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Identification of olive-tree cultivars with SCAR markers

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Summary

There is an urgent need for the development of early identification techniques in olive-trees due to the economic importance of cultivar identification in periods of expansion like now. We have been able to identify 22 olive-tree cultivars using only 10 different, specific, repeatable markers. These markers were designed by the cloning of significant RAPD bands obtained in PCR performed on bulked DNA to retain the genetic variability of each cultivar. Clones were partially or totally sequenced and new primers derived from these sequences were used to obtain Sequence Characterised Amplified Region (SCAR) fragments. We have demonstrated that the use of the 10 SCAR markers is enough to provide a simple, cheap, and reliable procedure to identify 22 geographically related olive-tree cultivars.

Abbreviations: AFLP – amplification fragment length polymorphism; AP-PCR – arbitrarily primed PCR; ASAP – allele-specific associated primers; CAPS – cleaved amplified polymorphic sequence; CTAB – N-cetyl-N,N,N-trimethyl-ammonium bromide; PCR – polymerase chain reaction; RAPD – random-amplified polymorphic DNA; RFLP – restriction fragment length polymorphism; SCAR – sequence-characterised amplified region; Ta – annealing temperature; UPGMA – unweighted pair group method with arithmetic averages

Introduction

Accurate and rapid identification of clones, varieties, or species is especially important in vegetatively propagated plants like olive-trees (*Olea europaea* L.). Hundreds of cultivars have been selected over the centuries for their adaptation to microclimates and soil types. As a result, there is considerable uncertainty about the names of many olive cultivars, becoming synonymous and homonymous a long-standing problem in olive-producing countries. This is an unliked scenario since olive-tree cultivar identification is of great economic importance in periods of expansion in its cultivation, like now. Differentiation among olive-tree cultivars is traditionally supported by numerous phenotypic traits in maturity for trunk, leaf, flower and fruit shape. More recently, workers have found allozymes to be useful markers for an objective cultivar identification. The weakness of both classifications has been put in evidence by the demonstration that chemical and morphological changes in olive-trees as well as other plants are induced by domestication, among other things. For example, agronomic selection for increased yield and growth rate has caused a reduction in plant defences (Massei & Hartley, 2000). Additionally, the long juvenile stage and the long non-productive period hamper a traditional method to identify the cultivar, which leads to an increase of plantation costs mainly in cases of cultivar miss-identification. Therefore, there is an urgent need for the development of methods that identify cultivars easily, rapidly and at an early stage of development.

Olive cultivar identification has been rapidly improved using molecular markers based on DNA since they provide an opportunity for direct comparison and identification of different genetic material independent

of any influences (Fabbri et al., 1995; Vergari et al., 1996; Wiesman et al., 1998; Mekuria et al., 1999; Claros et al., 2000; Sanz-Cortés et al., 2001). The number of molecular markers that can be generated is almost unlimited. These markers are independent of environment and developmental stages, which makes them potentially useful in the description of cultivars required for proprietary rights or labels of origin (Dulson et al., 1998).

PCR based techniques are generally quick and straight forward to perform, and the fact that PCR requires only small amounts of DNA makes it useful even with *in vitro* plantlets. Since there is little sequence information of the olive genome available, most of the studies have been performed using 'arbitrary' primers in a PCR reaction called RAPD (Williams et al., 1993) which is an efficient and relatively simple way to generate molecular markers that have enhanced the saturation of genomic regions near specific loci. It is quick, easy and requires no prior sequence information. Marker assisted selection with RAPDs is not always possible because RAPDs designed for one population are not always polymorphic for another, or since they lack reliability among laboratories (Jones et al., 1997; McGregor et al., 2000). The use of RAPD results for the characterisation and evaluation of germplasm and genetic resources – and for the identification of markers for use in breeding programmes at wide-scale – implies a standardisation to yield reproducible results since the error intrinsic to the procedure greatly compromises the value of the technique for marker-assisted selection and virtually precludes its use in seed quality control applications. Some attempts have been made to achieve such standardisation (Jones et al., 1997; Claros et al., 2000), but the best option to overcome these problems is the use of specific molecular markers.

Specific molecular markers can be deduced from unique, single-copy segments of the genome and can be considered co-dominant and can be used in closely related species (Shah et al., 2000), while non-specific markers like RAPD are dominant markers (the homozygote is not distinguishable from the heterozygote) and non-locus specific. There are three ways to obtain specific molecular markers: one type is deduced from microsatellite sequences that consists of tandemly repeated multicopies of small oligonucleotide motifs. Many microsatellite alleles are too large to be amplified, and the microsatellites identified so far are species specific and costly to develop. The second are markers deduced from gene sequences. This approach is the most recommended, although the paucity of olive-tree sequences makes this approach unlikely. The third way is to convert polymorphisms to a RFLP probe or a SCAR (Paran & Michelmore, 1993). SCAR markers are based on the sequencing of RAPD or AFLP fragments and further definition of more specific primers (Naqvi & Chatoo, 1996; Adam-Blondon et al., 1998; Negi et al., 2000). Hence, the PCR products allow reproducibility of the technique on a relatively wide range of reactive conditions and in different laboratories (Lawson et al., 1998). The use of restriction enzymes to detect polymorphic nucleotides by the loss or gain of a restriction enzyme recognition site is called CAPS (Konieczny & Ausubel 1993) and used to increase the number of polymorphisms detected by a single marker.

SCARs will allow for rapid marker development, even though they are not highly polymorphic. SCARs are advantageous over RAPD markers as (i) they detect only single, genetically defined loci, (ii) their amplification is less sensitive to reaction conditions, (iii) they can potentially be converted into codominant markers that will increase the available information in a marker-assisted selection program, (iv) they are not aware of the presence of introns that could eliminate the priming sites, (v) scoring results obtained by SCARs are more straightforward than other PCR-based markers. The ideal case is the one where SCARs can be used as an ASAP (presence/absence assay to detect the product (Gu et al., 1995)), eliminating the need for electrophoresis to resolve the amplifications as well as decreasing the cost and increasing the speed of the analysis. Consequently, SCAR markers offer the most practical method for screening numerous samples in a time and labour-saving manners, being accurate, feasible to use and cost efficient (Kasai et al., 2000).

In previous works, we have identified, characterised, and established relationships of 22 geographically related olive-tree cultivars in Spain (Claros et al., 2000). This approach was conducted with RAPD and AP-PCR markers and the resulting classification was used to support several Labels of Origin for the olive oil produced by some of these varieties. Since the reproducibility of polymorphisms described is essential for a guarantee certificate of accuracy, we have focused on the development of a minimal number of specific molecular markers (SCAR) that can identify the 22 cultivars in a reproducible and easy way. Indeed, the specific markers described here could be used in further assays of breeding and quality control.

Materials and methods

Plant material

The twenty-two different olive-tree cultivars defined in our previous work (Claros et al., 2000) were used. To represent the maximal genetic diversity occurring in a cultivar, 10 different trees (genotypes) were sampled from their natural sites.

Cloning and end-sequencing RAPD fragments

DNA extraction and RAPD analyses were performed as previously described (Claros et al., 2000). When polysaccharides were still present, the DNA sample was treated with CTAB (Murray & Thompson, 1980). The amplified products of the adequate RAPDs were excised from agarose gels. The gel slice was melted and diluted in water to decrease the agarose concentration at 0.4%. 1 μl of this solution was reamplified using the same primer that identified the RAPD polymorphism. After reamplification, the reaction mixture was adjusted to 5 mM $MgCl_2$, 1.5 mM $(NH_4)_2SO_4$, 10 mM 2-mercaptoethanol, 0.2 mM of each nucleotide triphosphate and 1 U of T4 DNA polymerase (Roche Biochemicals) were added. The reaction was incubated at 12 °C for 15 min and was stopped by adding 25 μl of TE and extracting with phenol/chloroform. The amplification was resolved on agarose gel electrophoresis and the DNA band was excised and purified by the phenol-freeze method (Bewsey et al., 1991). DNA was concentrated by precipitation with 0.3 M ammonium acetate and 2 ethanol volumes. DNA was blunt-end ligated into the pBluescript II SK^+ vector (Stratagene) that had been linearised with *Eco*RV. The ligation mixture also contains 2.5 U of *Eco*RV to prevent vector recircularisation (neither the vector nor the insert were dephosphorylated) to increase the ligation efficiency. The presence of the fragment in the clone was assessed by restriction enzyme digestion and by PCR screening the bacterial colonies using forward and reverse primers. DNA was introduced into *E. coli* DH5α by electroporation in an Eppendorf Mod 2510 following the manufacturer's instruction. The cloned fragments were end-sequenced by the dideoxy-chain termination method (Sequenase II kit, USB) using the T7, T3 or SP6 primers. The resulting markers (Table 1) were named starting by a 'P' if they are maintained in pBluescript, or by a 'G' if they were cloned in a pGEM-T (Promega) respecting the manufacturer's instructions. The two following characters correspond to the names of the RAPD primer that amplified the polymorphism, and the rest of characters are in function with the working name given to each band in a RAPD reaction.

SCAR primer design

For each cloned RAPD amplification product, two oligonucleotides (one from each end) were designed to be used as SCAR primers. Each primer contained the original 10 bases of the RAPD primer plus the next 10 internal bases from the end. In other cases, the 20 nucleotides were internal and no RAPD primer sequence was present. The GC contents of primers and the 3' end stability should also be considered to design repeatable primer sets (Vanichanon et al., 2000).

SCAR assay

Amplification of genomic DNA (1 ng/μl) with SCAR primers was carried out in a GeneAmp PCR system 2400 (Perkin-Elmer) in standard PCR reaction that consisted in 30 cycles of 1 min at 91 °C, 1 min at the annealing temperature, and 2.5 min at 72 °C. Amplification products were resolved electrophoretically in a 2% or 2.5% agarose gel. The polymerases used were EcoTaq (EcoGen) and Biotherm (GeneLabo) at 0.025 U/μl. The annealing temperature was first calculated as 4-fold the number of GC plus 2-fold the number of AT and decreasing the result by 4 °C. In cases where the two primers possess very different Ta, the lowest one was chosen as the one to optimise. Each primer pair was tested up to 5 °C over and under this annealing temperature to obtain the optimum. In all cases, the restriction enzymes *Eco*RI, *Hin*dIII, *Rsa*I, *Hin*fI, *Taq*I, *Hae*III and *Hpa*II were tested to try to increase the degree of polymorphism, the result being a CAPS analysis (Konieczny & Ausubel, 1993). Reproducibility of each experiment was confirmed at least twice.

Amplified bands generated by RAPD and AP-PCR were visually scored as 1 if present and 0 if absent. In cases where several polymorphisms were present, each one received a different correlative number (Table 2). The conversion of the multistate data matrix to a binary matrix was performed with CAFCA v1.5 software. The matrix was analysed with the quartet-puzzling method (Strimmer & von Haeseler 1996) implemented in Puzzle 4.0, and the dendrograms were obtained by the UPGMA method. The resulting dendrograms were displayed by TreeView (Page, 1996).

Table 1. Characteristics of the SCAR loci derived from RAPD markers selected in this study to identify olive-tree cultivars

RAPD polymorphism[a]	Primer sequence[b]	SCAR marker	Cultivar used as source	Ta[c]	CAPS enzyme	#[d]
OPF8-342	F83-t7: CTCTGCCGATGGTCTAGGC	PF83	Hojiblanca	63	–	2
	F83-t3: CTCTGCCTGAAACAGAATT					
OPF8-342-B	F80-t7: CTCTGCCTGAGTTCTAGTGA	PF80	Hojiblanca	65	–	2
	F80-t3: CTCTGCCTGAACCAACAGAA					
opf8-308	f84-T7: CTCTGCCTGAACATATTGCT	PF84	Verdial de la	60	*Hin*fI	3
	F84-t3: CTCTGCCTGAGAGATGCCTA		Axarquía			
OPF6-650[e]	F5-U: GGGAATTCCGCAGAACTAAA	PF6	Hojiblanca	63	–	2
	F6-R: GGGAATTCCGGCACATCCTT					
OPF10-488	F10-U: GTGCCAATTTCTGACATTTT	GF10	Verdial de la	57	–	1
	F10-R: ACCTGATAGGGAAGATTTAT		Axarquía			
OPJ1-512	J11-t7: CCCGGCATAAATTTCGGGCTC	PJ11	Verdial de la	63	–	2
	J11-t3: CCCGGCATAAAACCAAATATT		Axarquía			
OPJ6-905	J65-t7: TCGTTCCGCAAGTTAAGAAC	PJ65	Acebuche fino	63	–	3
	J65-t3: TCGTTCCGCATGTATTGTGG					
OPJ18-230	J18-U: TGGTCGCAGACGGTACGCGT	GJ18	Verdial de la	63	–	3
	J18-R: TGGTCGCAGAACACGATTAC		Axarquía			
OPX4-400	X4B-U: CACTTCAACAACTTTATCGA	GX4B	Aloreña	57	*Eco*RI	3
	X4B-R: TTCATCTTCTTCCCACAAAG				*Rsa*I	3
OPAH2-978	H1-U: TCCACATCATAGCCAACCTT	GH1	Blanqueta	55	–	2
	H1-R: CATGATTATATAGTCCAGGC					
OPAH2-720	H2-U: CACTTCCGCTCATTGAAGTA	PH2	Picudo	60	*Rsa*I	5
	H2-R: CACTTCCGCTGAAACTTAAT					

[a] The first 4–5 letters refer to the primer name (Operon Technologies) used to identify the progenitor marker. The number refers to the size in bp of the amplified product assessed in a previous work (Claros et al., 2000).
[b] The underlined nucleotides represent the sequence of the progenitor RAPD primers.
[c] Optimal annealing temperature.
[d] Number of polymorphisms obtained with a marker. A 1 refers to a monomorphic band.
[e] Not described in previous work.

Results

We have previously analysed 601 conspicuous, highly reproducible RAPD bands that provide 62 polymorphisms to identify and characterise 22 olive-tree cultivars (including two feral forms) using bulk DNA (Claros et al., 2000). The criteria followed to select the polymorphic bands to be cloned were: (i) their reproducibility, (ii) the amount of DNA obtained in a single RAPD reaction, (iii) the economic relevance of cultivars that possess the polymorphisms, and (iv) the absence of other bands in the proximity to avoid contamination. The polymorphisms that were cloned and sequenced are shown in Table 1. Ligation of the purified bands with the corresponding plasmid gave us plasmids with inserts of the same size as the original RAPD polymorphism. These markers except GF10 were able to differentiate all cultivars considered in this work.

Marker GF10 provided, with the same intensity in all cultivars, a single, constant band of 358 bp that was used as an internal positive control rather than a significant marker. The same result was obtained with other SCAR primers deduced from internal sequences of different RAPD fragments (results not shown). The difference in size respect to the RAPD polymorphism (Table 1) is a consequence of the absence of the progenitor RAPD primer in the SCAR primer. Additionally, it should be noted that GH1 marker also lacks the RAPD primer sequence but it was revealed specific of the two feral forms (‘acebuche fino’ and ‘acebuche basto’) when the DNA polymerase used was EcoTaq, but not when the enzyme was Biotherm (results not shown). The sequence of this marker, as well as markers PH2 and PJ65, was not completed since a single

Table 2. Multistate data matrix concerning the cultivars and the SCARs used in this work. Numbering over 1 indicates that there are several polymorphisms in addition to the basic presence/absence. The markers are ordered like in Table 1.

	PF83	PF80	PF84 *Hin*fI	PF6	GF10	PJ11	PJ65	GJ18	GX4B *Eco*RI	GX4B *Rsa*I	GH1	PH2 *Rsa*I
Acebuche fino	0	1	2	1	1	1	2	1	1	2	1	2
Acebuche basto	1	1	2	1	1	1	2	1	1	1	1	2
Blanqueta	1	1	2	1	1	1	3	1	1	1	0	1
Arbequina	0	1	2	1	1	1	3	1	1	3	0	3
Nevadillo Blanco	0	1	0	1	1	1	3	1	1	1	0	1
Picudo Axarquía	0	0	0	1	1	1	3	1	2	3	0	1
Verdial Axarquía	1	1	2	1	1	1	3	1	1	1	0	3
Aloreña	1	1	1	1	1	1	3	1	1	1	0	1
Zorzaleño	1	1	0	1	1	1	2	1	1	1	0	1
Lechín Granada	1	1	0	1	1	1	2	1	1	1	0	3
Lechín Sevilla	0	1	0	1	1	1	2	1	0	1	0	3
Gordal Antequera	0	1	0	1	1	1	2	2	1	1	0	1
Manzanilla Sevilla	1	1	0	1	1	1	3	1	1	1	0	1
Verdial Ronda	0	1	0	1	1	1	2	2	0	1	0	4
Hojiblanca	1	0	1	1	1	1	2	1	2	1	0	1
Hojiblanca Gaona	0	0	1	1	1	1	0	0	0	1	0	4
Picudo Baena	1	1	0	1	1	1	3	1	1	1	0	1
Picual	1	0	0	1	1	1	3	1	1	1	0	1
Picudo Ronda	1	1	0	1	1	1	2	1	1	1	0	4
Moriso	0	1	1	0	1	0	0	2	0	1	0	0
Chorrúo	1	1	2	1	1	1	2	1	1	1	0	1
Picudo El Burgo	0	1	0	0	1	0	2	1	1	1	0	1

end-sequencing was not enough to complete the insert size. The third marker that does not contain the RAPD primer is GX4B, which amplified a single band of 337 bp. Both *Eco*RI and *Rsa*I provide polymorphisms, but in different cultivars, that enable us to obtain two CAPS markers from one SCAR. PH2 provide good CAPS results with *Hae*III, *Hpa*II and *Rsa*I, the last being the one that provided clearer differences, although it did not amplify anything with 'morisco' DNA (Figure 1). Due to the ploidity of olive-trees and the codominant nature of SCARs, it is tempting to assume that PH2 marker might amplify two alleles (cultivars 'verdial de la Axarquía' and 'blanqueta'), and both alleles are present in wild type olive trees (both kinds of 'acebuche'). However, additional work should be done to verify this hypothesis.

PJ65 marker contained the same 5' and 3' end-sequences as the cloned and sequenced band OPJ6-1278 from 'aloreña'. Indeed, both bands of 978 and 1250 bp were present in SCARs using primers J65-t3 and J65-t7. However, there is important sequence divergence in the internal part of the two sequences although the 5' and 3' ends were identical (results not shown). PJ11 amplified the same band everywhere but in 'picudo de la Axarquía' and 'hojiblanca Gaona'. It is interesting to note that primer PJ11-t7 possessed a theoretical Ta = 54 °C while it seems to work fine at 63 °C. Marker PF6 is not able to amplify DNA from 'picudo de la Axarquía' and 'hojiblanca Gaona' like PJ11. It does not add more information, but increases the reliability of the identification. Marker PF80 was obtained as a contaminant band during the cloning of PF83. PF84 amplifies the expected band of 264 bp as well as a new band of 750 bp in 4 cultivars. The digestion with *Hin*fI modifies the pattern of the 264 bp and allows the identification of 3 polymorphisms. GJ18 provides the 225 bp band and another of around 50 bp that could be an internal rearrangement. The amplification was repeated in different conditions and the band of 50 bp was always present in the same cultivars, prompting us to use it as a polymorphism.

Since the only aim of such analysis was to confirm that the pattern of polymorphisms revealed by the defined SCAR and CAPS markers in Table 1 were

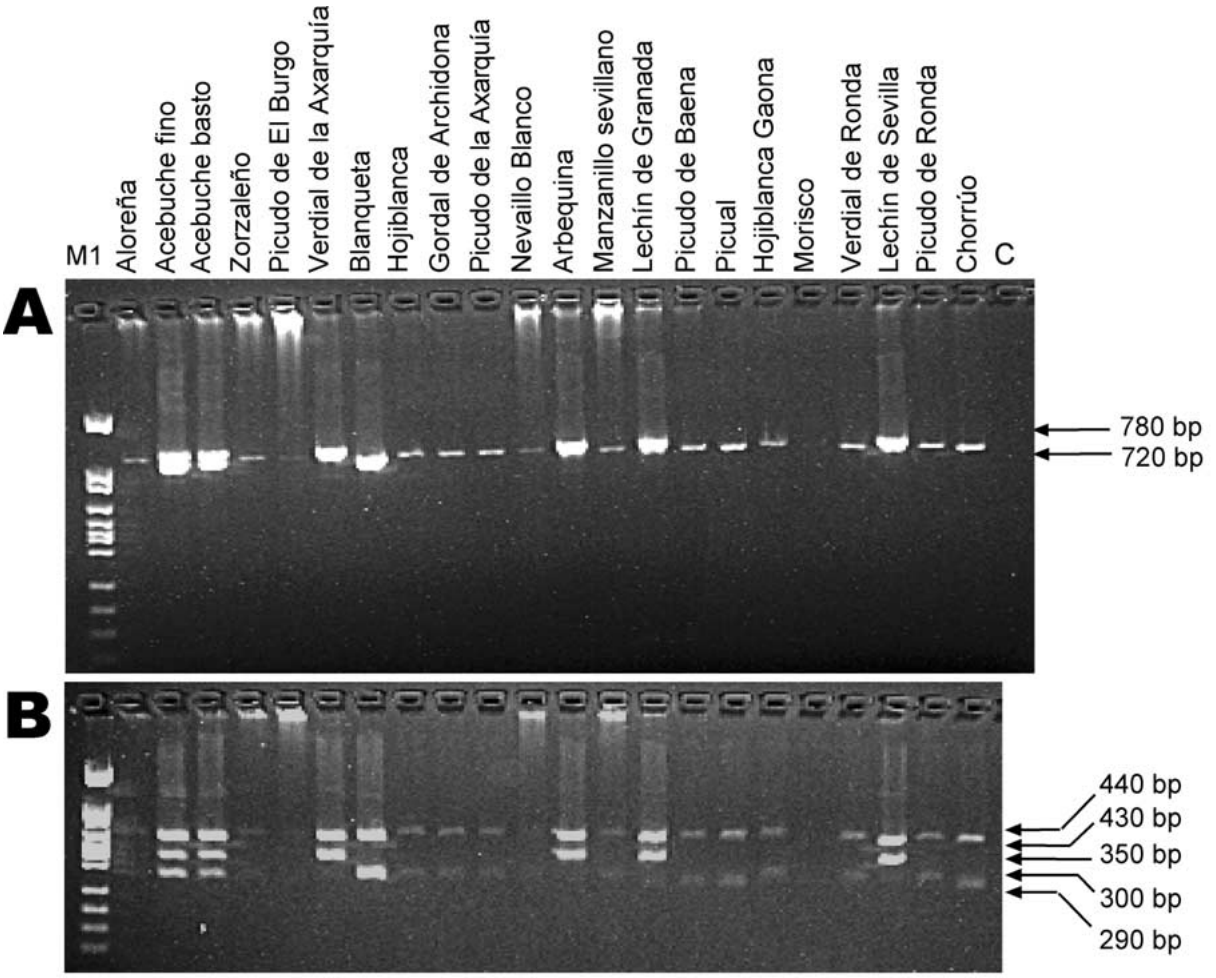

Figure 1. A) Result obtained with the 22 olive-tree varieties using the SCAR marker PH2 and a Ta of 60 °C. The apparent two polymorphic bands are signalled by arrows, indicating their molecular weight. B) CAPS performed with the restriction enzyme *Rsa*I on the previous SCAR amplificates. New polymorphisms, marked by arrows, are clearly distinguished.

enough to distinguish the cultivars as well as visualise the cultivars that are best and worst discriminated, we converted these data to a mulstistate matrix (Table 2) that served to build a dendrogram with the UPGMA method (Figure 2). Although cultivars were distinguished, it was not surprising that they were clustered in a meaningless way, even if they were subjected to a maximum likelihood analysis to obtain a matrix of distances between the cultivars. The arm length reflects the accuracy of the cultivar identification; hence, cultivars like 'hojiblanca Gaona', 'morisco' or 'picudo de El Borge' are the best differentiated, while others like the two feral forms ('acebuche'), 'zorzaleño' and 'chorrúo', or 'blanqueta' and 'picudo de Baena' will need more markers to provide a confident result.

Discussion

The analysis of DNA polymorphisms is critical to modern genetic research. We have found that the use of RAPDs and bulk DNA for the development of specific markers such as SCARs, provides a robust, efficient method for marker development. SCAR primers deduced from internal sequences were less polymorphic than those that included the initial RAPD primer sequence, suggesting that the polymorphism is mainly presented in this decamer sequence as reported in other plants (Parasnis et al., 2000). Although these results are in agreement with the finding that SCARs did not detect high level of variation (Paran & Michelmore, 1993), it is not in agreement with the hypothesis

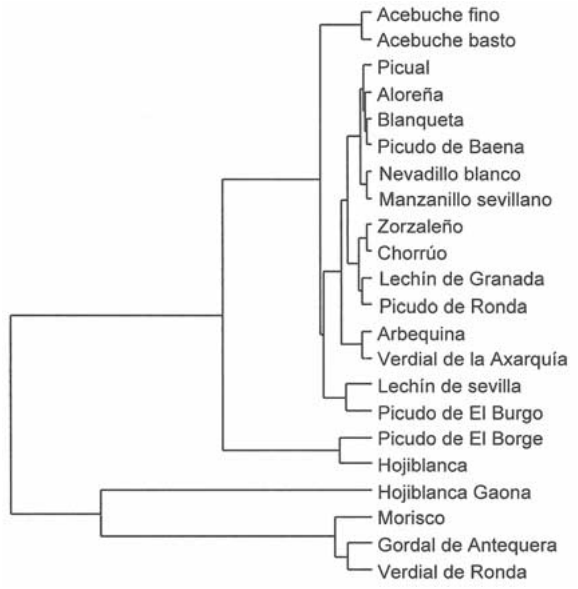

Figure 2. Dendrogram obtained by the UPGMA method on the distance matrix obtained by the maximum likelihood method. Branch length is proportional to the amount of markers that differentiate the cultivars.

that RAPD primers may not anneal with the exact genome sequence. On the contrary, it should be considered in accordance with results of laboratories that have prepared CAPS markers with oligonucleotides that do not contain RAPD polymorphisms (Bodénès et al., 1997; Moury et al., 2000). This can be explained considering that the predicted annealing temperature for the RAPD primers is 32 °C and the reactions are performed at 37 °C, which is more stringent. In fact, RAPD primers provide specific amplification even at 45 °C (Williams et al., 1993) and there are more and more laboratories that currently use 40–42 °C as PCR annealing temperature. Another explanation could be that the SCARs amplifications are present in more cultivars than the RAPD band used to obtain the sequence. In any case, converting RAPD fragments into SCAR markers did not lead to the same conclusions as the RAPD fragments themselves: this change does not affect the identification of loci involved in differentiation, but may modify the assignment of alleles (Bodénès et al., 1997).

The SCAR markers described here have some advantages over RAPD markers used to distinguish olive-trees. The use of RAPD markers allowed us to identify molecular markers within a few months, but due to their dominant nature, the amplification of multiple loci, and their sensitivity to reaction conditions, restricted their further use. SCARs are more independent to reaction conditions, and are usually codominant markers that amplify a single locus, making them robust and more reproducible assay than could be obtained with the short primers used for RAPD analysis. This is the reason why we have used primers a little shorter than the 24-mer described by Paran and Michelmore, since they can produce fragments in most genotypes (Gu et al., 1995).

The simplification introduced in the technology by SCARs results in a lack of phylogenetic information (Ruas et al., 1999). We have chosen the UPGMA algorithm since we only want to discover similarities among cultivars without any phylogenetic meaning. Comparing the current dendrogram (Figure 2) with the phylogram described by Claros and co-workers (2000) for the same cultivars can be concluded that phylogenetic information is better obtained with RAPD. This can be explained taking into account that RAPD gathers in the same experiment a lot of different sequences belonging to all chromosomes of the genome. In contrast, SCARs are very small pieces of sequence which cannot be considered representative of the complete genome.

Sequences around the RAPD priming sites of SCAR markers developed in this work are not polymorphic, because primers designed beyond the priming site provide less polymorphisms. We were obliged to use restriction enzymes to increase the polymorphism of some SCAR markers like GX4B or PF84 to have a reliable discrimination among cultivars with the least number of assays. It is not unusual that SCAR primers designed for a single, polymorphic band often lead to monomorphic amplifications and loss of the initial polymorphism (Paran & Michelmore, 1993; Bodénès et al., 1997).

In the aim of identifying olive-tree cultivars, the origin of the DNA used is not important provided that it can differentiate cultivars. For example, markers like PJ65 and PH2 seem to amplify two forms of the same or a different marker: when they amplify a single band, the pattern after an enzymatic digestion is simple while the presence of two bands reflects the mixture of two patterns (Figure 1). As we use bulked DNA, we cannot know if the different amplifications corresponded to different alleles from the same locus, or different alleles from different individuals, or different, independent loci. Hybridisations and genetic crosses should be performed to ellucidate this point, and this goes beyond the objective of this work.

The 22 olive cultivars can be distinguished using only 10 among the 12 SCAR markers described in Table 2 since GF10 provides the same result in all cultivars and PF6 and PJ11 provide identical polymorphisms in all cultivars. This simplify the former RAPD study that, repeating at least three times each reaction, quantified more than 500 bands to do the same task (Claros et al., 2000). Since commercial olives are propagated asexually, different accessions of individual cultivars would be expected to show a high degree of genetic similarity, as previously reported (Mekuria et al., 1999). Departures from this generality might occur due to somatic mutation or to misnaming of cultivars. Recently, it has been proved that domestication induces at least morphological changes (Massei & Hartley, 2000), and that cultivar selection has occurred in different genetic pools and in different areas (Besnard et al., 2001). In this context, the use of bulk DNA produces a series of markers that take into account the genetic diversity of a cultivar (Gemas et al., 2000). The markers described in this work, since derived from bulked DNA obtained from 10 different trees of the same cultivar, cannot be considered specific for a individual but a cultivar. A future added value of these SCAR markers is their utility as genetic markers to construct linkage maps. At the moment, this is very limited since there is no F_2 population identified to our knowledge. The long juvenile phase and the enormous culture extension that should be cultivated are slowing down this kind of analyses. The longer primers used in SCAR can tolerate mismatches that would not be in RAPD assays (Vanichanon et al., 2000).

In conclusion, we have been able to describe a cheap, reliable procedure to identify 22 geographically related olive-tree cultivars. More markers should be added to increase the confidence of the assay for cultivars like 'zorzaleño', 'chorrúo', 'blanqueta' or 'picudo de Baena'. In a near future, the markers ought to be adapted for an ASAP procedure or to be displayed as a microarray to simplify the technique.

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