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ORIGINAL PAPER

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Development, characterization and inheritance of new microsatellites in olive (*Olea europaea* L.) and evaluation of their usefulness in cultivar identification and genetic relationship studies

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Abstract Twelve new microsatellites have been developed in olive. For that purpose, a genomic library of the olive cultivar 'Arbequina' was enriched for GA, GT and ACT repeats. Two methods of screening yielded 27 sequences containing microsatellites out of the 119 clones sequenced. The GA repeat seems to be the most abundant motif. Among sequences containing microsatellites, 4 (14.8%) were redundant, 1 (3.7%) was previously described in the literature and 12 (44.4%) could not be used for primers design because the repeat motifs were incomplete. Suitable primer pairs were obtained for the remaining 10 (37.0%) sequences plus an additional 14 recovered from a formerly developed library. For the 24 primer pairs designed, 4 failed to amplify, 8 produced a complex bands pattern and 12 succeeded in giving amplification products. Considering these 12 primer pairs, 10 showed single locus amplification, whereas the other 2 revealed two loci each. This was demonstrated by studying allele segregation in two olive progenies. Sixty-eight alleles were detected for the 12 microsatellites when 51 olive cultivars were analysed. The number of alleles per locus ranged from 1 to 13. The expected heterozygosity varied between 0 and 0.83. All pairs of cultivars could be distinguished using only three microsatellites due to their great discrimination power value. The data coming from genotyping the 51 olive cultivars for 7 out of the 12 new microsatellites were used for constructing a dendrogram by unweighted pair group method with arithmetic mean cluster analysis using the Dice similarity coefficient. Cultivar association according to their geographical origin was observed.

Keywords Olive · Discrimination power · Enriched library · Multi-locus · SSR · Genetic similarity

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Introduction

Olive is one of the crops of the greatest cultural and economic importance in the Mediterranean Basin. Ninety-five per cent of the world surface devoted to olive cultivation is in this area (Civantos 1996).

The genetic diversity existing in the cultivated olive is enormous. To date, 2,600 different olive cultivars have been described (Rugini and Lavee 1992). This great genetic variability in cultivated forms of olive makes it necessary to develop reliable tools for the correct identification of cultivars. Initially, morphological traits were successfully used for discriminating different cultivars (Barranco and Rallo 1984), although they are influenced by environmental conditions. Isozymes have also been used in cultivar identification (Trujillo et al. 1995), but they might also have shown a differential expression depending on the environment, the type of tissue and other factors.

For some time now, scientists have made use of DNA-based markers for a better genetic knowledge of olive. Random amplified polymorphic DNAs (RAPDs) have been used for cultivar identification (Fabbri et al. 1995; Belaj et al. 2001); for estimating genetic distances among wild, feral and cultivated olives from the Mediterranean Basin (Belaj et al. 2002; Besnard and Bervillé 2000); and for following the spread of olive in Macaronesia (Hess et al. 2000). Wild, feral and cultivated olives and the

relationships between them have been studied using amplified fragment length polymorphisms (AFLPs) (Angiolillo et al. 1999).

However, microsatellites or 'simple sequence repeats' (SSRs) could be the markers of choice when the degree of genetic polymorphism required is high and the time and cost of their development are not prohibitive. Microsatellites are short (1–6 bp) tandemly repeated DNA motifs (Hamada et al. 1982) spread throughout the whole genome of eukaryotes. They are relatively abundant and supply good genome coverage. They are multi-allelic, hypervariable, codominant and amenable to automation by polymerase chain reaction (PCR) markers. Their utilization requires a small amount of starting DNA, and their characteristics of robustness and reproducibility make microsatellites suitable markers for comparing different laboratory results. Furthermore, they can be transferred to related species since microsatellites flanking sequences are highly conserved (De la Rosa et al. 2002; Rallo et al. 2003). In recent years, big efforts have been made to develop good, reliable molecular tools such as microsatellite markers that could shed some light on olive tree genetics (Rallo et al. 2000; Sefc et al. 2000; Carriero et al. 2002; Cipriani et al. 2002; De la Rosa et al. 2002). All of them have been used for cultivar identification. Additionally, microsatellites have been employed by Carriero et al. (2002) and by Belaj et al. (2003) for elucidating genetic relationships between several olive cultivars and by Cipriani et al. (2002) for detecting intra-cultivar variation. Microsatellites have revealed themselves to be very useful markers for checking parentage of olive progenies from controlled crossings (De la Rosa et al. 2004; Díaz et al. 2006). Recently, microsatellites developed in other species belonging to this same family have been employed, together with other markers, for elaborating linkage maps of the olive genome (De la Rosa et al. 2003; Wu et al. 2004). However, the scarcity of microsatellites used made it difficult to relate the two parental maps.

The number of microsatellites available until now in olive is still very low compared with the microsatellites developed in other important fruit crops like apple (Guilford et al. 1997; Liebhard et al. 2002) and peach trees (Cipriani et al. 1999; Testolin et al. 2000; Aranzana et al. 2002), and they are clearly insufficient for studying in any depth a complex species with 46 chromosomes ($2n$=23).

The goals of this paper were to report the development of new microsatellites in olive and to test their suitability for cultivar identification and genetic similarity studies. Additionally, the multi-locus nature of microsatellites was also studied for the first time in olive by checking their segregation in olive progenies.

Materials and methods

Plant material and DNA isolation

Young olive-tree leaves from 51 cultivars (Table 1) located at the World Olive Germplasm Bank of CIFA 'Alameda del Obispo' (Córdoba, Spain) were collected and stored at −70°C until their utilization. These cultivars had been previously identified using morphological traits (Barranco and Rallo 1984), RAPD (Belaj et al. 2001) and

Table 1 Olive cultivars included in this study, their countries of origin and their registration number (RN) at the World Olive Germplasm Bank CIFA 'Alameda del Obispo' (Córdoba, Spain)

Cultivar	Country of origin	RN	Cultivar	Country of origin	RN	Cultivar	Country of origin	RN
'Alfafara'	Spain	605	'Domat'	Turkey	94	'Manzanilla de Sevilla'	Spain	21
'Aloreña'	Spain	829	'Empeltre'	Spain	13	'Memeçik'	Turkey	93
'Arbequina'	Spain	231	'Farga'	Spain	12	'Meski'	Tunisia	115
'Ascolana Tenera'	Italy	62	'Frantoio'	Italy	80	'Moraiolo'	Italy	78
'Ayvalik'	Turkey	97	'Galega'	Portugal	128	'Morisca'	Spain	17
'Bical'	Spain	387	'Gemlik'	Turkey	92	'Morrut'	Spain	224
'Blanqueta'	Spain	11	'Gerboui'	Tunisia	538	'Picholine Marrocaine'	Morocco	101
'Cakir'	Turkey	96	'Gordal Sevillana'	Spain	234	'Picual'	Spain	9
'Carolea'	Italy	736	'Hojiblanca'	Spain	2	'Picudo'	Spain	3
'Castellana'	Spain	576	'Kaissy'	Syria	140	'Sevillenca'	Spain	227
'Changlot Real'	Spain	15	'Kalamon'	Greece	105	'Sourani'	Syria	787
'Chemlali'	Tunisia	744	'Konservolia'	Greece	219	'Uslu'	Turkey	95
'Chetoui'	Tunisia	113	'Koroneiki'	Greece	218	'Verdial de Badajoz'	Spain	988
'Cobrançosa'	Portugal	124	'Leccino'	Italy	82	'Verdial de Huévar'	Spain	6
'Coratina'	Italy	79	'Lechín de Granada'	Spain	54	'Verdial de Vélez-Málaga'	Spain	883
'Cordovil de Serpa'	Portugal	131	'Lechín de Sevilla'	Spain	5	'Villalonga'	Spain	364
'Cornicabra'	Spain	10	'Manzanilla Cacereña'	Spain	430	'Zaity'	Syria	788

microsatellite (Rallo et al. 2000) markers. Total genomic DNA was extracted according to Murray and Thompson (1980), with slight modifications (De la Rosa et al. 2002).

Microsatellite-enriched genomic library construction

The enrichment procedure for GA, GT and ACT repeats was performed according to the method employed by Rallo et al. (2000), with minor modifications described below. DNA from the cultivar 'Arbequina' was digested with two restriction enzymes, *Msp*I and *Sau*3AI (Roche), separately at 37°C overnight. The selection of fragments with sizes of between 200 and 700 bp was performed since the cloning procedure employed was more efficient in this length range. Phosphorylated linkers ('short': 5′-CGGAATTCTGGACTCAGTGCC-3′ annealed to '*Msp*I-long': 5′-CGGGCACTGAGTCCAGAATTCCG-3′ or '*Sau*3AI-long': 5′-GATCGGCACTGAGTCCAGAATTCCG-3′) were ligated to both ends using T4 ligase (Roche). Digested DNA was hybridised with an equimolar mix of three biotinylated-tagged probes—$(GA)_{13}$, $(GT)_{13}$ and $(ACT)_{10}$—for 5 h. Two hybridisation temperatures (45 and 65°C) were assayed. Streptoavidin-coated paramagnetic beads (Dynal) and a magnetic rack were employed for catching the fragments containing those repeats. The single-strand DNA recovered was duplicated by PCR using the short adaptor as a primer. The amplifications were carried out using a thermal cycler Gene Amp PCR system 9600 (Applied Biosystems) in 50-µl volumes containing 4 µl of the enriched mixes, 15 mM Tris–HCl pH 8.0, 50 mM KCl, 2.5 mM $MgCl_2$, 0.25 mM of each dNTP, 0.2 µM of short linker and 3.5 U of Ampli*Taq*-Gold polymerase (Applied Biosystems). The PCR profile used was: 94°C for 11 min, followed by 20 cycles of 94°C for 45 s, 59°C for 45 s and 72°C for 2 min, ending with a 7-min final extension at 72°C. PCR products were isolated with the QIAquick PCR Purification kit (Qiagen). The enrichment procedure was repeated, taking that double-stranded DNA previously denatured as template (Carriero et al. 2002).

Cloning and transformation steps were carried out with the PCR-Script Amp Cloning Kit (Stratagene) following the supplier's indications.

Screening for positive clones

The α-complementation strategy described by Sambrook et al. (1989), with slight modifications, was performed to discriminate bacteria transformed with recombinant plasmids. These bacteria were grown on Petri dishes with Luria–Bertani (LB) agar medium containing the antibiotic ampicillin at a concentration of 50 µg/ml, 100 µl of 10 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and 100 µl of 2% X-Gal (5-bromo-4-cloro-indolyl-β-D-galactoside). Colourless colonies were grown overnight in LB with ampicillin and subsequently in LB agar with the same antibiotic. Two colony-screening methods were tested, plaque hybridisation and PCR.

For the first one, colonies were transferred onto positively charged nylon membranes (Roche) and the cellular debris eliminated using Proteinase K (60 U/ml, Roche). The hybridisation was performed with a mixture of the three aforementioned probes—$(GA)_{13}$, $(GT)_{13}$ and $(ACT)_{10}$—labelled at 3′ end with digoxigenin-ddUTP (DIG-ddUTP, Roche) using terminal transferase (Roche). High temperatures (68–70°C) and salt concentrations (5× SSC, 0.2% SDS) were maintained during the 3 h of pre-hybridisation and the overnight hybridisation with the three probes. The first two washes were made under low stringency conditions, at room temperature and with high salt concentrations (2× SSC, 0.1% SDS), for 5 min to remove non-specifically bound probes. The following two washes were carried out at high temperatures (68–70°C) and with low salt concentrations (0.5× SSC, 0.1% SDS) to eliminate hybrids with a low homology. Probe-target hybrids were localized using anti-DIG-AP Fab fragments (Roche) and chromogenic reactives [nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP), Roche]. Coloured products are usually originated in a reaction catalyzed by the alkaline phosphatase conjugated to the antibodies bound to the positive clones.

The second approach used for colony screening consisted of two rounds of PCR. Colony PCR was made with all the white bacteria to detect the presence of recombinant plasmids by comparison with a negative control. For this purpose, M13 (−20) forward and reverse primers were employed since there is a specific binding site for them in the pPCR-Script Amp SK(+) cloning vector used. Only the DNAs obtained from bacteria transformed with recombinant vectors were used as templates in the following multiplex PCR, where the same primers mentioned before plus $(GA)_{13}$, $(GT)_{13}$ and $(ACT)_{10}$ oligos were employed.

Positive clones identified with both methods were purified with the QIAprep Spin Miniprep kit (Qiagen) and the TempliPhi Amplification kit (Amersham Biosciences) according to the manufacturers' protocols.

Microsatellite identification, primer design and amplification by PCR

Clones theoretically containing microsatellites were sequenced with a 3700 DNA Analyzer at the Centre for Biological Research, CSIC, in Madrid, Spain.

Redundant clones were discarded using Lasergene software for Mac OS, and all sequences were checked at the GenBank database in the NCBI internet site (http://www.ncbi.nlm.nih.gov/BLAST). Specific primers in the flanking regions of the microsatellite sequences obtained here and in an additional 14 previously found (Rallo 2001) were designed using OLIGO 6.45 software (National Biosciences, Inc., Plymouth, MN, USA). Primers were designed to be as long as possible since amplification with long primers is more reliable (Rallo et al. 2000). Product

length was limited to 100–300 bp because this size range is optimum for genotyping on an automatic sequencer. Oligonucleotides were synthesized by Sigma-Genosys Ltd.

After an optimisation task, amplifications were performed in 15-μl volume solutions containing 15 ng of genomic DNA, 15 mM Tris–HCl pH 8.0, 50 mM KCl, 2.5 mM $MgCl_2$, 0.25 mM of each dNTP, 0.2 μM of reverse and forward (fluorescently labelled with FAM, HEX or TET) primers and 1 U of Ampli*Taq*-Gold polymerase (Applied Biosystems). PCRs were carried out on a Gene Amp PCR system 9600 (Applied Biosystems) programmed with an initial denaturation step at 94°C for 11 min, followed by 35 cycles of 94°C for 30 s, the annealing temperature of each primer pair (Table 2) for 45 s and 72°C for 2 min, plus a final elongation step at 72°C for 7 min.

In order to accurately establish the size of the different alleles at each locus, samples were analysed on an

Table 2 Characterization of the newly developed microsatellites, including repeat motif, both forward and reverse sequences, size range and number of alleles (N_a), annealing temperature (T_a), observed (H_O) and expected (H_E) heterozygosity, discrimination power (D_j), number of genotypes (N_g) and number of distinguishable cultivars (N_{dc})

Locus (GenBank accession no.)	Repeat motif	Primer sequences (5′–3′) and fluorescent tag	T_a (°C)	Size range (bp)	N_a	H_O	H_E	D_j	N_g	N_{dc}
IAS-oli23	$(AG)_{18}$	FAM-CCCCCCATGTTTTTAATTCAATGAAAAAT TAAGTGG	55	213–238	13	0.82	0.81	0.94	23	16
AJ748719		ACCACCACCGGTCTGGAAGGGATG								
IAS-oli24	$T(A)_6T(A)_2$ $TTAC(A)_4T(A)_2$	TET-CACCCTCATTCAACGCATT	50	220–237	4	1	0.55	0.15	3	0
AJ748720		GGCGCACGTAGTAATTATATTGGA								
IAS-oli25	$(T)_{16}$	HEX-GGCGGTCGTTGACCATCTACCGT TAGCTG	50	118–126	6	1	0.71	0.68	6	2
AJ748721		CACACGTTCAGACGTTCTCCATCAAATCACC								
IAS-oli26	$(GA)_{14}$	HEX-TCGGAGAGTTGCTACAGAACA	50	168–210	13	0.43	0.83	0.92	22	14
AJ748722		CCCTTCCAAGTCGAGCAATAAA								
IAS-oli27	$(T)_5GA(T)_{10}CA$ $(T)_3$	FAM-GGTGGTATTGCCCTCGACA	45	115–140	9	0.49	0.74	0.88	16	9
AJ748723		CCGCCATCTATTCAACCATC								
IAS-oli28[a]	$(AT)_4(AC)_{18}$	HEX-CGGAATGCACGTTGTTGAAATATTCGAC	50	129–180	2	0.82	0.48	0.64	6	3
AJ748724		CCACCTCGAAGCTATAATATTTGTGATTGGA		176–223	4	0.45	0.37			
IAS-oli29	$(TGG)_{18\ imp}$	TET-GGCTGCTAGCAGGGTAAGGAA TATTGGTGGAC	72[b]	227–258	6	0.44	0.74	0.76	9	3
AJ748725		CCAAAACACAAATGACCACCACAAATCCAAC								
IAS-oli30	$(GT)_{6\ imp}$	HEX-CGAGCTATCGACTATAACCTAGCGCTGA	55	252	1	0	0	0	1	0
AJ748726		AAGCCTAACCATACTGAACATCTCTGCAC								
IAS-oli31	$(TG)_7TA(TG)_2$, $(CT)_5$	HEX-ATGCCAACATGACATCGAA	55	203	1	0	0	0	1	0
AJ748727		CGGACAAGAGAAATATGAGTGCAAAA								
IAS-oli32	$(TC)_4$, $(CA)_{6\ imp}$	HEX-CGGCAATAATAGAGCTCCAA	50	162	1	0	0	0	1	0
AJ748728		ATCCGTGCATTCTATGGTTT								
IAS-oli33	$(G)_6$, $(GA)_5$	FAM-AAGGGACGGAGAAGAAGAGTTATACAG GA	60	157	1	0	0	0	1	0
AJ748729		GCGCATCATCATATCTAGCTCTCTTGG								
IAS-oli34[a]	$(TGG)_{8\ imp}$, $(TC)_{5\ imp}$	TET-GCGGCTGTGGTTGTCAATCTCT CATCTTCCTTC	60	185–192	3	0.77	0.53	0.88	14	7
AJ748730		CCACCACCAAATCATGCGCAGTTGCAGT		198–206[c]	4	0.33	0.58			

Data were obtained from the evaluation of 51 olive cultivars
[a]Two-loci microsatellite
[b]A two-temperature profile was employed for the amplification
[c]Null allele was observed

automatic sequencer ABI 310 (Applied Biosystems). The Genescan software version 3.1 for Mac OS was employed for a result analysis.

Evaluation of microsatellites for polymorphism and genetic relationships in a set of 51 olive cultivars, data analysis and segregation studies

For studying the informative potential of the microsatellites, the observed (H_O) and expected (H_E) heterozygosities were calculated using the data obtained in the set of 51 cultivars (Tables 2 and 3). H_E values were estimated using the formula (Nei 1973):

$$H_E = 1 - \sum p_i^2,$$

where p_i is the frequency of the ith allele.

The usefulness of the microsatellites for cultivar identification purposes was estimated by the calculation of the discrimination power (Dj) value, according to Tessier et al. (1999):

$$D_j = 1 - C_j = \sum_{i=1}^{I} c_i = \sum_{i=1}^{I} p_i \frac{(Np_i - 1)}{N - 1},$$

where c_i and C_j are the confusion probability for the ith genotype of the given jth microsatellite and the confusion probability for the jth microsatellite, respectively; p_i is the ith genotype frequency; N is the number of individuals analysed.

The numbers of different genotypes and unequivocally distinguishable cultivars per each primer were estimated. The abundances of the different sorts of motifs and of the microsatellite categories were also studied.

In order to evaluate the genetic relationships between the cultivars considered, a similarity matrix was constructed scoring the amplified fragments as present or absent in each cultivar and using the SIMQUAL programme and the DICE similarity coefficient (Dice 1945). Only data coming from polymorphic microsatellites were employed. IAS-oli34 was not taken into account due to the presence of a null allele revealed by the homozygotic genotype of some of the cultivars analysed, which made it difficult to discriminate between homozygotic and heterozygotic genotypes for the remaining allelic variants. A dendrogram was generated with this coefficient and the unweighted pair group method with arithmetic mean (UPGMA) cluster analysis of the similarity using the SAHN-clustering and TREE programmes of the NTSYS-pc ver. 2.02j package (Exeter Software, Setauket, NY). Additionally, the correlation coefficient between the similarity matrix and the cophenetic values matrix was calculated to test the goodness of fit of the cluster analysis. The relationship between the groupings obtained and the geographical origin was studied.

The inheritance of two putatively multi-locus microsatellites, IAS-oli28 and IAS-oli34, was tested by genotyping 'Manzanilla de Sevilla' × 'Arbequina' (32 seedlings) and 'Picual' × 'Arbequina' (20 seedlings) progenies, respectively.

Results

Identification of positive clones

The DNA obtained in the hybridisation with paramagnetic beads at 45°C was used for the cloning procedure due to the greater yield observed compared with the results at 65°C. The first method used for detecting clones with microsatellite motifs was colony hybridisation. All the colonies gave a coloured signal, and among those showing a more intense colour, 22 were sequenced. Ten of them did not even contain any inserts, seven incorporated a plasmid with insert but without any microsatellite sequence, and finally, five clones (22.7%) had at least one microsatellite inside with five or more repeats. The second method of screening (PCR) displayed 51 positive clones, only 12 (23.5%) of which actually had a microsatellite included in their sequence. Ten clones initially discarded were recovered and confirmed as containing microsatellites, although four of these clones could not be considered as being false negatives because the repeat motif contained was different from the three probes employed for the detection.

Therefore, among a total of 119 clones sequenced (positive clones plus ambiguous ones), 27 contained microsatellites with at least five single repeats (some of the clones had several different motifs). All the repeated sequences contained less than 22 units: 21 (65.6%) had between 5 and 10 repeats, and 11 (34.4%) had between 11 and 21 repeats. The mean repeat number of the mono-, di-, tri- and tetranucleotide motifs found ranged from 6 in G/C to 13 in TGG/CCA (Table 4). The longest uninterrupted stretch (56 bp) was found in a compound microsatellite with the motifs GT and GA, both of them used in the enrichment procedure. The GA repeat appeared to be the most abundant motif in the developed library. The simple imperfect microsatellite was the most frequent type among clones obtained from the enriched genomic library constructed and those sequences coming from the former library that gave scorable PCR products.

Strangely enough, the highest levels of homology showed by the olive sequences studied here were associated, in most cases, with plant cytoplasmic (chloroplastic or mitochondrial) DNA (data not shown). Only one of the amino acid sequences derived from a DNA sequence with repeat motifs was similar to described proteins (three hypothetical proteins of *Zea mays*), whereas several clones that did not contain any microsatellite resulted in being homeologous to some known plant proteins (data not shown). This is not surprising since most of the microsatellites are thought to be located in non-coding regions (Metzgar et al. 2000).

Table 3 Allelic composition of each of the 51 olive cultivars for the eight polymorphic microsatellite markers developed in the present study

Cultivar	SSR name							
	IAS-oli23	IAS-oli24	IAS-oli25	IAS-oli26[a]	IAS-oli27	IAS-oli28	IAS-oli29[a]	IAS-oli34[a,b]
'Alfafara'	220/229	221/237	119/123	192/199	116/122	129/180, 176/223	227/248/258	185/190, 206/206
'Aloreña'	221/229	221/237	118/123	186/203	116/125	129/180, 176/176	250/250	185/190, 200/200
'Arbequina'	219/233	220/236	119/123	187/190	124/125	129/180, 176/176	247/247	190/190, null/200
'Ascolana Tenera'	216/229	221/237	119/123	190/190	115/115	129/180, 176/176	250/250	185/190, null/null
'Ayvalik'	220/233	221/236	120/123	168/186	124/124	129/180, 176/176	227/232	185/190, 200/206
'Bical'	221/229	221/237	118/123	187/190/199	116/124	129/180, 176/223	227/248	185/190, null/null
'Blanqueta'	216/233	221/237	119/124	195/195	126/126	129/180, 176/176	247/247	185/190, 200/206
'Cakir'	221/229	221/237	118/123	186/186	124/125	129/180, 176/223	227/248/258	190/190, 200/200
'Carolea'	217/233	221/237	118/123	199/199	116/127	129/180, 176/176	250/250	185/190, null/null
'Castellana'	217/219	221/237	119/123	186/186	116/116	129/129, 176/223	250/250	185/190, null/null
'Changlot Real'	217/228	221/237	118/123	199/199	115/140	129/180, 176/223	247/247	190/190, 200/200
'Chemlali'	217/229	221/237	119/124	210/210	124/124	129/180, 176/176	250/250	190/192, 200/200
'Chetoui'	217/229	221/237	119/124	210/210	116/124	129/180, 176/176	250/250	190/192, 200/200
'Cobrançosa'	217/217	221/237	118/123	192/199	116/125	129/129, 176/223	250/250	185/190, 200/206
'Coratina'	216/228	221/237	118/123	168/186	124/124	129/180, 176/223	247/250	185/192, 200/200
'Cordovil de Serpa'	221/229	221/237	119/123	168/186	124/124	129/180, 176/223	227/248/258	190/190, 200/200
'Cornicabra'	217/229	221/237	118/123	192/199	116/125	129/180, 176/176	250/250	185/190, 200/206
'Domat'	217/233	221/237	118/123	195/195	116/125	129/180, 176/176	227/248/258	190/190, 200/200
'Empeltre'	229/238	221/237	118/123	184/184	125/125	129/180, 176/223	227/248	185/190, 200/200
'Farga'	229/233	221/237	119/123	192/192	124/124	129/180, 176/176	247/247	190/192, 200/200
'Frantoio'	229/233	221/237	119/124	168/188	124/124	129/180, 176/223	247/247	190/192, 198/200
'Galega'	217/231	221/236	119/123	188/191	124/124	129/180, 176/176	247/250	185/190/192, 198/200
'Gemlik'	217/233	221/237	119/123	199/199	116/124	129/180, 176/176	250/250	185/190, 200/200
'Gerboui'	221/229	221/237	119/124	186/186	125/125	129/180, 176/223	227/248/258	185/190, null/null
'Gordal Sevillana'	217/229	221/237	119/123	199/199	116/125	129/180, 176/176	250/250	185/190, 200/200
'Hojiblanca'	217/217	221/237	119/124	192/192	124/124	129/129, 176/223	250/250	185/190, 200/206
'Kaissy'	217/233	221/237	119/123	184/186	116/116	129/180, 176/176	227/248	185/190, 200/200
'Kalamon'	216/216	221/237	118/123	186/186	123/123	129/180, 176/176	250/250	185/190, 200/200
'Konservolia'	217/217	221/237	119/123	199/199	116/116	129/180, 176/176	250/250	185/190, 200/206
'Koroneiki'	221/237	221/237	119/123	186/186	116/123	129/180, 176/223	227/232	190/192, 200/200
'Leccino'	217/217	221/237	118/123	184/192	125/125	129/129, 177/223	250/250	185/190, 200/206
'Lechín de Granada'	221/229	221/237	118/124	192/199	124/124	129/180, 176/223	227/248/258	190/190, 200/206
'Lechín de Sevilla'	216/216	221/237	119/123	192/199	123/123	129/129, 176/223	227/248	190/192, null/null
'Manzanilla Cacereña'	229/229	221/237	119/124	190/199	116/116	129/180, 176/223	250/250	185/190, 200/200
'Manzanilla de Sevilla'	217/217	221/237	119/123	199/199	116/124	129/129, 176/223	227/248	185/190, 200/206
'Memeçik'	217/233	221/237	118/123	199/199	116/125	129/180, 176/176	227/248	185/190, 200/200
'Meski'	221/233	221/237	119/123	186/186	124/124	129/180, 176/176	250/250	185/190, 200/206
'Moraiolo'	232/238	221/237	119/123	192/192	124/124	129/129, 176/176	232/232	190/190, 200/206
'Morisca'	217/229	221/237	118/123	192/192	116/125	129/180, 176/176	250/250	185/190, null/null
'Morrut'	219/229	220/236	119/123	190/190	116/124	129/180, 176/176	247/247	190/190, 200/200
'Picholine Marrocaine'	217/229	221/237	118/123	199/199	124/124	129/180, 176/176	250/250	185/190, 200/200
'Picual'	217/229	221/237	118/123	187/192/199	116/125	129/180, 176/223	250/250	185/190, 200/206
'Picudo'	217/219	221/237	118/124	199/199	116/124	129/129, 176/223	247/247	185/190, 200/206
'Sevillenca'	228/232	221/237	118/123	186/195	116/124	129/180, 176/176	227/247	190/190, 200/206
'Sourani'	213/233	221/237	118/123	186/186	116/126	129/180, 176/176	227/248/258	185/190, 206/206
'Uslu'	217/229	221/237	119/123	186/186	116/140	129/180, 176/176	227/248	190/190, 200/200
'Verdial de Badajoz'	217/219	221/237	118/123	192/192	116/125	129/180, 176/223	250/250	190/190, null/null
'Verdial de Huévar'	217/219	221/237	118/123	192/192	116/116	129/129, 176/223	250/250	190/190, 206/206
'Verdial de Vélez-Málaga'	229/233	221/237	119/126	186/191	115/126	129/180, 176/176	227/232/248	185/190, 206/206
'Villalonga'	217/217	221/237	118/123	186/186	116/116	129/180, 176/176	247/247	185/190, null/null

Table 3 (continued)

Cultivar	SSR name							
	IAS-oli23	IAS-oli24	IAS-oli25	IAS-oli26[a]	IAS-oli27	IAS-oli28	IAS-oli29[a]	IAS-oli34[a,b]
'Zaity'	217/229	221/237	119/123	178/186/190	116/116	129/180, 176/219	250/250	185/190, 200/200

[a]Extra amplification products found
[b]Any cultivar is assumed to be homozygotous when only an allele is observed at the second locus because it was not possible to discriminate between them and the heterozygotic genotypes for the null allele, except in those cases where the segregation analysis was carried out. This probably causes an underestimation of the heterozygosity values

Design of suitable primers and amplification

Four out of the 27 sequences containing microsatellites were redundant, and another one turned out to be the locus described by Rallo et al. (2000) as IAS-oli06. Primers could not be designed in 12 sequences since the repeat motifs were incomplete or too close to one end. The high occurrence of these interrupted microsatellites is due to the relatively low insert length (average value of 259 bp). Primer pairs were designed for the remaining 10 sequences and for additional 14 ones coming from a former library (Rallo 2001). Four of these 24 primer pairs failed in amplification, 8 produced spurious bands or complex pattern of bands and 12 succeeded in giving scorable PCR products.

Characterization of the microsatellites

The 12 microsatellites were characterized with 51 olive cultivars (Tables 2 and 3). A total of 68 alleles were detected, ranging from 1 (in IAS-oli30, 31, 32 and 33) to 13 (IAS-oli23 and 26) alleles per locus. In markers studied here, no correlation was found between the length of the microsatellite and the average number of alleles per locus. The same result was obtained when the microsatellites were grouped in simple and compound ones. Although a great length of the microsatellite is not enough for the existence of a considerable number of alleles, this seems to be a necessary prerequisite as has been previously reported in other trees like *Castanea sativa* (Mill.) (Marinoni et al. 2003). Simple microsatellites showed more alleles per locus on average (7.4) than the compound ones (1.9).

The expected (H_E) and observed (H_O) heterozygosities varied between 0 and 0.83 (0.47±0.32) and 0 and 1 (0.53±0.35), respectively. The observed heterozygosity was higher than the expected one for seven out of the 14 microsatellite loci (Table 2). Simple and perfect microsatellite was the only type in which the average of both H_O and H_E was higher than the means obtained from total microsatellites and seems to be the most informative one. Ninety-two per cent of the cultivars showed the same heterozygotic genotype for IAS-oli24 (Table 3). In the case of IAS-oli25, only two different heterozygous profiles represented 78.4% of the cultivars analysed. In IAS-oli34, the values of both heterozygosities were probably underestimated due to the presence of a null allele that made it difficult to discriminate between homozygous and heterozygous.

Table 4 Occurrence and mean and standard deviation (SD) of repeat number of particular motifs belonging to the different types of microsatellites found

Type	Motif	Occurrence[a]	Repeat number	
			Mean	SD
Mono	T/A	9	9.44	4.14
	G/C	1	6.00	–
Di	GA/TC	12	12.50	6.13
	GT/AC	6	8.33	3.94
Tri	TGG/CCA	2	13	5
	TCA/TGA	1	10	–
Tetra	CACT/AGTG	1	9	–

[a]All tandemly repeated DNA motifs included even those belonging to the same compound microsatellite and those that could not be transformed into scorable markers

Identification and genetic relationships between cultivars

All the pairs of cultivars could be distinguished using only three microsatellites (IAS-oli23, IAS-oli26 and IAS-oli27; Tables 2 and 3). This was due to the high presence of unique alleles (7 out of 35 in those three microsatellites and 10 out of 68 when all the markers were considered) and genotypes (44 out of 66 in those three microsatellites and 59 out of 108 for the complete set of loci). As expected, these three microsatellites were among the four with the highest discrimination power values.

The data coming from genotyping 51 olive cultivars with seven SSRs were employed for elucidating the genetic relationships between them by constructing a dendrogram using UPGMA cluster analysis (Fig. 1). The highest similarity coefficients were obtained between 'Cornicabra' and 'Morisca', 'Chemlali' and 'Chetoui' (0.96 in both cases), 'Cornicabra' and 'Picual' and 'Verdial de Badajoz' and 'Verdial de Huévar' (0.93 and 0.92, respectively) cultivars. For a similarity coefficient of over 0.55, three main groups can be distinguished. Seven out of the 25 Spanish cultivars ('Alfafara', 'Lechín de Sevilla', 'Manzanilla de Sevilla' 'Bical', 'Lechín de Granada', 'Empeltre' and 'Verdial de Vélez-Málaga'), four out of the six Turkish

varieties (‘Cakir’, ‘Domat’, ‘Memeçik’ and ‘Uslu’), two Syrian varieties (‘Sourani’ and ‘Kaissy’) and one representative from Portugal (‘Cordovil de Serpa’), Tunisia (‘Gerboui’) and Greece (‘Koroneiki’) are included in group I. Six out of the nine cultivars belonging to the closest countries to the hypothetical olive origin centre (Turkey and Syria) comprised a subgroup of cluster I. Group II includes most of the Spanish cultivars (‘Aloreña’, ‘Gordal Sevillana’, ‘Verdial de Badajoz’, ‘Verdial de Huévar’, ‘Cornicabra’, ‘Morisca’, ‘Picual’, ‘Castellana’, ‘Manzanilla Cacereña’, ‘Hojiblanca’, ‘Changlot Real’, ‘Picudo’, ‘Sevillenca’ and ‘Villalonga’), four out of the six Italian cultivars (‘Carolea’, ‘Ascolana Tenera’, ‘Coratina’ and ‘Leccino’), two out of the five Tunisian cultivars (‘Chemlali’ and ‘Chetoui’), two varieties from Greece (‘Konservolia’ and ‘Kalamon’) and one cultivar from Morocco (‘Picholine Marrocaine’), Portugal (‘Cobrançosa’) and Syria (‘Zaity’). Two Spanish cultivars (‘Blanqueta’ and ‘Farga’), two Italian cultivars (‘Frantoio’ and ‘Moraiolo’) and one cultivar coming from Tunisia (‘Meski’) constitute group III. Two Spanish cultivars (‘Arbequina’ and ‘Morrut’), along with a cultivar from Portugal (‘Galega’) and another from Turkey (‘Ayvalik’), fall clearly apart from the three main groups.

Inheritance studies

Two out of the 12 microsatellites developed, IAS-oli28 and IAS-oli34, showed multi-locus amplification (Tables 2 and 3). This was confirmed by testing their segregation in ‘Manzanilla de Sevilla’ × ‘Arbequina’ and ‘Picual’ × ‘Arbequina’ progenies, respectively. All the expected classes were found in the segregation of the four loci evaluated. According to the χ^2 test, one of the IAS-oli34 loci did not show any Mendelian inheritance. This locus was studied in the progeny of a cross in which one parent carried a null allele. For IAS-oli28, although the size ranges of alleles of the two loci overlapped, they were easily distinguishable thanks to the segregation analysis.

Discussion

Since one of the first constraints in microsatellite development methods is the high proportion of false-positive colonies, the screening strategy followed may play an important role in reducing the number of sequencing reactions and improving the detection of real-positive clones. In this sense, two different strategies have been tried out: plaque hybridisation and PCR. Apparently, the efficiency of both approaches seems to be comparable

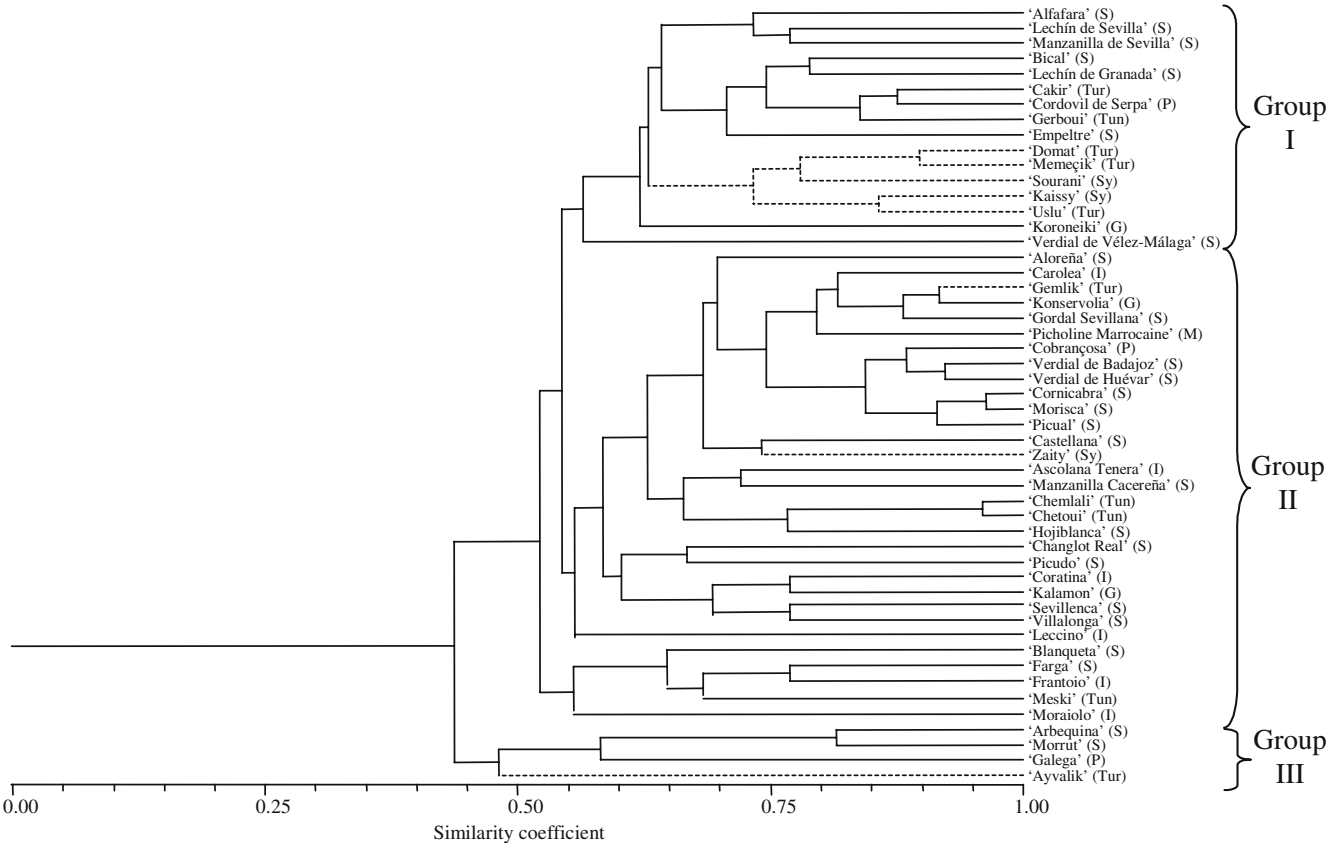

Fig. 1 UPGMA dendrogram created using seven SSRs and the DICE similarity coefficient in a set of 51 olive cultivars coming from eight different countries: Greece (*G*), Italy (*I*), Morocco (*M*), Portugal (*P*), Spain (*S*), Syria (*Sy*), Tunisia (*Tun*), Turkey (*Tur*). Continuous lines (—) correspond to olive propagation areas and broken ones (- - -) to cultivars close to the hypothetical centre of origin

since the percentages of true-positive clones obtained with them were similar. However, the second method proved to be better for detecting the desirable repeat sequences. In fact, the number of clones containing unexpected repeats was much higher with the first detection method (four out of the five clones with microsatellites) compared with the PCR strategy (one out of 12). The results obtained in the screening of the enriched library by plaque hybridisation agreed with those described by Carriero et al. (2002) since the number of false positives was very high even among the colonies showing the strongest signals. The low stringency during the hybridisation of the DNA with paramagnetic beads at 45°C could be the explanation for the abundance of false positives. Anyway, these efficiency values are higher than those obtained in non-enriched libraries, for instance, in peach tree (1%) by Cipriani et al. (1999). Moreover, the relatively high redundancy observed in this library appears to be a common event in enriched libraries as reported by Fisher et al. (1998).

Both GA and GT repeats have been found in the developed library as expected. It may seem surprising that inserts with the repeat ACT, also employed in the enrichment steps, had not been obtained, whereas the mononucleotide $(T/A)_n$, which was not used in such processes, was one of the most frequent motifs. Nevertheless, Scotti et al. (2002) reported that trinucleotide microsatellites were less abundant than dinucleotide ones, and Wang et al. (1994) stated that the most numerous microsatellite type in plants is $(AT)_n$ followed by $(A)_n$. Compound motifs seem to be also relatively frequent in the olive genome as has been previously reported (Rallo et al. 2000; Sefc et al. 2000; Carriero et al. 2002; Cipriani et al. 2002; De la Rosa et al. 2002) and has been confirmed here.

All microsatellites with less than 14 repeats had a lower number of alleles than average (Table 2). The high number of different alleles (13) found in some of the loci isolated (IAS-oli23 and 26) contrasts with the monomorphic loci IAS-oli30, 31, 32 and 33. The 'birth' of a new microsatellite (Messier et al. 1996) could be the explanation for the low level of polymorphism found in IAS-oli33 due to the limited number of repeats found in this locus. In the case of IAS-oli30, 31 and 32, the phenomenon described as 'microsatellite death' (Taylor et al. 1999) could happen due to the interrupted structure of their repeats. In this sense, the lack of correlation between the length of the microsatellite and the average number of alleles per locus, observed here and in a previous study (Rallo et al. 2000), is probably due to the appearance of a new allele being caused by the gain or the loss of any repetitive unit. A possible explanation for the higher degree of polymorphism of simple microsatellites compared with compound ones is that the increase in the number of tandem repeats in a particular motif could be counteracted by the decrease in another motif belonging to the same compound microsatellite. In these cases, the polymorphism remains concealed, and it is impossible to detect it.

On the whole, the microsatellites developed in olive seem to be characterized by medium levels of heterozygosity, i.e. never higher than 0.66 (Rallo et al. 2000; Cipriani et al. 2002; De la Rosa et al. 2002), including those obtained in the present study. This coincides with the results given in other trees like conifers in which the average observed heterozygosity was 0.51 (Hodgetts et al. 2001).

The expected heterozygosity is a suitable indicator of the informative potential of a marker in cultivar identification studies since the highest values of this parameter were displayed by the three microsatellites that allowed us to distinguish the total number of cultivars considered here.

Null alleles have been found in one locus of the microsatellite IAS-oli34. This allele may have appeared because of the loss of a chromosomic segment with a deleterious or partially lethal effect. This hypothesis could explain why in this locus the observed segregation does not fit a Mendelian ratio. Null alleles have already been described in olive microsatellites (Rallo et al. 2000). These types of alleles are frequently found when segregation analyses are carried out. Fisher et al. (1998) observed null alleles in 35% of loci studied in the coniferous species *Pinus radiata* D. Don.

Two out of the three closest associations between two cultivars ('Chemlali'–'Chetoui' and 'Cornicabra'–'Picual') have been previously described (Belaj et al. 2001, 2002; Rallo et al. 2003; Sanz-Cortés et al. 2001). Their remarkably high similarity coefficients suggest the existence of a common ancestor for every one of these pairs of cultivars. In general, the groups obtained here are consistent with those reported by Belaj et al. (2001) for the same set of cultivars using RAPD markers.

A certain tendency to group together depending on their geographical origin can be perceived here. In this sense, most of the cultivars coming from the most western areas, Morocco and the Iberian Peninsula, are agglutinated in cluster II. On the other hand, the cultivars from the eastern Mediterranean Basin, Turkey and Syria, are mostly integrated in one of the two subgroups belonging to cluster I. Although small associations of cultivars from the central Mediterranean countries Italy, Greece and Tunisia can be observed, in general, they appear as being scattered all along the dendrogram. Similar results were obtained when a dendrogram was constructed using only the Spanish varieties (data not shown), in which the cultivars were grouped together based on their growing areas. These results reflect the low dispersion of olive tree in Spain, where the same local varieties have been cultivated for centuries with low levels of exchange between the different crop zones. Several authors have also described similar clusters of cultivars according to their geographical origin (Bandelj et al. 2004, 2003; Rallo et al. 2003).

The cophenetic coefficient of the data derived from the 51 cultivars was 0.71, which was similar to that obtained by Bandelj et al. (2004) in a study carried out with SSRs in 19 olive cultivars. The relatively high value of this coefficient is indicative of the good fit of the clustering to the group of data.

The existence of microsatellite markers that generated multiple products have also been previously reported in olive. Of the microsatellites developed by Cipriani et al.

(2002) and Rallo et al. (2000), 17 and 30.8%, respectively, belonged to this category. However, the presence of duplicated loci was not confirmed by segregation studies in those previous works. To our knowledge, this is the first time that the segregation of multi-locus microsatellites has been tested and confirmed in olive progenies. These multiple microsatellites could be very useful tools in future studies of cultivar identification since a small number of them would permit discrimination between different cultivars.

In conclusion, taking into account the low number of specific markers available in olive, the eight polymorphic new microsatellites developed in this study could be of great use in cultivar identification, genetic mapping, marker-assisted selection within a breeding programme of the species and in evolutionary, population structure and phylogenetic studies. The great discrimination capacity of some of these microsatellites make them particularly interesting for cultivar identification since a high proportion of unique alleles has been observed. Furthermore, the existence of confirmed and distinguishable duplicated loci in two of them may supply extra useful information for genetic studies. The availability of these new microsatellites constitutes a valuable opportunity for exchanging data with other laboratories, which may result in being highly productive considering that *Olea europaea* L. is a sparsely genetically studied species whose nomenclature is still confusing.

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