVMD5	226:238
VVMD6	212:214
VVMD7	243:263
VVMD24	210:214
VVMD25	243:259
VVMD27	175:189
VVMD28	239:253
VVMD31	206:210
VVMD32	241:-
VVMD34	240:-
VMC2a3	164:182
VMC2c7	132:146:163:170
VMC2f12	192:248
VMC3a9	98:108
VMC3e12	146:-
VMC5g7	190:208
VMC5g8	288:298
VMC8g9	169:181
VMC9a2-1	175:198
VVS2	136:144

The "standard genotype" was compared with that obtained for some other cepages from Bordeaux, showing that Carmenère shared one allele with Cabernet franc in every locus analysed (data not shown). This result strongly suggests that 'Cabernet franc' is one of its parents, and is also consistent with their well-known morphological similarity (Viala and Vermorel 1901). As 'Cabernet franc' is one of the parents of 'Cabernet Sauvignon' (Bowers and Meredith 1997), this fact is evidence that members of the 'Carmenet' *sortotype* are closely related.

Genetic diversity of 'Carmenère' analysed at 20 microsatellite loci: Genetic diversity between 'Carmenère' clones was studied upon the "standard genotype" deduced. Only two out of 20 loci (10 %) were polymorphic, VVMD7 and VMC5g7. With VVMD7, two allelic combinations were found, sizing 243:263 (65 % of the samples) and 243:263:**265** (35 %). Interestingly, this same marker was the only one able to differentiate two synonymous cultivars, 'Black Currant' and 'Mavri Corinthiaki', of 16 microsatellite loci tested in a previous study (Ibáñez *et al.* 2000). The analysis of nine 'Carmenère' accessions from the INRA-Bordeaux collection revealed that all shared the diallelic genotype (243:263) at VVMD7, except one genotype (accession # 1205, alleles 243:263:**265**). This finding suggests that mutation at VVMD7 occurred in France before the propagation of the 'Carmenère' material to Chile. The lower mutation frequency exhibited by the French material was unexpected since France is the centre of origin of this cultivar. However, the original frequency of genetic variants could have suffered distortion in France after *Phylloxera*, resulting in genetic erosion. At the same time, the over-representation of this particular mutation amongst accessions collected in Chile could correspond to the original frequency present in France at pre-phylloxeric times; these accessions would have been passively propagated after their arrival in Chile, maintaining the original proportion.

The VMC5g7 locus was polymorphic in only one of the 26 samples (accession Cm23, obtained from University of Talca collection), showing a shift from the standard 'Carmenère' pattern (alleles 190:208) to 190:208:**210**. None of the French clones from INRA-Bordeaux had the polymorphic genotype at this locus, suggesting that the mutation could have arisen in Chile, or again it could be a case of genetic erosion in France. Polymorphism at this locus has been previously reported for other cultivars such as 'Chardonnay' (Riaz *et al.* 2002; Bertsch *et al.* 2003), 'Pinot gris' (Hocquigny *et al.* 2004) and 'Cabernet Sauvignon' (Moncada *et al.*, 2005). In 'Cabernet Sauvignon' the mutation detected was from 194:196 to 194:196:**198**, a mutation present in every Chilean 'Cabernet Sauvignon' clone

but less frequent in the French material of the same cultivar (MONCADA *et al*. 2006). The detection of mutations at the VMC5g7 locus in different cultivars suggests that it is an unstable locus, becoming a good candidate at which to look for mutations in clone populations of other cultivars. These results also emphasise that clonal genetic diversity could differ from region to region. This is being revealed by ongoing molecular analyses made for clonal selection and maintenance.

Clustering analysis by UPGMA of the 26 'Carmenère' accessions detected three genotypes of the four that are possible, after two mutations (data not shown). The "standard genotype" deduced previously was considered as Genotype I being the most common, comprising 16 clones (62 %) without mutations, including the Italian accession. Genotype II was possessed by nine clones with the mutation at VVMD7, while genotype III was represented only by the single clone mutated at VMC5g7. French clones were not included in the analysis, but eight of them had the genotype I and only one exhibited the genotype II (accession # 1205). The simplest theoretical shift undergone by the two new alleles found was equivalent to the addition of one dinucleotidic unit in the microsatellite (Tab. 3), suggesting that *slippage* was the mutation mechanism (ELLEGREN 2004). For both loci, the new allelic pattern was triallelic. This is considered an evidence of chimeras, a mechanism of genetic diversification occurring in grapevines clones proposed by HOCQUIGNY *et al*. (2004). At present, chimeras has been described for 'Pinot' (FRANKS *et al.* 2002, HOCQUIGNY *et al.* 2004), 'Chardonnay' (RIAZ *et al*. 2002, BERTSCH *et al.* 2003), 'Cabernet Sauvignon' (MONCADA *et al*. 2006) and other cultivars (CRESPAN 2003). In the case of the IBÁÑEZ *et al.* (2000) study, it was concluded that 'Black Currant' was 240:246 and 'Mavri Corinthiaki' was 240:248 at VVMD7, but a more careful inspection of their data strongly suggests that 'Mavri Corinthiaki' was 240:246:**248**, this pattern also corresponding to a chimerical mutation.

Genetic diversity in 'Carmenère' determined by AFLP: The AFLP analyses were made on the same 26 clones analysed with microsatellites, using 11 primer combinations. On average, 31 amplicons per AFLP reaction were obtained, totalling 344 fragments analysed, seven of these polymorphic with *Eco*RI-AAC/*Mse*I-GCG and *Eco*RI-ACG/*Mse*I-GCG primer combinations (Tab. 4). This gave an overall 2 % of polymorphism. The polymorphic index found amongst 'Carmenère' accessions based on AFLP was intermediate between the values previously reported in wine cultivars. It was high compared to 11 'Primitivo' clones studied with 50 primer combinations (FANIZZA *et al.* 2005), where only nine polymorphisms over ca. 3,000 AFLP amplicons were detected, and was low in comparison to 24 'Traminer' clones (IMAZIO *et al.* 2002), with 40 polymorphisms over 117 AFLP bands obtained with just three primer combinations.

The UPGMA-based clustering revealed five groups (dendrogram not shown), with a main cluster of 22 non-differentiated clones and four single groups for clones Cm10, Cm36, CmIt and CmPUC26. The coefficient of genetic similarity obtained for AFLP analysis was at the order of 0.90 (except for clone CmPUC26, which was 0.87), similar to the studies of CERVERA *et al.* (1998), which proposed a value of genetic similarity >0.90 amongst genotypes belonging to the same variety.

Comparison between both molecular marker tools: 'Carmenère' displayed two polymorphic loci (10 %) in 20 microsatellite loci analysed with 26 samples revealing three genotypes. This diversity is lower

Table 3

New alleles found in Carmenère: Polymorphic loci motives and the putative shift undergone by allele mutation

Locus	Motive	Allele putative shift (pb)	New motives	Total new alleles
VVMD7	CT	263→265	1	1
VMC5g7	GA	208→210	1	1

Table 4

Summary of data obtained with AFLP

Primer combinations	Total number of amplified fragments	Total number of polymorphic fragments
*Eco*RI-AAC/*Mse*I-GTA	40	0
*Eco*RI-AAC/*Mse*I-GCG	9	2
*Eco*RI-AAC/*Mse*I-GAC	27	0
*Eco*RI-AAC/*Mse*I-GGT	21	0
*Eco*RI-AAC/*Mse*I-GTT	40	0
*Eco*RI-AAC/*Mse*I-GTC	28	0
*Eco*RI-AAC/*Mse*I-GCA	62	0
*Eco*RI-AAC/*Mse*I-GAG	33	0
*Eco*RI-AAC/*Mse*I-GAT	42	0
*Eco*RI-ACG/*Mse*I-GTA	12	0
*Eco*RI-ACG/*Mse*I-GCG	30	5
Total	344	7

than the 21.4 % found in 'Cabernet Sauvignon', where 59 clones analysed at 84 microsatellite loci presented 18 polymorphic loci, displaying 22 genotypes (Moncada *et al.* 2006) and also lower than the 22.4 % found in the 'Pinot' group, where 145 clones analysed at 49 loci presented 11 polymorhic loci displaying 14 genotypes (Hocquigny *et al.* 2004). In addition, AFLP analysis showed only five identity groups, one of these composed of 22 clones without differentiation. Even when the number of informative polymorphisms amongst 'Carmenère' clones and accessions is very limited, the combination of both SSR and AFLP markers could be used to identify some of the clones studied. A clustering analysis combining both SSR and AFLP data (see Figure) showed that five out of 26 clones (19 %) could be differentiated. This is a very promising percentage considering the reduced number of markers evaluated.

Each molecular marker technique showed different groups and genotypes which were not coincident. Moreover, the three genotypes revealed by microsatellites were included in the cluster of 22 clones obtained by the AFLP (group 1 in the Figure). This is not a case of inconsistency, considering that SSR and AFLP are techniques with different sequence targets, one focused to specific hypervariable regions of the genome (microsatellites), while the other is based on point mutations in restriction enzyme sites or indels, being a massive, anonymous and dominant approach (AFLP).

Mutations detected by microsatellites could allow expression of genetic diversity in evolutive terms in 'Carmenère'. The genetic diversification undergone by accessions as the result of independent mutations in L1 or L2 cell layers occurred in genotype I which resulted in the new genotypes II and III, that is proposed in a phylogenetic diagram (see insert in the Figure). From there, it can

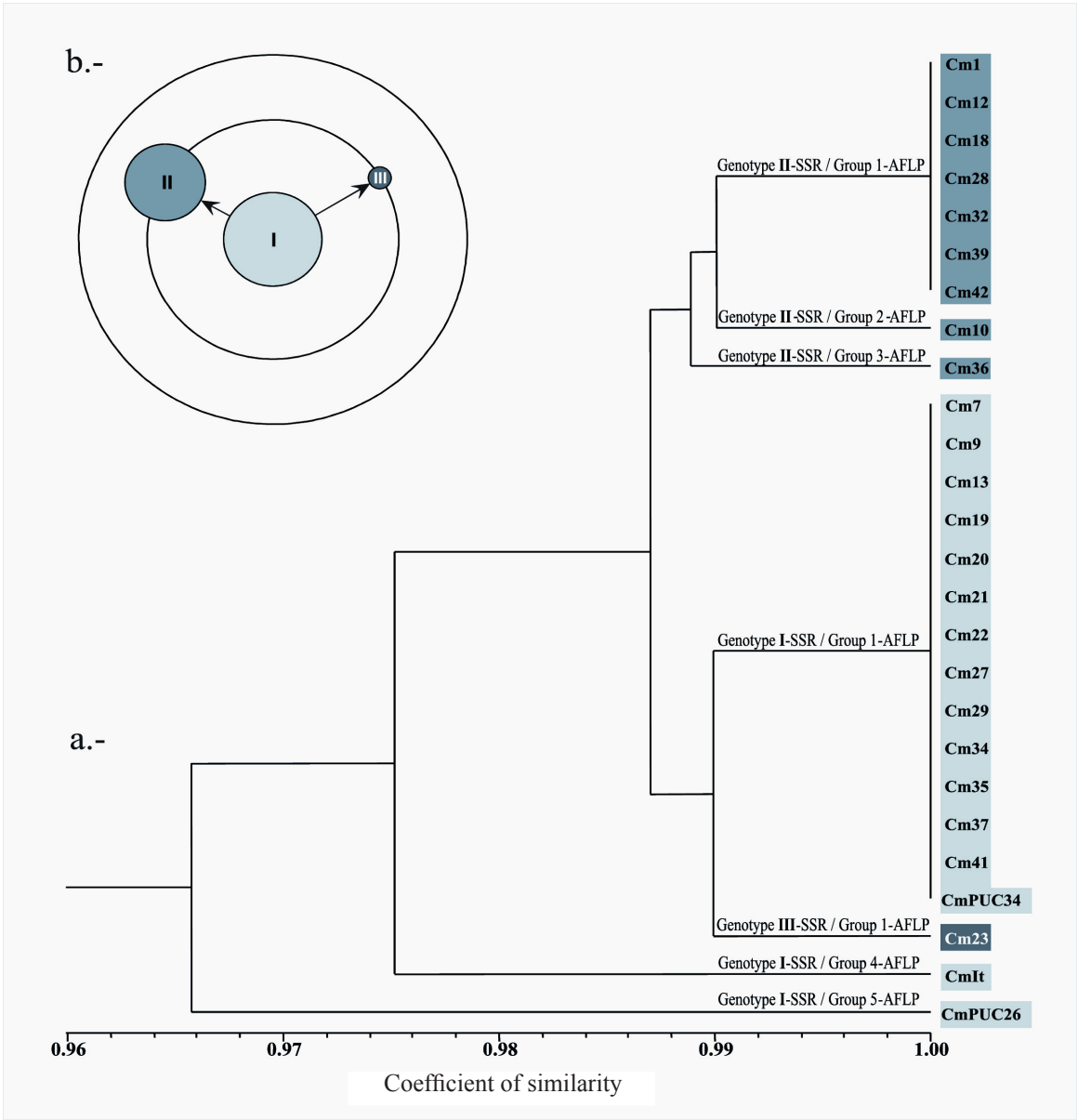

Figure: (**a**): Dendrogram representing genetic similarity in 'Carmenère' clones combining information from 20 SSR loci and 11 AFLP primer combinations. On each final branch the name of the genotype or group obtained respectively by SSR or AFLP is indicated. Insert (**b**): Diagram illustrating phylogenetic relationships among 'Carmenère' genotypes detected by SSR. Each genotype is represented by one circle: genotype I (16 clones), genotype II (nine clones) and genotype III (one clone). Correspondence of genotypes and clusters in the dendrogram is shown with different shadings.

be predicted that new genotypes and mutations would arise when testing a larger set of microsatellite loci on these 'Carmenère' clones, structuring new ways of diversification. The complementary nature of the results obtained with AFLP and SSR as a whole, suggest that 'Carmenère' has a low level of genetic diversity in comparison with other wine cultivars. Causes for this lower diversity are unknown at this time but possible explanations are that the cultivar is young as it has not yet accumulated a large number of somatic mutations. More relevant than the age of the cultivar could be its reduced dispersion in cultivated areas, a factor that could have hampered somatic mutation events. All these antecedents might be considered in clonal selection assays for this cultivar, where low morphological diversity is detected.

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