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Development of SCAR markers for germplasm characterisation in olive tree (*Olea europea* L.)

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Abstract

A set of 14 SCAR markers were developed starting from RAPD, AFLP and SAMPL analysis of several olive germplasm accessions. Eight RAPD, two AFLP and four SAMPL fragments were converted into dominant and codominant SCARs by cloning and sequencing the selected fragments. The markers obtained were evaluated on forty different olive cultivars from different Italian production areas (mainly from Liguria). The combined use of these SCARs made possible to univocally identify 26 cultivars while the remaining 14 will require the development of further markers since most of them are placed in a main group containing six genetically similar cultivars (among which Frantoio and Taggiasca) and four minor groups containing two cultivars each. A total of 31 different haplotypes were identified and the analysis of several individual plants indicated no intra-cultivar variability. Considering the SCAR polymorphism two alleles were scored for each markers with the only exception of markers IGPS3 and IGPS4 showing 4 alleles with 7 recognised groups and 5 alleles with 4 groups, respectively. Though less polymorphic in comparison with other markers like SSRs, the developed SCARs proved useful in genotype identification. In addition, they could potentially be used for breeding applications and forensic analysis.

Introduction

The olive tree is one of the most ancient fruit tree species cultivated in the Mediterranean area. Its domestication probably occurred in the Near-East some 5500–5700 years ago. The distribution of cultivar mitotypes has shown that the prevailing displacement of cultivars occurred from East to West (Besnard and Bervillé 2000). Nevertheless these cultivar transfers did not exclude an original breeding effort within the western countries, from the locally available wild olive trees (Besnard et al. 2001).

In modern times, the olive tree is mostly found around the Mediterranean basin and the cultivars in this area hold great genetic variation. Bartolini et al. (1998) lists 1200 cultivars with more than 3000 different common names in germplasm collections located in 24 countries. This diversity is mainly due to local selection of outstanding genotypes and subsequent cloning.

The identification of olive cultivar is a topic of great economic importance especially when its cultivation is expanding and there is growing commercial interest for quality products.

The identification of olive-tree cultivars has been traditionally carried out by morphological and agronomic traits. Identification based on the analysis of isozymes has also been used (Trujillo et al. 1995). Although these markers provide a

very useful tool for cultivar identification, they have limitations because of the small number of polymorphisms detected and of environmental and cultivation influence.

The introduction of Polymerase Chain Reaction (PCR; Mullis and Faloona 1987) and the subsequent development of DNA fingerprinting methods have introduced the possibility to univocally identify a cultivar or a clone from a specific area. Some of these molecular techniques have already been successfully applied to the classification and identification of olive cultivars. Among them, Random Amplified Polymorphic DNA (RAPD; Fabbri et al. 1995; Belaj et al. 2001), Amplified Fragment Length Polymorphism (AFLP; Angiolillo et al. 1999), and Simple Sequence Repeat (SSR; Rallo et al. 2000; Sefc et al. 2000; Carriero et al. 2002; Cipriani et al. 2002) have been widely applied.

The conversion of AFLP, RAPD and other molecular markers to Sequence Characterised Amplified Regions (SCARs; Paran and Michelmore 1993), based on sequence data, significantly improves the reproducibility and reliability of PCR assays, and therefore their utility for many applications, such as marker assisted selection (MAS) and cultivar identification. Unfortunately, a high percentage of these markers become monomorphic when primers are extended based on cloned sequences (Hernández et al. 1999) and therefore the development of SCAR is demanding work. Hernández et al. (2001) reported the development of SCAR markers by directly sequencing RAPD products and they showed that the generated markers were useful for the MAS of the high flesh/stone ratio and suitable for further applications such as cultivar identification and mapping.

In the present paper we are reporting on the development of a set of molecular SCAR markers obtained by cloning and sequencing some polymorphic RAPD, AFLP and Selective Amplification of Microsatellite Polymorphic Loci (SAMPL; Morgante and Vogel 1994) DNA fragments usable for cultivar identification.

Material and methods

Plant material

Forty *O. europea* L. cultivars were analysed (Table 1). Some varieties were sampled from an olive germplasm conservation field (Villa Pratola, Santo Stefano Magra, La Spezia, Italy) held by the Consiglio Regionale Liguria, from 1 to 3 individual samples were considered for each variety. Other genotypes were provided by partners of Oliv-Track project (QLK1-CT-2002-02386), from the olive germplasm bank of Córdoba, Spain. The remaining genotypes were sampled from individual plants in specific areas in Italy.

DNA extraction

Total DNA extraction from leaf tissue followed the CTAB method by Doyle and Doyle (1987) with the only modification concerning the concentration of CTAB. Five grams of leaves were ground in liquid nitrogen and incubated in 20 ml of 10× CTAB buffer (10% CTAB, 75 mM Tris–HCl pH 8.0, 15 mM EDTA pH 8.0, 1.4 M NaCl, 0.2% β-mercatptoethanol) at 65 °C for 1 h. The other extraction steps were performed as previously reported.

RAPD analysis

Five random 10-mer primers were used (individually and in combination) for amplification: PLT250 TCGGCAACCA (AH1, Fabbri et al. 1995), PLT253 ACGGCGATGA (AI11), IGP820 GAAACGGGTG (Operon kit A7), IGP821 CCGTCGGTAG (Operon kit AB1), and IGP824 CACCGTATCC (Operon kit D12). The volume of the final reaction (20 μl) was made up of 1× buffer (buffer A, InCura), 3.5 mM MgCl2, 200 μM dNTPs, 1 μM primer (0.5 μM when two primers were uesd), 1.5 U *Taq* DNA polymerase (InCura) and 20 ng of template DNA.

Amplifications were performed in an Applied Biosystems 9700 thermal cycler with an initial denaturing step of 5 min at 94 °C, followed by 45 cycles of 40 s at 94 °C, 1 min at 35 °C, 2 min at 72 °C, and a final extension step of 5 min at 72 °C. PCR products were run on 2.5% agarose gel and DNA bands were visualised by ethidium bromide staining. As it is documented in literature (Pejic et al. 1998), RAPD markers suffer from a lack of reproducibility. Consequently, to confirm the electrophoretic patterns and the obtained polymorphic bands, every PCR was

Table 1. List of the studied genotypes and their origins. The coordinates refer to the field position of the genotypes collected at the olive germplasm field in Liguria. Each coordinate refers to a single plant, two or more coordinates in the same position refer to more individuals sampled for that cultivar in different field positions.

Genotype	Field position	Localities or collections
Castelnovina	D27	OGCF Liguria region (Italy)
Davide	G24	OGCF Liguria region (Italy)
Frantoio	A14–B15	OGCF Liguria region (Italy)
Lantesca	G28	OGCF Liguria region (Italy)
Lavagnina	C21–F15	OGCF Liguria region (Italy)
Leccino	A18–B24	OGCF Liguria region (Italy)
Liccione	L17–L19	OGCF Liguria region (Italy)
Mortina	H26–H27	OGCF Liguria region (Italy)
Negrea	I21–I22	OGCF Liguria region (Italy)
Merlina	H16	OGCF Liguria region (Italy)
Olivastrone	M9–M10	OGCF Liguria region (Italy)
Olivotto	H22	OGCF Liguria region (Italy)
Pignola	L14–L15	OGCF Liguria region (Italy)
Pignola SV	H6–H7	OGCF Liguria region (Italy)
Pinola	L23–L24	OGCF Liguria region (Italy)
Prempesa SP	E27	OGCF Liguria region (Italy)
Razzola SP	D19–E26–F24	OGCF Liguria region (Italy)
Rossese	C27	OGCF Liguria region (Italy)
Taggiasca	H12–H14	OGCF Liguria region (Italy)
Taggiasca IM	D15–E22	OGCF Liguria region (Italy)
Maurino		CNR Florence
Frantoio		CNR Florence
Biancolilla		OGB Cordoba
Canino		OGB Cordoba
Carolea		OGB Cordoba
Dolce Agogia		OGB Cordoba
Dritta		OGB Cordoba
Frantoio		OGB Cordoba
Leccino		OGB Cordoba
Gentile di Chieti		OGB Cordoba
Ottobratica		OGB Cordoba
Tonda Iblea		OGB Cordoba
Ogliarola Leccese		OGB Cordoba
Nocellara del Belice		OGB Cordoba
Bosana		OGB Cordoba
Sinopolese		OGB Cordoba
Cellina di Nardò		OGB Cordoba
Lezzo		Lake Garda (Italy)
Leccio		Lake Garda (Italy)
Casaliva		Lake Garda (Italy)
Moraiolo		Assisi (Umbria, Italy)
Frantoio		Assisi (Umbria, Italy)
San Felice		Giano (Umbria, Italy)
Taggiasca		Imperia (Liguria, Italy)
Cassanese		Sibari (Calabria, Italy)
Dolce di Rossano		Rossano Calabro (Calabria, Italy)
Leccino		Assisi (Umbria, Italy)
Tondina		Sibari (Calabria, Italy)

Legend: SV – Savona (Liguria, Italy); SP – La Spezia (Liguria, Italy); IM – Imperia (Liguria, Italy). OGCF – Olive Germplasm Conservation Field Liguria Region. OGB, Olive Germplasm Bank Cordoba. CNR, Centro Nazionale Ricerche, Firenze, Italy.

repeated twice under the same conditions of composition of reaction volume, amplification profile and handled thermalcycler (AB GeneAmp PCR System 9700). Selected fragments were excised and purified (gene-clean III kit, Bio101) from the agarose gel.

AFLP analysis

AFLP marker analysis was performed according to Vos et al. (1995). The following primer combinations were used: *Eco*RI + AGC/*Mse*I + AGC, *Eco*RI + AGC/*Mse*I + ACG, *Eco*RI primers were labelled with 33P. The amplified products from selective PCR were resolved by electrophoresis on 6% denaturing polyacrilamide gels and visualised by exposing a BioMax film for 24–48 h. Selected fragments were excised and purified from the acrylamide gel.

SAMPL analysis

The SAMPL analysis was performed according to Morgante and Vogel (1994) with some modifications. Four μl of a 15-fold diluted AFLP preselective PCR, carried out with *Eco*RI + A and *Mse*I + C preselective primers, were used as template for the amplifications. The final volume of the reaction (20 μl) was made up of 1× buffer (buffer A, InCura), 2.5 mM MgCl2, 200 μM dNTPs 0.5 μM of each primers and 1.5 U *Taq* DNA polymerase (InCura). The following primers combination were used: *Mse*I + CTA/IGP569 (TTGACTACGAATTCACACACA) and *Eco*RI + ACT/IGP569. PCR reactions and amplification products electrophoresis were performed using the same conditions as for RAPD.

Cloning and sequencing of the polymorphic fragments

The selected fragments were ligated into the pGEM-T easy vector (Promega) and then transformed by electroporation into *E. coli* strain DH5α. PCR reactions for cycle-sequencing were performed using the following profile: 35 cycles of 20 s at 94 °C, 15 s at 50 °C, and 1.5 min at 60 °C. The final volume of the reaction (20 μl) was made up of 2 μl of sequencing reagent premix (DYEnamic ET Terminator Cycle Sequencing Kit, Amersham Pharmacia), 6 μl 5× sequencing Buffer (400 mM Tris–HCl, 10 mM MgCl2, pH 9.0) 5 pmol of primers (T7 and SP6) and DNA (10–100 ng). Unincorporated dye terminators were removed by ethanol precipitation and sequences were loaded on the ABI Prism 3100 Genetic Analyser (Applied Biosystems). Refer to the manual for electrophoresis procedures.

SCAR analysis

Based on the sequence of the cloned fragments, we designed the pairs of oligonucleotide primers reported in Table 2. PCR reactions were carried out in a final volume of 20 μl, containing 20 ng DNA, 1× buffer (buffer A, InCura), 2 mM MgCl2, 150 μM of each dNTP, 5 pmol of each primer, and 1 U *Taq* DNA polymerase (InCura). The following cycle was applied: 5 min denaturation at 95 °C, 35 cycles of 30 s denaturation at 95 °C, 40 s at the appropriate annealing temperature (Table 2), 1 min 30 s extension at 72 °C. The amplified products were resolved by electrophoresis on agarose gel and visualised by ethidium bromide staining. Primer IGP317 was dye labelled (6FAM) and the electrophoresis for primer pair IGP317/318 was carried out using the ABI Prism 3100 Genetic Analyser.

Results and discussion

Three different molecular analysis methods (RAPD, AFLP and SAMPL) were performed in search for DNA polymorphisms, which can be used for generating informative PCR-based molecular SCAR markers and defining the individual cultivars. Five RAPD primers (see material and methods) were used, individually or in combination.

Only the fragments confirmed by repeated amplifications were considered useful for generating SCAR markers. The most interesting bands were the cultivar-specific ones, characteristic of the individual varieties. Alternatively, we also considered the fragments present in a restricted number of cultivars (Figure 1a, b).

At the end of RAPD analysis we noticed that, on the one hand, the obtained electrophoretic patterns enabled us to identify many of the cultivars, as reported in other works (Belaj et al. 2001), while on the other hand the number of cultivar specific polymorphic fragments was very low. The developed SCAR markers are reported in Table 2.

Starting from polymorphic RAPD fragments, the following SCAR markers were developed: IGPS3, IGPS5, IGPS6, IGPS9, IGPS11, IGPS12, IGPS13, and IGPS14.

Table 2. Primer sequences, type of polymorphism, annealing temperature and expected size for each markers.

Locus	Polymorphism	Primer	Annealing temperature	Expected size
IGPS1	Presence/absence	IGP700 CTATATTAGCTTGACTCGTTGC IGP701 AATTCACACACATATTCCACAAG	56 °C	591 bp
IGPS2	Presence/absence	IGP422 AGACAGGTCCAAAACTCAGC IGP423 GCTTATAAGGCTGCCATAGC	60 °C	137 bp
IGPS3	Different dimensions	IGP317 TCGGCAACCATGTGGTAGTG IGP318 TGACCACCATCACCCAAAAAC	60 °C	432 bp
IGPS4	SNP	IGP706 CACTGGGAGTACAAATTCAT IGP707 CACACACAGAGTCAGAG	50 °C	202 bp
IGPS5	Double/single band	IGP558 ACGGCGATGATGATAACAGCC IGP559 ACGGCGATGACCAAGCG	56 °C	364 bp
IGPS6	Presence/absence	IGP738 CCAATGCAGGAAGAAAGTCGG IGP739 ATGAGAGCGAGGATGAGGATG	From 62 to 56 °C	481 bp
IGPS7	Double/single band	IGP386 GCCACAAAATCTGCAAGGAC IGP387 GCAGGTACTGTAGAAAATGG	56 °C	113 bp
IGPS8	Presence/absence	IGP702 CACTACAATCAATAGCAA IGP715 ACATAACGGCGTGTATAG	56 °C	104 bp
IGPS9	Double/single band	IGP876 CCGTCGGTAGGGCACATAG IGP877 GAAACGGGTGGAGGCTCTC	56 °C	364 bp
IGPS10	Presence/absence	IGP753 CACACACACCAAAAAAATCCC IGP754 AAGAGAGGCCATAGGTGATAC	56 °C	261 bp
IGPS11	Presence/absence	IGP878 CACCGTATCCCCCTGTATGT IGP879 CACCGTATCCAGAGTCGGG	56 °C	458 bp
IGPS12	Presence/absence	IGP882 GAAACGGGTGCCCACCTAC IGP883 GAAACGGGTGATAGTATATAGTA	56 °C	399 bp
IGPS13	Presence/absence	IGP884 GAAACGGGTGGGATAGGGG IGP885 GGGTGCTACGAGACCAGCA	58 °C	430 bp
IGPS14	Presence/absence	IGP889 GAAACGGGTGGGAATATCTATAC IGP890 CCGTCGGTAGATCTGCAATATT	60 °C	653 bp

In two cases the conversion from RAPD to SCAR, by the development of longer and more specific primers, maintains the original RAPD polymorphism among the cultivars: IGPS6 amplifies only in cultivar Cassanese and IGPS13 in cultivar Ogliarola Leccese. In the remaining markers the original RAPD polymorphism is lost. Marker IGPS5 and IGPS9 amplify in all the cultivars but a second discriminating band is always present in some genotypes (Table 3). For markers IGPS11, IGPS12 and IGPS14 the amplification products are observed in more than one cultivar (Table 3). Marker IGPS12 recognises a cluster of cultivars that are genetically similar: Frantoio, Razzola, Taggiasca, Lavagnina, Davide, and Casaliva. Additionally, the amplification product is always present, even if more weakly, in cultivar Leccino (Figure 2a, b).

The electrophoretic pattern of IGPS14 for some cultivars is reported (Figure 3).

Marker IGPS3 amplifies in all the utilised cultivars; however, it shows a high degree of polymorphism in the dimension of the amplification products, showing 4 different alleles that define 7 different groups (Table 3); cultivar Tonda Iblea and Carolea are characterised by single haplotypes and therefore are distinguishable from the remaining genotypes.

Starting from AFLP polymorphic fragments, the SCAR markers IGPS2 and IGPS7 were developed. Marker IGPS2 amplifies in several cultivars and IGPS7 amplifies in all the cultivars, but varieties San Felice and Dritta show two bands in the amplification product. The remaining SCAR markers were developed starting from SAMPL polymorphic fragments. For markers IGPS1 and IGPS8 the products of PCR are present in several samples. Marker IGPS4 (from SAMPL fragment specific for cultivar Moraiolo) amplifies in all the cultivars. However, two polymorphisms are present in the sequence (SNP, Single Nucleotide Polymorphism). A first SNP is found in position 89 from primer IGP706. Such polymorphism consists of a heterozigosity G/T, while T is found in all the remaining cultivars. The second SNP is found in position 123, the three possible alleles

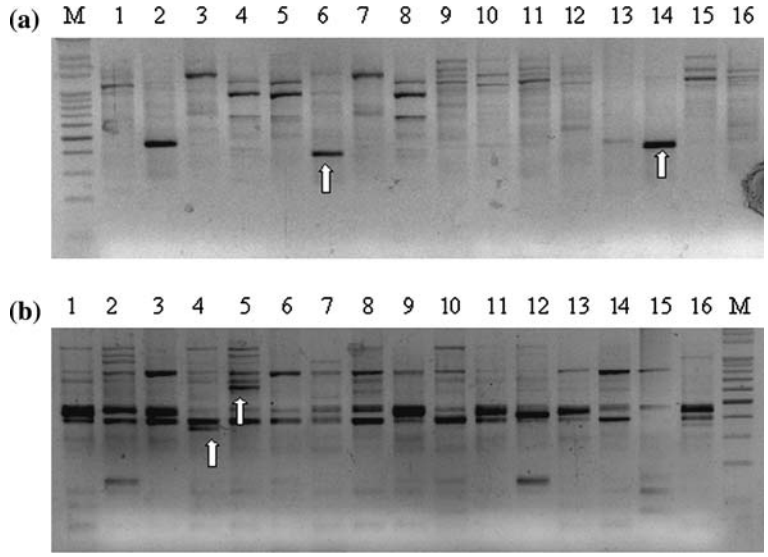

Figure 1. (a, b) Electrophoretic pattern for RAPD markers. (a) IGP820 RAPD marker: (M) 100 bp DNA molecular marker (InCura), (1) Biancolilla, (2) Canino, (3) Carolea, (4) Castelnovina, (5) Dritta, (6) Franotio, (7) Dolce Agogia, (8) Leccino, (9) Ottobratica, (10) Nocellara del Belice, (11) Cellina di Nardò, (12) Tonda Iblea, (13) Sinopolese, (14) Ogliarola Leccese (15) Gentile di Chieti, (16) Negrea. IGPS12 and IGPS13 markers were developed starting from the RAPD fragments present respectively in sample number 6 (S12) and number 14 (S13) pointed by the arrows. (b) IGP820/IGP821 RAPD marker combination: (1) Biancolilla, (2) Canino, (3) Carolea, (4) Cellina di Nardò, (5) Dolce Agogia, (6) Dritta, (7) Frantoio, (8) Gentile di Chieti, (9) Leccino, (10) Ottobratica, (11) Nocellara del Belice, (12) Ogliarola Leccese, (13) TondaIblea, (14) Bosana, (15) Sinopolese, (16) Castelnovina, (M) 100 bp DNA molecular marker (Incura). The pointers indicate some of the fragments used for the conversion in SCAR markers.

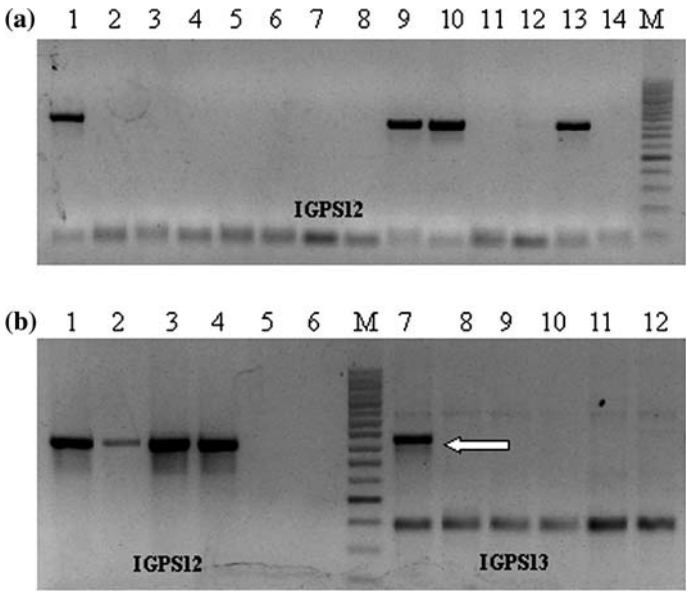

Figure 2. (a, b) Electrophoretic patterns of SCARs IGPS12 (2a, b) and IGPS13 (2b). (a): (M) 50 bp DNA molecular marker (InCura), (1) Frantoio, (2) Maurino, (3) Prempesa, (4) Castelnovina, (5) Merlina, (6) Pinola, (7) Pignola, (8) Moraiolo, (9) Razzola, (10) Lavagnina, (11) Olivastrone, 12) Olivotto, 13) Davide, (14) Mortina. (b): (1) Franotio, (2) Leccino, (3) Razzola, (4) Taggiasca, (5) Lantesca, (6) Rossese, the amplification product is from SCAR IGPS12. (M) 50 bp DNA molecular marker (InCura), samples (7) Ogliarola Leccese, (8) Leccino, (9) Frantoio, (10) Taggiasca, (11) Lantesca, (12) Rossese refer to SCAR IGPS13 the expected signal is present only in cultivar Ogliarola Leccese.

Table 3. Cultivar IGP markers profile.

Cultivars	IGP – Markers													
	S1	S2	S3	S4	S5	S6	S7	S8	S9	S11	S12	S13	S14	Haplotypes
Lezzo	–	–	B	TG	α	–	β	–	β	–	–	–	–	1
Leccio	–	–	B	TG	α	–	β	–	β	–	–	–	–	1
Leccino	–	–	B	TG	α	–	β	–	β	–	+	–	–	2
SanFelice	–	–	B	TG	n.a.	–	α	–	n.a.	n.a.	–	–	n.a.	n.a
Dritta	–	–	B	TG	β	–	α	–	β	–	–	–	–	3
Casaliva	–	–	B	TA	β	–	β	–	β	–	+	–	–	4
Taggiasca	–	–	B	TA	β	–	β	–	β	–	+	–	–	4
Razzola	–	–	B	TA	β	–	β	–	β	–	+	–	–	4
*Lavagnin*a	–	–	B	TA	β	–	β	–	β	–	+	–	–	4
Davide	–	–	B	TA	β	–	β	–	β	–	+	–	–	4
Frantoio	–	–	B	TA	β	–	β	–	β	–	+	–	–	4
Moraiolo	–	+	A	TR	β	–	β	+	β	–	–	–	+	5
Prempesa	–	+	A	TR	β	–	β	+	β	–	–	–	+	5
Castelnov.	–	+	A	TA	β	–	β	–	α	–	–	–	+	6
Merlina	–	+	A	TA	β	–	β	+	β	–	–	–	+	7
Canino	+	–	B	TA	β	–	β	–	β	–	–	–	+	8
DolceRoss.	+	–	B	TG	α	–	β	–	β	–	–	–	+	9
Maurino	–	–	C	TA	α	–	β	–	β	+	–	–	+	10
Sinopolese	–	–	C	TA	β	–	β	+	β	–	–	–	+	11
Cassanese	–	–	C	TR	β	+	β	–	n.a.	n.a.	–	–	n.a.	12
Tondina	–	–	C	TG	β	–	β	+	α	–	–	–	–	13
Lantesca	–	+	B	TG	β	–	β	+	β	–	–	–	–	14
Ogliarola	+	+	E	TA	β	–	β	–	β	+	–	+	+	15
Rossese	–	+	E	KG	β	–	β	+	β	–	–	–	–	16
Mortina	–	+	E	KG	β	–	β	+	β	–	–	–	–	16
Negrea	+	+	E	TA	α	–	β	+	α	–	–	–	+	17
Pinola	–	–	D	TR	β	–	β	+	β	–	–	–	+	18
Olivotto	–	–	D	TA	β	–	β	–	β	–	–	–	–	19
Bosana	–	–	D	TR	β	–	β	–	β	–	–	–	–	20
Biancolilla	–	–	D	TG	β	–	β	+	β	–	–	–	+	21
Olivastra	–	–	D	TG	β	–	β	–	α	–	–	–	–	22
Ottobratica	–	–	D	TR	β	–	β	–	α	+	–	–	+	23
GentileCh.	–	–	D	KG	β	–	β	–	β	–	–	–	–	24
Pignola	–	–	D	TG	β	–	β	–	β	–	–	–	+	25
Liccione	–	–	D	TA	β	–	β	+	α	–	–	–	–	26
Carolea	–	+	G	TG	α	–	β	+	β	–	–	–	+	27
DolceAgog	–	+	C	KG	α	–	β	–	β	–	–	–	+	28
Noc.Belice	–	+	E	TG	β	–	β	+	β	–	–	–	+	29
TondaIblea	–	–	F	KG	β	–	β	+	β	–	–	–	+	30
CellinaNar	+	–	B	TA	β	–	β	+	α	+	–	–	+	31

Legend: n.a. not assigned; IGPS1, IGPS2, IGPS6, IGPS8, IGPS11, IGPS12, IGPS13, IGPS14: + = Presence, – = Absence; IGPS3 different letters refer to the different allelic forms detected; IGPS4 different letters refer to the different allelic forms detected: TA = T83, A123, TR = T83, A/G123 (code IUPAC, A/G = R), TG = T83, G123, KG = G/T83 (code IUPAC, T/G = K), G123; IGPS5, IGPS7, IGPS9: α = Double Band, β = Single Band. Liguria cultivars are underlined. Numbers from 1 to 31 refer to the different haplotypes, varieties with an identical SCAR pattern have the same number.

being A, A/G and G. The marker detects 4 groups based on the different combinations of the SNP polymorphisms present in the sequence.

Marker IGPS10 (developed from SAMPL fragment specific for cultivar San Felice) maintains its specificity if the product of an AFLP preselective PCR is used as template for the PCR, while no amplification is obtained starting from genomic DNA.

The combined use of the markers IGPS1, IGPS2, IGPS3, IGPS4, IGPS5, IGPS8, IGPS9, IGPS12, and IGPS14 enable us to divide the 16 starting Ligurian cultivars into 12 clusters. Almost all the cultivars are recognised univocally

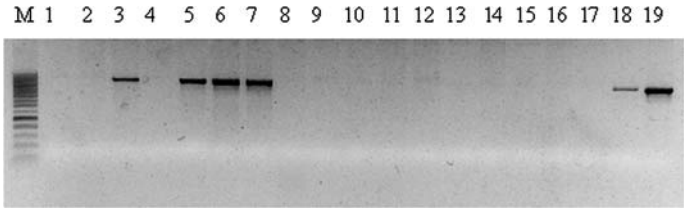

Figure 3. Electrophoretic pattern of SCAR IGPS14 for some varieties. This marker was developed starting from the RAPD fragment in sample number 5 (Dolce Agogia) indicated by a pointer in Figure 1b. (M) 50 bp DNA molecular marker (InCura), (1) Leccino, (2) Frantoio, (3) Dolce Agogia, (4) Rossese, (5) Carolea, (6) Maurino, (7)Pinola, (8) Taggiasca, (9) Razzola, (10) Davide, (11) Lantesca, (12) Lavagnina, (13) Tondina, (14) Bosana, (15) Lezzo, (16) Leccio, (17) Casaliva, (18), Moraiolo, (19) Merlina.

(Table 3). Two groups have not been resolved yet. The main group contains some cultivars that are genetically similar: Taggiasca, Razzola, Lavagnina, and Davide. A second group contains the cultivars Rossese and Mortina. If all 40 cultivars are considered, using the 9 markers previously reported with the addition of marker IGPS7, the samples can be divided into 31 different clusters. The main group is the same as the one for the Ligurian varieties, which, in addition to the already mentioned cultivars, also includes cultivars Frantoio and Casaliva (often considered to be very similar, Cipriani et al. 2002). The same group is obtained also with other classes of more polymorphic markers (SSRs and AFLPs) and always the genetic differences result very low (data not shown). Additionally, we obtain three minor groups, respectively including cultivars Lezzo and Leccio, Moraiolo, and Prempesa, Dritta and San Felice (Table 3). The haplotype is not determined for cultivar San Felice because there was no DNA available to carry out all the amplifications. However for all the tested markers we obtain the same results as for cultivar Dritta, which is considered as synonim of San Felice in other works (Marchionni et al. 1999) Figure 3).

No intra-cultivar variability is found when more than one plant per cultivar is analysed. Among the others, for cultivars Frantoio and Leccino, samples are considered from Cordoba olive germplasm bank, Liguria olive germplasm conservation field, and a production site in Italy (Table 1). Again, no differences are found, which, even though the number of sampled individuals is small (respectively 5 for Frantoio and 4 for Leccino), indicates homogeneity in the plant material. However, besides the small number of individuals, we should also consider the fact that usually the SCAR markers do not reveal high levels of polymorphism for single locus. Other classes of more polymorphic molecular markers, in particular SSRs, are preferred when searching for intra-cultivar variability.

As far as the degree of polymorphism is concerned, the most developed markers show two allelic forms. This is observed both for the markers characterised by presence/absence of the amplification product and for those (IGPS5, IGPS7 and IGPS9) that always amplify but with differences in the number of bands of the resulting product. The only exceptions are observed with markers IGPS3 and IGPS4.

IGPS3 is a separate case, to wit it reveals a number of alleles similar to that of some SSR markers. Carriero et al. (2002) developed and used some SSR markers to analyse some Italian cultivars. In this case the number of detected alleles, not considering the monomorphic ones, is in the range 2–9. This marker shows a repeated motif (GGCTATn) in the sequence. Variations in the number of repetitions of this exanucleotide motif might determine the marker polymorphism; in some cultivars, such as Moraiolo, this motif is repeated 5 times, in other cultivars only 3 times.

The classes of markers which are generally used for genotyping, thanks to the high level of polymorphism revealed for single locus or single assay, are RAPDs, SSRs, and AFLPs. Belaj et al. (2004) found that a combination of three highly polymorphic RAPD primers was optimal to discriminate among 103 cultivars with low cumulative confusion probability. SSRs are capable of revealing many allelic forms and more information for single locus, while AFLPs detect the highest number of polymorphisms in a single assay (Pejic

et al. 1998). In spite of the lower level of polymorphism found at the end, if compared to the listed markers, the developed SCARs show their utility for genotyping olive trees, despite the small number of different genotypes, a substantial number of haplotypes (31) were identified. Two markers are diagnostic of single genotypes (IGPS6 and 13) in our sample set, while the others recognise more than one genotype.

Not all fourteen markers are needed to obtain the highest number of haplotypes; the result can be achieved with 10 markers, while IGPS6, 10, 11, and 13 are not necessary. In order to determine the discriminating power of the markers, *D* value was evaluate as defined by Tessier et al. (1999). Since the most markers are dominant, when considered individually, the obtained values are low, ranging from 0.05 for diagnostic SCARs to 0.7857 for IGPS3. The mean value is low, 0.32913 when all 14 markers are considered and 0.40614 if only the ten markers needed to define the 31 haplotypes are considered. As expected, the markers showing the lowest *D* values are the two cultivar specific ones, whose discriminating level is very low, but which could prove very useful for the definition of individual varieties, for certain purposes, like breeding programmes, forensic analysis, and olive oil traceability. There is a sharp increase in the *D* value if two or more markers are amplified simultaneously. Considering the different amplification conditions, we have started to develop three different multiplex PCRs, with 2 (IGPS4 and S8), 3 (IGPS2, S3 and S14), and 5 (IGPS1, S5, S7, S9, and S12) markers respectively. Up to now good results have been obtained with the first two reactions, while the last one is still being developed. The reaction with S4 and S8 defines 8 different haplotypes with $D = 0.8552$; the reaction with S2, S3 and S14 defines 14 haplotypes with $D = 0.8923$; the last multiPCR defines 10 haplotypes with $D = 0.7888$ (data not shown). These values for *D* are comparable with those reported by Belaj et al. (2004) for RAPD markers.

The assay efficiency of single locus markers like SSRs (Heyen et al. 1997) can be increased if multiplex PCR reactions and gel-running procedures are adopted, where several markers are simultaneosly amplified and co-electrophoresed using multicolour fluorescent technologies; the same is true also for SCARs.

These SCARs could potentially be used in breeding programmes; examples of this are the cultivar specific SCARs and by SCAR IGPS12 that is present in a cluster of genetically similar cultivars including Frantoio that is frequently used as pollen donor in the breeding programmes. For this reason it could be used for paternity testing of F1s when this variety is used as the male parent on crosses with females lacking this amplification product (Hernández et al. 2001).

The same markers can be considered in searching for associations, with agronomic valuable traits to be used as early selection techniques in the MAS. Finally, they are suitable for more specific applications such as mapping and olive oil traceability. As far as traceability is concerned, the application of SCARs on the DNA extracted from olive oil is under investigation.

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