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Microsatellite markers isolated in olive (*Olea europaea* L.) are suitable for individual fingerprinting and reveal polymorphism within ancient cultivars

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Abstract We have isolated and sequenced 52 microsatellites or simple sequence repeats (SSRs) from nearly 60 positive clones obtained from two 'Frantoio' olive genomic libraries enriched in (AC/GT) and (AG/CT) repeats, respectively. The repeat-containing fragments obtained from genomic DNA restricted with *Tsp509*I were separated using a biotinylated probe bound to streptavidin-coated paramagnetic beads. Fragments were then cloned into lambda ZAPII vector and sequenced. Thirty of the 36 primer pairs which gave correct re-amplification in the source genome were used to assay the polymorphism of 12 olive cultivars, namely four well-known cultivars ('Coratina', 'Frantoio', 'Leccino', 'Pendolino') and eight ancient cultivars grown locally near Lake Garda ('Casaliva', 'Favarol', 'Fort', 'Grignan', 'Less', 'Raza', 'Rossanel', 'Trep'). The local cultivars were each represented by two to four long-lived individuals. The analysis was carried out using ^{33}P-labelled primers and 6% polyacrylamide sequencing gels. All except two microsatellites showed polymorphism, the number of alleles varying from 1 to 5. The average genetic diversity (*H*) was 0.55. The power of discrimination (*PD*) was 0.60. All cultivars, including the local ones, were easily separated from each other. Variations in the SSR pattern were observed among individual plants of the same cultivar in four out of the eight local cultivars analysed. Several primer pairs (17%) amplified more than one locus.

Keywords SSR · Simple sequence repeat · Molecular markers · DNA polymorphism · Genetics

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Introduction

Microsatellite DNA (simple sequence repeat or SSR) is becoming a popular tool for genetic analysis in plants. It consists of short (1–6-bp long) stretches of DNA tandemly repeated several times. These short repeats are abundant and frequently interspersed in eukaryotic genomes, highly polymorphic and easily amenable to PCR-based assays using primers complementary to the repeat flanking regions. These positive features make microsatellites the markers of choice in studies on population genetics and for the fingerprinting of individuals (Rafalski et al. 1996). The major drawbacks of this class of molecular markers are the time required for their isolation and the costs involved, but these drawbacks have been recently overcome by the introduction of library enrichment procedures and automatic sequencing (Morgante et al. 1998).

The olive (*Olea europaea* L.) is an ancient crop which spread from the Middle East towards the West. The Romans extended its cultivation from the Greek islands throughout the Mediterranean basin, mainly along the African and European coasts but also near the great alpine lakes, such as Lakes Iseo, Como, Lugano and Garda, where the large volumes of water ameliorate the climate. Olives have a protracted juvenility and in the past this has discouraged programmes of plant breeding and improvement. Olive germplasm therefore developed through the accumulation of seedlings identified by chance and vegetatively propagated for centuries by local populations.

Morphological and biometric characters have been widely used to describe olive germplasm (see references in Cantini et al. 1999). Recently, biochemical. and molecular markers have been added to the tools, such as isozymes (Ouazzani et al. 1993; Trujillo and Rallo 1995), RAPDs (Fabbri et al. 1995; Vergari et al. 1998; Hess et al. 2000), AFLPs (Angiolillo et al. 1999), and ISSR (Hess et al. 2000), that have been used to characterise cultivars. More recently, several microsatellites have been isolated from olives (Rallo et al. 2000; Sefc et al.

2000). In addition, three microsatellites, isolated from ash (*Fraxinus excelsior* L.), have also been successfully applied to the olive (Lefort et al. 1999).

We report, in the present paper, 30 microsatellites isolated from olive, using genomic libraries enriched in (AC) or (AG) repeats, and tested a panel of 13 cultivars representative of different regions of Italy in order to evaluate the degree of polymorphism of these markers. Cultivars from the Lake Garda region, where the olive industry is based on centuries-old trees, were also assessed for the microsatellite somatic stability, using several individual trees of the same cultivar.

Material and methods

Microsatellite identification

Total DNA was extracted from young leaves of the olive cultivar 'Frantoio' according to a procedure slightly modified from that of Doyle and Doyle (1990). Genomic DNA was digested with *Tsp509*I, a frequent-cutter restriction enzyme which recognises the AATT sequence. Fragments were fractionated through agarose gel and those of about 200–700 bp long were eluted onto a nylon membrane (Sambrook et al. 1989), ligated to adapters, denatured and hybridised to either $(GT)_{13}$ or $(CT)_{13}$ biotinylated probes bound to streptavidin-coated paramagnetic beads (Dynal, Oslo, Norway). Single-strand DNA fragments recovered by the beads were then used as a templates for the PCR reactions carried out with a primer complementary to one of the adapters initially ligated to the restriction fragments. Fragments were then cloned into the Lambda Zap II vector (Stratagene, Amsterdam, The Netherlands) and sequenced. The detailed protocols of library enrichment, cloning and sequencing of positive plaques are described in Morgante et al. (1998).

All sequences were checked against each other using the FASTA program (accessed at http://www.icgeb.trieste.it/ ~netsrv/) and against the GenBank database, with the microsatellite portion excised, using the BLASTA program (accessed at http://www.ncbi.nlm.nih.gov/BLAST/). Unique sequences were then retained for PCR primer design in the repeat flanking regions using Primer 3 (Whitehead Institute of Biochemical Research, Cambridge MA, USA).

Individual fingerprinting

Eight ancient olive cultivars from Lake Garda identified by Bargioni (1962) as 'Casaliva', 'Favarol', 'Fort', 'Grignan', 'Less', 'Raza', 'Rossanel', and 'Trep', and four cultivars typical of different Italian growing areas ('Coratina', 'Frantoio', 'Leccino', and 'Pendolino'), were analysed at 30 microsatellite loci selected from the 52 isolated from the 'Frantoio' libraries because of their good resolution and low stuttering on gel. The cultivars from Lake Garda were each represented by 2 to 4 long-lived individuals.

DNA was extracted from 1 g of young leaves collected in spring and stored at –80°C until used, following the protocol of Doyle and Doyle (1990) modified as above. PCR reactions were performed in a volume of 25 µl containing 10 mM Tris-HCl pH 9.0, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM each dNTP, 0.2 µM each primer labelled with γ-^{33}P-ATP using polynucleotide kinase (Sambrook et al. 1989), 100 ng genomic DNA and 0.1 U of Taq polymerase (Amersham Pharmacia Biotech AB, Sweden) adopting the temperature profile: 95°C for 5 min, then 35 cycles of (94°C for 45 s, 57°C for 45 s and 72°C for 45 s), finishing with 72°C for 8 min.

PCR products were separated on a 6% denaturing polyacrylamide gel (Long Ranger™, FMC BioProducts, USA) containing 7 M urea and run with 1.2×TBE buffer at a constant power of 55 W. The gels were then dried and autoradiographed on X-ray film using standard procedures. A reference molecular size marker was prepared from pUC18 plasmid DNA, that was sequenced using the M13 universal primer (Amersham Pharmacia Biotech AB, Sweden), radioactively-labelled as above and the four sequencing reactions were loaded onto the gel. Allele scoring and sizing was done by eye using the plasmid sequence lanes as a length reference. All PCRs and electrophoreses were repeated at least twice. Moreover, each gel was scored independently by three people.

Analysis of microsatellite information content

The information content of microsatellite loci was estimated, using the profile of one individual per cultivar, either by *gene diversity* (*h*) (Nei 1973) according to the formula

$$h=1-\Sigma p_i^2$$

where p_i is the frequency of the i^{th} allele or by the *power of discrimination* (*PD*) (Kloosterman et al. 1993), according to the formula above, but with allele frequency replaced by the genotype frequency. Cultivars showing only one fragment amplified by a pair of primers were conventionally considered to be homozygous at that locus. As a consequence the genetic diversity reported here could be underestimated, if null alleles occurred.

Results

Isolation and characterisation of microsatellites from olive enriched-libraries

The library enrichment in microsatellite repeats resulted in 4% of positive plaques. This percentage is very low in comparison with those reaching 50% that we have obtained with other species (Cipriani et al. 2000).

Of the 57 positive plaques recovered and sequenced, 52 (91%) contained the sought repeat, 44 (77%) were amenable to the design of primers with the remainder being discarded because they were duplicated or the repeat was too close to the cloning site, and 36 (63%) were correctly re-amplified from the 'Frantoio' genomic DNA.

Three clones showed high homology with GenBank sequences. One clone was homologous to the accession coded as "chloroplast psbA, trnH, trnL genes" of tobacco (emb/Z00044), which do not include the $(CA)_{11}$ repeat found in olive. Since primers did not give PCR products, the sequence was probably a cloning artefact. The two remaining clones were homologous to olive tandem repeats (emb/AJ002765,emb/AJ131699.1; Katsiotis et al. 1998).

Microsatellite polymorphism and cultivar identification

Of the 36 primer pairs available for fingerprinting, six were discarded because they yielded unreadable patterns. The remaining 30 were screened in the panel of cultivars (Table 1).

All microsatellites were polymorphic, except UDO99–003 and UDO99–022 which were monomorphic showing one and two bands respectively. Five primer pairs (17%) generated multiple bands, possibly because of the simultaneous amplification of two different

Table 1 Primer sequencess, repeat motif and sequence length of 30 microsatellites sequenced in the olive cultivar 'Frantoio'

Locus	Primer sequences (5'→3')	Repeat motif	Length (bp)
UDO99–001	AAAAATCACTTCTATTTTTGTTAG GCTCTCACGAGGAAGACTAT	$(CA)_{11}$	144
UDO99–003	TCTTCTGTTTGGGCTCCATT TGCGGCTCTCGTTAGAATTT	$(AC)_{15}$	137
UDO99–004	TTTGCCCTGGATTGGTACA AGCTTGAGCATCATCTGTGAG	$(AC)_{10}$	148
UDO99–005	TTCATAATAGTTCTCTTTTGTGGATGA TGGAGAAGGACACAGGGAAG	$(AC)_{9}$	139
UDO99–006	TCAGTTTGTTGCCTTTAGTGGA TTGTAATATGCCATGTAACTCGAT	$(GT)_5(AT)_6G(GT)_9$	172
UDO99–007	TGTGTTCTTTATTTGAAGGAATCTT TCGCTTTTGTGTTACATATTCG	$(GT)_{21}$	120
UDO99–008	AAAAACACAACCCGTGCAAT AAATTCCTCCAAGCCGATCT	$(AC)_{13}$	159
UDO99–009	TTGATTTCACATTGCTGACCA CATAGGGAAGAGCTGCAAGG	$(AG)_{16}$	119
UDO99–011	TGACTCCCTTTAAACTCATCAGG TGCGCATGTAGATGTGAATATG	$(CT)_7(CA)_{10}(CT)_2(CA)_2$ $CT(CA)_2CT(CA)_9$	115
UDO99–012	TCACCATTCTTAACTTCACACCA TCAAGCAATTCCACGCTATG	$(GT)_{10}$	164
UDO99–014	TTCCCCTTATTCAATGTGAACC ACTGCAGTTTGGGAATCAAA	$(GT)_{10}$	103
UDO99–015	ATTCATCTATGGGCCGCTTC TCAACACAACCTACTAGCCTACCA	$(TG)_{12}$	109
UDO99–017	GCCCACAAACTCTTTGAACC GCGATTTTTCCCTGTATTTAGGT	$(TG)_{11}$	164
UDO99–019	TCCCTTGTAGCCTCGTCTTG GGCCTGATCATCGATACCTC	$(GT)_{20}(AT)_5$	165
UDO99–020	TGAGGAGCCTTTGAACACTTT CGGACCCTGAAGTGATGATT	$(AC)_{10}TT(AC)_5$	197
UDO99–022	AATTGATCTACACTCCATCGAA ATGTTCCGAAGCCACATACT	$(CACG)_2(CA)_6$	196
UDO99–024	GGATTTATTAAAAGCAAAACATACAAA CAATAACAAATGAGCATGATAAGACA	$(CA)_{11}(TA)_2(CA)_4$	188
UDO99–025	AACATGCCGTTGCATTTTTA GGCATCAATCTACTTCCCACA	$(AC)_{16}(AT)_5$	158
UDO99–026	AATTGACACCTACACACACACA ACCTATTTCATGGTTTGCAC	$(AC)_{20}$	114
UDO99–027	TCCGTGCAAACCATGAAATA TTGATGACTAGCACACATGTAGGA	$(A)_{19}$	122
UDO99–028	CTGCAGCTTCTGCCCATAC GCAGATCATCATTTGGCACT	$(CA)_{23}(TA)_3$	152
UDO99–031	TATCCTCTATGTGGCGATG TTGGTTAAAAGGATTGATACA	$(TG)_{21}(TATG)_6$	151
UDO99–034	CTCTCGGGCATGTATCATTT TTGCATATTTGTATGATTCATTT	$(TG)_{23}$	117
UDO99–035	AATTTAATGGTCACACACAC ATTGCGAAATAGATCTACGA	$(CA)_{15}$	145
UDO99–036	AACACTGTGCCACCTCAACA GAACCCAACCCCCATCTTAC	$(GT)_{19}(AG)_5$	164
UDO99–039	AATTACCATGGGCAGAGGAG CCCCAAAAGCTCCATTATTGT	$(AT)_5(GT)_{11}$	170
UDO99–041	TTCTTTAGGCCGGCTAATACC AGATTGGTTCGTTTGAGATGC	$(CA)_{18}$	100
UDO99–042	CGAGCACAACATGTGGAATC CATCTGTCTCCGCTAACAATTT	$(CA)_{13}$	151
UDO99–043	TCGGCTTTACAACCCATTTC TGCCAATTATGGGGCTAACT	$(GT)_{12}$	174
UDO99–044	AATTCCGACAAGTTGTGTGTG CACAGCACCCAACCAGATTT	$(TG)_7G(TG)_2(G)_3(TG)_2$ $(TGCG)_5$	149

loci (Table 2). As a consequence, 35 loci were amplified by the 30 primer pairs.

The average number of alleles per locus was 3.0, ranging from 1 to 5 according to the locus, with the number of alleles per locus not being ascertainable in the duplicated loci (Table 2). The frequency for each allele was generally low with 17% of alleles being detected in only one cultivar (data not shown). The *gene diversity* (*h*) ranged from 0 in monomorphic loci to 0.77 (average 0.55). The *power of discrimination* (*PD*) followed, as

Table 2 Number of alleles, size range, *gene diversity* (*h*), *power of discrimination* (*PD*), and profile of the cultivar 'Frantoio' at 30 microsatellite loci sequenced in olive (*O. europaea* L.) and screened in 12 olive cultivars. Each cultivar was represented by a single genotype

Locus	No. alleles	Size range	*h*	PD	'Frantoio' profile
UDO99–001	2	141–145	0.44	0.44	145
UDO99–003	1	138	0	0	138
UDO99–004	2	146–150	0.448	0.57	146/150
UDO99–005	2	138–140	0.1524	0.28	140
UDO99–006	3	150–174	0.502	0.68	150/174
UDO99–007(1)	7	94–141	–	0.67	108/120
UDO99–008	3	160–170	0.59	0.75	160/165
UDO99–009(1)	6	83–119	–	0.79	103/119
UDO99–011	5	105–132	0.71	0.75	115/124
UDO99–012	3	157–167	0.665	0.75	165/167
UDO99–014	3	95–105	0.579	0.74	95/105
UDO99–015	3	103–111	0.54	0.65	111
UDO99–017	3	157–165	0.523	0.68	165
UDO99–019	4	101–169	0.71	0.72	133/169
UDO99–020	2	199–200	0.49	0.49	200
UDO99–022(1)	2	198–202	–	0	198/202
UDO99–024	3	175–189	0.645	0.71	183/189
UDO99–025	2	159–163	0.44	0.44	163
UDO99–026	2	99–117	0.49	0.61	99/117
UDO99–027	2	122–193	0.49	0.61	122/193
UDO99–028	4	136–172	0.68	0.74	154
UDO99–031	5	114–155	0.774	0.85	147/155
UDO99–034(1)	5	80–122	–	0.64	80/95/120
UDO99–035	5	136–168	0.71	0.62	136/148
UDO99–036(1)	5	145–167	–	0.69	149/155/167
UDO99–039	5	172–183	0.68	0.81	172
UDO99–041	3	73–98	0.57	0.28	73/98
UDO99–042	3	144–152	0.56	0.57	152
UDO99–043	5	179–219	0.72	0.75	179/219
UDO99–044	4	128–150	0.65	0.74	128/150
Overall mean	*3.0*		*0.55*	*0.60*	

(1) Duplicated locus. The attribution of alleles to either locus has not been attempted, in the absence of segregation data

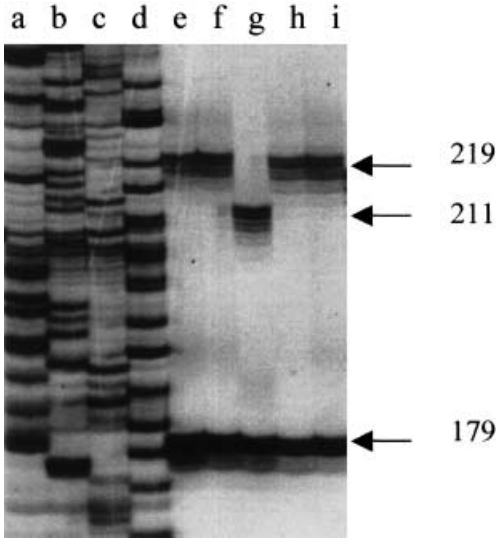

Fig. 1 Autoradiogram of a polyacrylamide-gel separation of the ^{33}P-labelled PCR-amplified microsatellite UDO99–043 showing evidence of within-cultivar polymorphism in 'Casaliva'. *Lanes* from left are: *a–d* the four sequencing lanes of the plasmid pUC18 used as a reference ladder for sizing; *e*='Frantoio', an olive cultivar from Tuscany from which 'Casaliva' is said to be derived; *f–i*=four long-lived individuals of 'Casaliva'. A 219→211 mutation is present in the individual of *lane g*

expected, *the gene diversity*, ranging from 0 to 0.85 with a mean value of 0.60.

All cultivars were readily separated from each other. We were also able to solve two problems of synonymy. 'Frantoio' and 'Casaliva' are often considered to be very similar. We confirmed that this is indeed the case: the individual coded as "Casaliva 3" had the same profile as 'Frantoio' at all loci; "Casaliva 0" and "Casaliva 2" each showed a different mutation at the locus UDO99–007; "Casaliva 1" showed a mutated allele at each of the loci UDO99–028 and UDO99–043 (Fig. 1). 'Casaliva' can therefore be considered a sport mutation of 'Frantoio'. The cultivars 'Leccino' and 'Less' are also sometimes considered to be one cultivar. This possibility can be excluded because of the differences shown at 22 of the 28 polymorphic loci considered (data not shown).

Variations within cultivars

We found frequent intra-cultivar polymorphism when two or more individuals of the traditional cultivars of the Lake Garda region were tested.

Sometimes this was due simply to misidentification of the plants. One genotype identified as 'Favarol' differed from three other genotypes identified as belonging to the same cultivar at 14 out of the 28 polymorphic loci

analysed. A similar difference at 50% of the polymorphic loci was found in one of three genotypes identified as 'Rossanel' and in the two 'Trep' genotypes. Olive cultivars are often very similar morphologically and in these three cases we have concluded that some individuals were misidentified.

On the other hand, we did detect real clonal or intra-cultivar variation in the cultivars 'Casaliva', 'Fort', 'Raza' and 'Rossanel' (data not shown) with differences at one, and in one case, two loci.

Discussion

The library enrichment in microsatellite repeats resulted in a low percentage (4%) of positive plaques. This value is close to that previously reported for olive by Rallo et al. (2000), who adopted our enrichment protocols, but much lower than those values previously recorded in our laboratory for other species (Cipriani et al. 2000). Moreover, four out of the 57 clones sequences were found duplicated. We believe that this low efficiency was due to technical problems that occurred during the enrichment procedure rather than to a low absolute number of repeat motifs in the olive genome. We noted that two of our sequences were already recorded in GenBank as tandem repeats (Katsiotis et al. 1998).

There are some discrepancies between the allele sizes calculated from the cloned 'Frantoio' sequences and reported in Table 1 and the allele sizes estimated in the 'Frantoio' PCR products run in the polyacrylamide gels and reported in Table 2. Moreover, the values reported in Table 2 are the most common ones, since we obtained slightly different estimates with different PCRs and electrophoreses (Cipriani et al. 2000). These discrepancies, which we are unable to resolve, could be due to non-template addition by the polymerase, denaturing conditions during electrophoresis, different base compositions in microsatellite sequences and the plasmid sequence used for sizing, and other possible drawbacks. We have previously discussed this problem (Testolin et al. 2000), which could make the results obtained under different conditions and in different laboratories not comparable, and we recommended using the cultivar from which the microsatellite had been originallyisolated and sequenced as a reference in all gels. The length of new alleles should be calculated from the length of the reference allele, which should be taken as reported for the cloned sequence, irrespective of the result of the actual electrophoretic run. In this way, the reference allele acts as an internal standard in any gel.

Of the 30 primer pairs processed in the whole panel of cultivars, five (17%) amplified two different loci. Duplicated genes are often found in eukaryotes, but when the frequency of duplicated DNA regions is high, complete genome duplication can be postulated. We do not know whether the domesticated olive (*O. europaea* L.) is diploid or polyploid, and our results are not sufficient to solve the question. There is some evidence that the olive could be an ancient polyploid. Breviglieri and Battaglia (1954) suggested that it is an allopolyploid and this is supported by the chromosome C-banding analyses of Falistocco and Tosti (1996).

We found a low number of alleles per locus, and this could be explained by the low number of cultivars assayed and/or by the possible coancestry of the cultivars studied. The high values for *gene diversity (h)* and *power of discrimination* (*PD*) found in most microsatellite loci render these markers a valid tool for discriminating between the currently propagated cultivars.

We discovered genetic variability in most cultivars from which we analysed two or more individuals. Within-cultivar differences fell into two clear-cut classes: the first included pairs of individuals with differences at ≥14 of the 30 loci analysed. We concluded that these were clear cases of missidentification because so many differences can be explained only by assuming that they were derived by sexual reproduction. The second class included pairs of individuals with differences at ≤2 of the 30 loci analysed. The olive is predominantly a cross-pollinating species and the percentage of heterozygous loci we found was rather high, ranging from 50% in 'Favarol' to 70% in 'Raza'. Differences at only one or two loci among the 30 analysed are too few to have originated through sexual reproduction. They are more likely due to somatic mutations occurring in such long-lived trees and reproduced through vegetative propagation. The occurrence of clonal genetic variation has been reported for other species, such as grape (*Vitis* spp.), where very ancient cultivars are still being propagated (Sensi et al. 1996, Cervera et al. 1998). What is novel is that we found genetic variability within olive cultivars by screening relatively few markers, whereas clonal variations were detected in grape only after several hundred AFLP and/or ISTR markers were screened.

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