Theor Appl Genet (2000) 101:1194–1201 © Springer-Verlag 2000

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Design of grapevine (*Vitis vinifera* L.) cultivar-specific SCAR primers for PCR fingerprinting

Received: 9 March 2000 / Accepted: 31 March 2000

Abstract Among 34 grapevine cultivars (*Vitis vinifera* L.), eight putative genotype-specific RAPD markers, from 'Albariño', 'Caíño blanco', 'Chardonnay', 'Folle blanche', 'Grenache blanc', 'Malvasía Sitges', 'Torrontés' and 'Treixadura' respectively, were selected to transform into SCAR markers. Of these, seven markers were cloned and then five which showed a positive specific hybridization signal were sequenced. For these five markers, 30 sequence-specific primers ranging from 14 to 29 bases were designed to amplify genomic DNA from 64 grapevine cultivars under more-stringent PCR conditions. Only, two primer pairs, $OpA11_{1175}$p17R/p17F and $OpD10_{800}$p14R/p14F, still produced a specific SCAR marker, the 'Folle blanche' $ScA11_{1175}$ and the 'Malvasía Sitges' $ScD10_{800}$ respectively. Moreover, the $ScA11_{1175}$ marker was amplified only in 'Folle blanche' among the 64 cultivars tested with a large annealing temperature range using either two different *Taq* DNA polymerases or two separate thermocyclers. In addition, we discuss the initial polymorphism originated by the RAPD technique and suggest a new design of SCAR primers to obtain reliable cultivar-specific SCAR markers from single PCR-based bands for identification purposes.

Keywords 'Folle blanche' · Hybridization · RAPD · SCAR · Sequence-specific primer pair · *Vitis vinifera* L

Communicated by H.F. Linskens

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Introduction

Since the polymerase chain reaction (PCR) technique was published, many workers have recognized its potential utility in the genetic characterization of organisms as compared with the restriction fragment length polymorphism (RFLP) technique. Later, other derived PCR-based techniques were developed which either need a previous knowledge of the genomic DNA sequence to design specific primers such as SSRs (simple sequence repeats) (Weber 1991) and CAPS (cleaved amplified polymorphism sequences) (Bourquin et al. 1995) or do not by using arbitrary primers such as RAPDs (random amplified polymorphic DNAs) (Williams et al. 1990) and AFLPs (Vos et al. 1995). Most of these techniques have been successfully applied to fungi and plants for different purposes (Weising et al. 1995).

These several techniques were adapted to resolve many viticulture problems, especially grapevine characterization, when ampelographic methods either could not be used or were too time consuming. The choice of using the fingerprinting technique depends on its application. Thus, many grapevine species (*Vitis* sp), cultivars and rootstocks, were studied for purposes such as identification, genetic relationship and gene mapping using RAPD (Jean-Jacques et al. 1993), SSR (Bowers et al. 1996) and AFLP (Cervera et al. 1998) strategies. For identification purposes, RAPD and AFLP techniques generate relatively complex patterns, making the analysis of gels tedious and time consuming. The AFLP method typically produces in each reaction between 50 to 100 bands, whereas the RAPD method often detects between 5 to 10, but can produce some false positives and negatives (Lamboy

1994) due to its competitive reaction (Heun and Helentjaris 1993). Alternatively, SSRs are highly specific but are sparsely distributed in plant genomes (Weber 1991). Therefore, efficient methods are still required for single and accurate identification of grapevine cultivars in routine procedures.

The development of sequence characterized amplified regions (SCARs) (Paran and Michelmore 1993) and allele-specific associated primers (ASAPs) (Gu et al. 1995), derived from RAPD fragments, introduced the possibility of obtaining reliable and single DNA markers linked to genes in lettuce and beans, respectively. In a similar way, Mondon et al. (1997) defined the sequence-specific DNA Primer (SSDP) from RAPD markers for the molecular typing of *Aspergillus fumigatus* strains. SCAR markers have been recently developed for many crops including grapes. Based on this technique, Xu et al. (1995) successfully fingerprinted grape rootstocks and Lahogue et al. (1998) identified a co-dominant SCAR marker linked to the seedless character in grapevine.

The identification of molecular markers specific to a single grape cultivar would be of great interest for regulatory agencies as well as for breeders and nurseries. In a previous paper concerning genetic relationships among 32 European grapevine cultivars, 14 theoretically genotype-specific RAPD markers were scored (Vidal et al. 1999a). In the present work we cloned and sequenced some of these RAPD markers in order to design cultivar-specific primers which could allow the amplification of a unique DNA fragment only from the expected cultivar. PCR amplifications, under more stringent conditions, allowed us to obtain two genotype-specific SCAR markers (for ‘Folle blanche’ and ‘Malvasía Sitges’) which could be easily visualized as a single band on an agarose gel. To our knowledge, this study is the first to describe the transformation of single grapevine cultivar-specific RAPD markers into reliable SCAR markers for use in cultivar identification.

Materials and methods

Plant material and DNA extraction

Thirty two white grapevine cultivars were initially used to obtain genotype-specific RAPD markers (Vidal et al. 1999a). Later, other red and white cultivars were added to check their specificity. Plant material was obtained from three germplasm collections (Table 1). Fully expanded young leaves were harvested from healthy shoots during early growth stages and kept in paper bags for 2 or 3 days at 4°C prior to DNA extraction. DNA was isolated following the protocol of the DNeasy Plant kit (QIAGEN) with some modifica-

Table 1 List of grapevine cultivars tested in this study. Berry color is noted

RAPD and SCAR amplification				SCAR amplification			
Code	Cultivar	Berry color	Source[a]	Code	Cultivar	Berry color	Source[a]
1	Albariño	White	MBG	**35**	Gros Manseng	White	ITV
2	Caíño blanco	White	MBG	**36**	Petit Manseng	White	ITV
3	Godello	White	MBG	**37**	Pinot noir	Red	ITV
4	Lado	White	MBG	**38**	Syrah	Red	ITV
5	Loureiro blanco	White	MBG	**39**	Merlot	Red	ITV
6	Torrontés	White	MBG	**40**	Grenache noir	Red	ITV
7	Treixadura	White	MBG	**41**	Gamay	Red	ITV
8	Airén	White	ENCIN	**42**	Cabernet Franc	Red	ITV
9	Albillo	White	ENCIN	**43**	Cot	White	ITV
10	Albillo blanco	White	ENCIN	**44**	Petit Courbu	White	ITV
11	Palomino (Jerez)	White	ENCIN	**45**	Gros Courbu	White	ITV
12	Malvar	White	ENCIN	**46**	Silveiriña	White	MBG
13	Malvasía	White	ENCIN	**47**	Dona branca	White	MBG
14	Sitges	White	ENCIN	**48**	Branco lexítimo	White	MBG
15	Viura	White	ENCIN	**49**	Agudelo	White	MBG
16	Verdejo	White	ENCIN	**50**	Verdello	White	MBG
17	Melon	White	ITV	**51**	Moscatel branco	White	MBG
18	Folle blanche	White	ITV	**52**	Mantilla	White	MBG
19	Sauvignon	White	ITV	**53**	Cumbrao	White	MBG
20	Chardonnay	White	ITV	**54**	Gran negro	Red	MBG
21	Chenin	White	ITV	**55**	Loureira	Red	MBG
22	Riesling	White	ITV	**56**	Alicante	Red	MBG
23	Gewurztraminer	White	ITV	**57**	Espadeiro	Red	MBG
24	Sylvaner	White	ITV	**58**	Mencía	Red	MBG
25	Aligoté	White	ITV	**59**	Merenzao	Red	MBG
26	Semillon	White	ITV	**60**	Caíño gordo	Red	MBG
27	Clairette	White	ITV	**61**	Verdello tinto	Red	MBG
28	Muscadelle	White	ITV	**62**	Tintilla	Red	MBG
29	Bourboulenc	White	ITV	**63**	Moscatel negro	Red	MBG
30	Grenache blanc	White	ITV	**64**	Moscatel rubio	Red	MBG
31	Colombard	White	ITV				
32	Vermentino	White	ITV				
33	Ratiño	White	MBG				
34	Viognier	White	ITV				

[a] MBG: Misión Biológica de Galicia (CSIC). Pontevedra, Spain. ENCIN: El Encín (Comunidad de Madrid). Alcalá de Henares, Spain. ITV: Institute Technique de la Vigne et du Vin. Unités Regionais, France

tions to avoid viscous grapevine samples. Seventy five milligrams of plant tissue were used and 500 µl of buffer AP1 and 150 µl of buffer AP2 were added at protocol steps two and four, respectively. DNA concentration was determined in a UV/VIS spectrophotometer (Perkin Elmer). DNA was diluted to 1 ng/µl in ultrapure sterile water for PCR analysis.

RAPD analysis

The RAPD reactions and detection were carried out as previously reported (Vidal et al. 1999b). However, 1 U of *Taq* DNA polymerase (Appligene-Oncor, France) per reaction was used. Some primers (Operon Technologies, Alameda, Calif.), which revealed clear cultivar-specific RAPD markers, were selected for the development of SCARs (see Table 2). Amplifications were performed twice on the first 34 samples shown in Table 1. The stained gels were first viewed on a UV transilluminator and later either photographed with a camera (Pentax) or recorded with a Gel Doc 1000 camera using the Molecular Analyst program version 2.1 (Bio-Rad).

Cloning and sequencing RAPD products

Each cultivar-specific RAPD band was excised from 1.5% standard agarose gels (Eurogentec) with a sterile cutter and the DNA was purified using the QIAquick Gel Extraction kit (QIAGEN). The fragment was then ligated into the pCR2.1 vector and cloned into a competent *Escherichia coli* strain, INVαF′, using the Original TA Cloning kit (Invitrogen). The selection of transformed clones was performed by PCR analysis using white colonies directly as DNA templates which were amplified with pCR 2.1-specific primers. Positive colonies were grown overnight in 10 ml of Luria Bertani liquid medium containing 50 µg/ml of kanamycin. For each cloned marker, purification of plasmid DNA was carried out from two independent transformed clones using the QIAprep Spin Miniprep kit (QIAGEN). The size of both DNA inserts was checked by *Eco*RI enzyme digestion (Boehringer Mannheim) followed by separation in a 2% agarose gel. The complete sequence of each cloned fragment was obtained from each end by Cybergene (France) using the T7 PRO and REV universal primers. For the $OpA11_{1175}$ marker it was only possible to sequence both ends, and not the entire fragment. Sequence analyses were carried out using the Mac DNASIS software system (Hitachi Software Engineering).

Hybridization analysis

*Eco*RI inserts were excised from the gel and purified as described above. Fragments were then random-prime DNA labeled using the DIG DNA Labeling kit (Boehringer Mannheim). The labeling reaction was performed overnight using 2 U of Klenow enzyme according to the manufacturer's instructions. Estimating the yield of DIG-labeled probes was carried out by dot blotting following the manufacturer's instructions. Putative genotype-specific RAPD markers were confirmed twice by using the labeled inserts to probe Southern blots of the original RAPD gels. Southern transfers were performed overnight using Qiabrane-charged nylon membranes (QIAGEN) and alkaline capillary transfer as described in the DIG System manual. After DNA transfer, the membranes were either exposed to UV light for 2 min 30 s for cross linking or incubated in an oven at 120°C for 30 min. Pre-hybridization for 1 h and overnight hybridization with constant rotation of the tubes were carried out at 42°C using DIG Easy Hyb buffer (Boehringer Mannheim). One microliter of DIG-labeled probe was added per ml of hybridization buffer. The membranes were washed twice and Digoxigenin-labeled DNA was detected by chemiluminescence according to the manufacturer's instructions. The blot was exposed to X-ray films (Kodak) from 5 min to 4 h.

SCAR design and analysis

For each sequenced RAPD marker, three pairs of oligonucleotides were designed to be tested as SCAR primers. Each primer contained the original 10-mer RAPD primer as described by Paran and Michelmore (1993); however, only the next four, six or eight internal bases from the end were added. The primer design for 'Folle blanche' was different (see Table 3). Sequence-specific primers were synthesized by Isoprim s.a. (France). The PCR reaction (25 µml) contained 5 ng of genomic DNA, 1 x reaction buffer, 1.5 mM of $MgCl_2$, 200 µM of dNTPs, 0.4 pmol of each primer and 0.5 U of *Taq* DNA polymerase. Two separate *Taq* polymerases (Appligene-Oncor and Eurogentec) were assayed to ensure reproducible results. Amplifications were also conducted in two different thermocyclers (models 480 and 2400 from Perkin Elmer) and programmed for one initial step of 5 min at 95°C followed by 30 cycles of (30 s at 95°C, 45 s at the annealing temperature and 1 min at 72°C) and then 10 min at 72°C. The optimal annealing temperature to achieve cultivar-specific amplification was determined by gradually increasing this parameter in successive trial runs. Sixteen microliters of SCAR products were resolved by electrophoresis using 2% agarose gels and then stained with 0.5 µg/ml of ethidium bromide solution for 15 min. Finally, SCAR profiles were recorded as described above.

Results

RAPD amplification and cloning

Seven cultivar-specific RAPD markers were initially selected for transformation into SCAR markers (Table 2). In addition, the $OpD10_{550}$ band from 'Chardonnay', which was not scored as a specific marker in a previous study on 32 cultivars, was selected because it produced an intense band from 'Chardonnay' DNA compared to the faint bands revealed from two cultivars, 'Riesling' and 'Colombard', and no band from the rest. Freshly isolated DNA from the first 34 cultivars listed in Table 1 was amplified under RAPD conditions using the selected primers. All band patterns were reproducible for each RAPD primer and each cultivar-specific marker was maintained (Table 2) in spite of the use of two additional cultivars, Ratiño and Viognier (Fig. 1a).

Out of eight reproducible markers, seven were successfully cloned (Table 2). Despite several attempts, the fragment size of 'Caíño blanco' obtained from both PCR screening and *Eco*RI enzyme digestion was always smaller than the original RAPD marker ($OpD11_{1125}$). However, an additional effort was not undertaken and not all putative positive clones were checked. Therefore, this marker was discarded for further analysis. For the others, two white colonies per marker were analyzed and the fragment size checked by both PCR screening and *Eco*RI digestion methods. As a result, one *Eco*RI site was revealed inside the 'Albariño' and 'Treixadura' fragments.

Hybridization analysis

The cloned markers were DIG-labeled and the efficiency of each probe was checked by dot-blotting on the RAPD

Table 2 Sequential relation of analyses performed in the transformation of RAPD markers into SCAR markers

Variety	RAPD[a] marker	Cloning	Dot blotting	Southern-blot analysis[b]			Sequence bp	SCAR[c] analysis
				Fn	Ab	Sh		
Albariño	$OpF07_{325}$	Yes	Positive	18	1	No	317	10
Caíño blanco	$OpD11_{1125}$	No	–	–	–	–	–	–
Chardonnay	$OpD10_{550}$	Yes	Positive	2	0	No	538	4
Folle blanche	$OpA11_{1175}$	Yes	Positive	2	2	No	?[d]	1
Grenache blanc	$OpF16_{1050}$	Yes	Nt[e]	0	2	Yes	1056	29
Malvasía Sitges	$OpD10_{800}$	Yes	Positive	16	1	No	797	1
Torrontés	$OpD11_{550}$	Yes	Negative	–	–	–	–	–
Treixadura	$OpF07_{625}$	Yes	Positive	4	3	No	Nt[e]	–

[a] Cultivar-specific RAPD markers. The letters and numbers refer to the primer used (Op=Operon Technologies). The subscripts indicate the size of the marker in base pair (bp)
[b] Hybridization results
Fn: false negative indicates hybridizing bands at the same molecular size in other cultivars
Ab: additional band indicates other hybridization bands at a different molecular size in addition to the expected signal
Sh: specific Hybridization signal
[c] Number of cultivars that were amplified using sequence-specific primer pairs
[d] ?: only the sequence of both ends was determined
[e] Nt: not tested

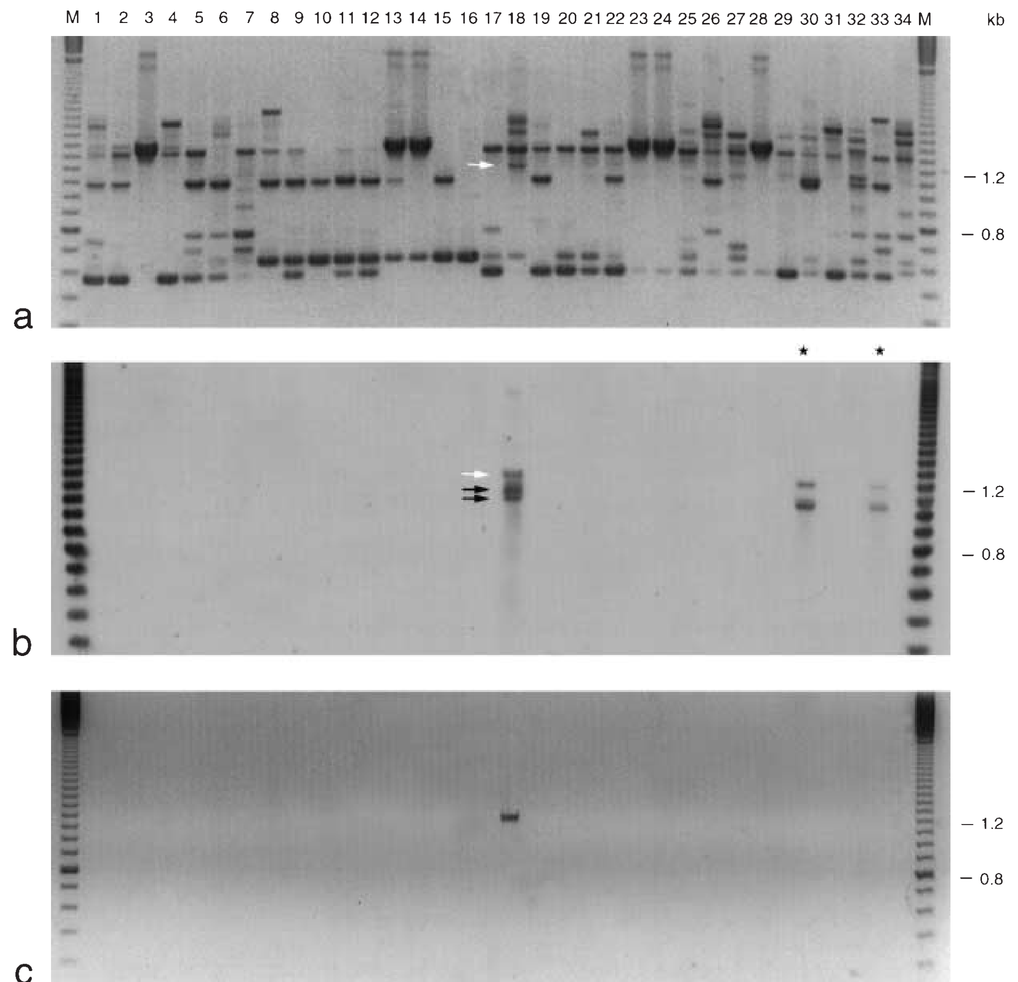

Fig. 1a–c PCR-based electrophoretic profiles and hybridization patterns. Cultivars from left to right correspond to the first 34 genotypes listed in Table 1. The first and last lane is a 100 bp molecular-weight ladder. The *numbers* on the right of the figures indicate the DNA size markers in kilobases (kb). **a** RAPD amplification from genomic DNA using the 10-mer primer OpA11. The RAPD marker $OpA11_{1175}$ specific to 'Folle blanche' is indicated by a *white arrow*. **b** Hybridization of the cloned and DIG-labeled RAPD fragment $OpA11_{1175}$ (*white arrow*) to a Southern blot of the RAPD gel. Cultivars marked with *asterisks* had been scored negative for the RAPD marker. The presence of hybridization bands in addition to the expected RAPD band are indicated by *black arrows*. **c** SCAR amplification from genomic DNA using the 17-mer sequence-specific primer pair $OpA11_{1175}$p17/p17. The unique amplification of a single SCAR marker $ScA11_{1175}$ on 'Folle blanche' was expected

product of five cultivars. This study allowed us to discard, prior to Southern-blot experiments, probes which hybridized in a non-specific way (Table 2). As a result, the probe $OpD11_{550}$ of 'Torrontés' hybridized more intensively on the 'Albariño' blot than the 'Torrontés' one (data not shown), and therefore was discarded for Southern hybridization.

Blotted RAPD gels (34 cultivars) were probed with the dot-blot positive probes and hybridization patterns were compared with the banding patterns based on ethid-

Table 3 Sequence-specific oligonucleotide primers derived from cloned RAPD fragments. The optimum primer pair and annealing temperature for initial specific amplification are noted

Cultivar	SCAR primer[a]	5′ to 3′ Sequence[b]	Cultivars screened for optimal SCAR conditions		Optimal primer pair	Optimal annealing
Albariño	OpF07R$_{325}$ OpF07F$_{325}$	CCG ATA TCC CGA ACG AAG CCG ATA TCC CAT TAT CAA	Caíño blanco Chardonnay	Loureiro blanco Bourboulenc	p18/p18	56°C
Chardonnay	OpD10R$_{550}$ OpD10F$_{550}$	GGT CTA CAC CGG TGC TTT GGT CTA CAC CCA TCT CGA	Chenin Melon	Folle blanche Sauvignon	p16R/p18F	61°C
Folle blanche[c]	OpA11R$_{1175}$ OpA11F$_{1175}$	CAA TCG CCG TTG GAT TAG ATG CTC T CAA TCG CCG TTT AAC CTT [TTT]$_3$ TC	Albariño Ratiño	Grenache blanc Riesling	p17/p17	58°C
Grenache blanc	OpF16R$_{1050}$ OpF16F$_{1050}$	GGA GTA CTG GCC TTA CTC GGA GTA CTG GTT CAC TAG	Bourboulenc Verdejo	Malvasía Viura	p18/p18	62°C
Malvasía Sitges	OpD10R$_{800}$ OpD10F$_{800}$	GGT CTA CAC CTA TAC GAT GGT CTA CAC CGA AAA CGG	Chardonnay Malvasía	Malvar Viura	p14/p14	50°C

[a] The letters and numbers preceding the R (Reverse) and F (Forward) refer to the progenitor primer used (Op=Operon Technologies). The subscript indicates the size of the marker in bp
[b] SCAR primers (p) of 14, 16 and 18 oligonucleotides were designed. The underlined sequences represent the original sequence of the progenitor RAPD primer
[c] Folle blanche SCAR primers were 17, 21 and 24 oligonucleotides for OpA11R and 17, 25 and 29 nucleotides for OpA11F

ium bromide staining. In all cases, for each probe, the hybridization signal corresponded to the expected RAPD band, indicating that the cloned fragments were derived from the amplified RAPD products and therefore the initial RAPD bands were not false positives. However, some false negatives at the same molecular size were detected in other cultivars, especially corresponding to the primers that revealed putative specific markers for 'Albariño', 'Malvasía Sitges' and 'Treixadura' (Table 2). With the probe OpD10$_{550}$, a hybridization signal was obtained in 'Chardonnay' (expected), 'Riesling' and 'Colombard'. With the probe OpA11$_{1175}$, hybridization was observed in 'Folle blanche' (expected) and also in 'Grenache blanc' and 'Ratiño' (Fig. 1b). Moreover, some hybridization signals at different molecular sizes in addition to the size of the expected RAPD band were observed (Table 2; Fig. 1b). After a long exposure of the X-ray film, the expected OpF16$_{1050}$ marker of 'Grenache blanc' was the only marker that showed a specific hybridization signal. However, in the other cases, the hybridization signal was slightly weaker in the cultivars that were initially scored as false negatives than in the expected cultivar (Fig. 1b). This result indicated that these putative false negatives probably did not share total homology with the cloned probe. Therefore, with the exception of 'Treixadura' marker OpF07$_{625}$, these cloned markers were sequenced to design sequence-specific primers.

Sequence of RAPD fragments

The sequence of these five cloned markers showed neither an open reading frame nor homology with other sequences in the databases (data not shown). Complete sequencing was obtained for four markers (size in Table 2), whereas only sequences of the extremities were determined for the 'Folle blanche' marker. It should be noted that the RAPD primer OpD10 amplified two specific bands, the OpD10$_{550}$ and OpD10$_{800}$ markers, that belong to the 'Chardonnay' and 'Malvasía Sitges' respectively. However, these two RAPD markers did not share sequence homology internal to the priming sites. This result was confirmed by cross hybridization on RAPD profiles of the probes and sequence analysis.

Amplification using sequence-specific primers

For each putative genotype-specific marker, an initial test of PCR conditions was performed using the genomic DNA of five cultivars with each primer pair from the set. This preliminary screening determined the optimal primer pairs and the annealing temperature for specific amplification (Table 3). Then, the selected primer pairs were used to amplify the genomic DNA of the first 34 cultivars listed in Table 1.

In each case, except for the 'Albariño' marker, a single band of the same size as the progenitor RAPD fragment was amplified. However, only two primer pairs, OpA11$_{1175}$p17/17 and OpD10$_{800}$p14/14, produced a single and specific marker on the expected cultivars, 'Folle blanche' and 'Malvasía Sitges' respectively. For the 'Folle blanche' primer pair, results were reproducible at a 58°C±2°C annealing temperature by using two different *Taq* polymerases (Appligene and Eurogentec) and thermocyclers (480 and 2400 Perkin Elmer) (Fig. 1c). A larger annealing temperature range was achieved (58°C±4°C) if only one *Taq* polymerase or thermocycler was used. Moreover, the 'Folle blanche'-specific SCAR marker ScA11$_{1175}$ was maintained even when 30 additional cultivars (Table 1) were used as templates for PCR (data not shown). Specificity was confirmed by hybridization between the blotted SCAR gel and the probed

fragment. By contrast, for the 'Malvasía Sitges' primer pair both up and down changes in annealing temperature induced a loss of specificity.

In the other cases, amplifications involving the primer pairs $OpF07_{325}p18/p18$, $OpD10_{550}p16R/p18F$ and $OpF16_{1050}p18/p18$ resulted in a loss of specificity because the putative specific RAPD markers of 'Albariño', 'Chardonnay' and 'Grenache blanc' amplified at the same molecular size on other cultivars (Table 2). The marker corresponding to the putative 'Chardonnay'-specific RAPD marker ($OpD10_{550}$) was also amplified under SCAR conditions in 'Riesling', 'Colombard' and 'Gamay'. Sequencing of these four amplification products (538 bp) revealed a near-total sequence homology (data not shown). Only two nucleotide divergences were found at 35 bp with 'Riesling' and at 64 bp with 'Colombard.' With the primer pair $OpF07_{325}p18/p18$, which originated from the 'Albariño' marker, length variants were obtained. Amplifications occurred in 'Albariño' and five other cultivars at the same size. However, in another four cultivars a slightly larger band was amplified that could indicate a case of allelism (data not shown). This result would allow us to transform a dominant RAPD marker into a co-dominant SCAR marker. However, the pedigree among the majority of grapevine (*Vitis vinifera* L.) cultivars is unknown; therefore, allelism can not be demonstrated.

Discussion

Worldwide, the species *V. vinifera* L. contains about 6500 cultivars and some intracultivar clones are also assumed to occur (Thomas et al. 1993). With this great biodiversity, the identification of genotypes with reliable and specific DNA markers is desired for the industry. In a previous RAPD study, we were able to identify some cultivars by specific markers, but these markers were amplified and visualized into patterns with other RAPD bands. Moreover, the RAPD technique has been questioned due to its lack of total reproducibility (Büscher et al. 1993; Lamboy 1994) which is an important pitfall both to cultivar identification in routine procedures and to data exchange among laboratories. In the present study, our objective was to clone eight putative specific RAPD markers and to transform them into single SCAR markers. The reliability of SCAR markers linked to a unique locus has been reported in some important crops (Naqvi and Chattoo 1996; Barret et al. 1998) as well as in grapevine (Lahogue et al. 1998).

The hybridization assays allowed us to confirm that we cloned the expected RAPD markers. Unexpected hybridization signals (false negatives), especially with 'Albariño'- and 'Malvasía Sitges'-specific probes, point out the limitations of the RAPD technique. Mismatches at the priming sites may permit band amplifications but in amounts too low to be detected by ethidium bromide staining. Similar results were also found by Xu et al. (1995) in grape (*Vitis*) rootstock. However, in our study, in most instances, the hybridization signal was more intense in the expected cultivar than in the putative false negatives. Hybridization also showed that in a RAPD pattern, bands of different molecular weight may have partial sequence homology. At this point, we should indicate that RAPD products often contain repetitive DNA sequences, therefore they are not ideal to use as hybridization probes (Paran and Michelmore 1993). In a previous study using *Vitis* cvs, Lodhi et al. (1997) probed both genomic and amplified DNA using RAPD bands as probes. A high percentage of repetitive genomic DNA was detected and some internal priming sites on amplified RAPD fragments were suggested. We have carried out re-amplification from four excised RAPD fragments. The fragment $OpF07_{325}$ did not produce additional bands whereas the fragments $OpD11_{1125}$, $OpA11_{1175}$ and $OpD11_{550}$ produced additional bands of smaller molecular size. In our study, the analyses of the sequences did not reveal repetitive DNA or internal priming sites. However, some annealing sites with partial complementation at the 3′ end of the primer were noticed. Therefore, we supposed that these additional hybridizing bands (from 1 to 3) correspond to products from the amplification of these weak internal priming sites, which did not allow enough amplification runs to be visible in ethidium bromide staining.

Amplification of targeted bands on additional cultivars was the main problem found using SCAR primers. Out of five markers sequenced, only two specific SCAR markers, $ScA11_{1175}$ and $ScD10_{800}$, were obtained. However, a larger screening on other grape cultivars should be performed to confirm these to be cultivar-specific SCAR markers. Although with the other SCAR primer pairs, the initial cultivar-specific RAPD markers were amplified on other cultivars, in all instances a polymorphic band was maintained. Moreover, as also found by Xu and Bakalinsky (1996), length variants were generated by the primer pair $OpF07_{325}p18/p18$ which provided additional polymorphism. In agreement with previous studies, our result indicated that the priming sites had major nucleotide divergences and that adding some nucleotides at the 3′ end of the RAPD primer may overcome the initial mismatches (Xu et al. 1995). In our study, the SCAR marker $ScD10_{550}$ was amplified in 'Chardonnay', 'Riesling', 'Colombard' and 'Gamay.' Recently, a close relationship between 'Chardonnay' (white berries) and 'Gamay' (red berries) was reported (Bowers et al. 1999). This SCAR marker showed minor nucleotide divergences between primer target sequences among the four cultivars. However, major divergences must exist at the priming sites because the RAPD marker was only unambiguous for 'Chardonnay' (data not shown). When we sequence an amplified fragment only the sequence between the priming sites belongs to genomic DNA, the ten bases of each termini corresponding to the original RAPD primer sequence (Paran and Michelmore 1993).

One source of polymorphism generated by the RAPD technique is the divergence in the primer target sequence

(Williams et al. 1990). Mismatches at the 3′ end, at one or both termini, may prevent amplification. As previously noted by Xu et al. (1995), adding 10–14 bases to the 3′ terminus of the 10-mer primers in a non-polymorphic region may provide enough homology to overcome the original mismatch and to permit annealing and amplification. To avoid this problem, we designed 30 shorter SCAR primers, by adding between four and eight nucleotides at the 3′ end from 10-mer primers, and then performed a screening to determine the optimal primer pair and the annealing temperature for specific amplification. However, only two primer pairs, $OpA11_{1175}$p17/p17 and $OpD10_{800}$p14/p14, were cultivar-specific. Other reports also demonstrated that longer SCAR primers showed increased amplification sensitivity when compared with the RAPD primers (Melotto et al. 1996).

Therefore, different strategies are suggested for further development of cultivar-specific markers in grape. If sequences corresponding to the RAPD primer annealing site and its upstream flanking region can be determined, then long SCAR primers can be designed by base addition at the 5′ end priming site, allowing the initial 3′ mismatch to be saved. This new design could also improve the reliability of current SCAR markers linked to genes and alleles. Other PCR products can also be used to design SCAR primers. However, stricter conditions should be applied at the beginning in order to obtain a more reliable specific DNA marker, such as the use of longer primers (Ye et al. 1996) or pairwise combinations (Hernández et al. 1999), and to select a higher annealing temperature (Atienzar et al. 2000). For the vine industry, to have cultivar-specific primers would allow a fast and accurate genotype identification which is especially important in ancient vine-growing areas (i.e. Appellation Controlée) where a great number of cultivars are grown and the price varies broadly among grapes. Regulatory agencies would also then have an efficient tool to avoid fraud. The extra work in cloning and sequencing DNA markers to design cultivar-specific primers would be justified to obtain a rapid and efficient method for cultivar identification. Savings, especially in situations requiring large sample numbers, could be realized by direct detection of amplification products in the test tube (Gu et al. 1995; Dedryver et al. 1996).

Acknowledgements We are grateful to three organizations [Misión Biológica de Galicia (Pontevedra, Spain); El Encín (Alcalá de Henares, Spain) and Centre Technique de la Vigne et du Vin (Unités regionales, France)] for supplying the plant materials. We thank Dr. B.I. Reisch and Dr. I. Paran for their critical review of the manuscript and Patricia Wallace for careful check of the English. This study was financially supported by the project 40225MC from ITV France and by a postdoctoral grant to the first author from 'Fundación Caixa Galicia.' The authors declare that the experiments performed comply with the current laws of the European Union.

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