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Development of simple sequence repeats (SSRs) in olive tree (*Olea europaea* L.)

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Abstract We report the development of microsatellites or simple sequence repeats (SSRs) in the olive tree (*Olea europaea* L.). Forty three positive clones obtained by the screening of a GA-enriched genomic library were sequenced and primers were designed for 13 microsatellite loci. Five primer pairs amplified polymorphic products of the expected size range. SSR polymorphism was explored in a set of 46 olive cultivars. A total of 26 alleles were detected for the five loci. Heterozygosity ranged from 0.46 to 0.71. Ninety one per cent of the cultivars had unique multilocus genotypes. Microsatellite segregation was studied in a complex population from a cross between the commercial cultivars ‘Leccino’ and ‘Dolce Agogia’.

Key words Microsatellites · Variable number of tandem repeats (VNTRs) · Enriched library · Hexamer repeat · Null allele · Marker-assisted selection (MAS)

Introduction

The olive tree (*Olea europea* L.) is a subtropical crop that has been cultivated for millennia in the Mediterranean basin, where it accounts to be the most important oleaginous crop in the area. Olive oil is becoming a very important product thanks to its nutritional and healthy advantages in relation to other vegetable oils.

The olive genetic patrimony is very rich. The long-living character of the tree and its low breeding pressure have contributed to the preservation of variability within the species. Nevertheless, although cultivar diversity is very high, these cultivars are mainly local and old, having a limited diffusion area (Barranco and Rallo 1985; Barranco 1997). Present economical trends towards a global market and the increasing olive oil demand reveal a serious need for developing new cultivars that meet the requirements of new competitive production systems.

Nevertheless, up to the present time, olive breeding has been limited to clonal or varietal selection and induced mutagenesis (Panelli et al. 1990; Guerriero et al. 1994; Rugini and Panelli 1994; Tous et al. 1998; Rossetto et al. 1999). Olive breeding, as in many other tree species, is hampered by the long juvenile period (15 years), difficulties in tree manipulation, as well as the level of genetic complexity of a species with 46 chromosomes ($n = 23$) and a lack of genetic knowledge.

The development of markers as a tool for olive germplasm characterization and early progeny selection is absolutely necessary to enable an effective breeding program for the species. In the recent years, different kinds of markers have been successfully used in olive spp. Ouazzani et al. (1993) and Trujillo et al. (1995) found a high level of isozyme polymorphism for cultivar identification. Random amplified polymorphic DNA (RAPD) has also been performed (Bogani et al. 1994; Fabbri et al. 1995; Vergari et al. 1996). More recently, the relation among cultivated olive, wild forms and related species have been explored through amplified fragment length polymorphism (AFLP) (Angliolillo et al. 1999). Nevertheless, the dominant character or the lack of reproducibility of some of these markers represent serious limitations when applied for breeding purposes.

Simple sequences repeats (SSRs) or microsatelites are short tandem repeats of DNA. They are also known as variable number of tandem repeats (VNTRs). Microsatellite polymorphism is based on the different numbers of a short repeated motif at a given locus. SSRs are becoming the markers of choice in many plant breeding programs because they are transferable, multi-allelic co-dominant markers, PCR-based, easily reproducible, randomly and widely distributed along the genome, and

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their analysis may be automated (Rafalski et al. 1995). To our knowledge, no microsatellite marker has been yet reported in olive, maybe due to the large initial effort that these markers require. The aim of this paper is to develop the first SSRs in olive and explore their potential as reliable markers for germplasm characterization and marker-assisted selection.

Materials and methods

Plant material and DNA isolation

Forty six cultivars from the olive Germplasm Bank at Córdoba (Spain), previously analyzed with RAPD markers (Belaj 1998), were selected for testing microsatellite performance and polymorphism (Table 1). Ninety six genotypes from a cross between ‘Leccino’ and ‘Dolce Agogia’ were also used to check Mendelian segregation.

DNA from these genotypes was isolated from young leaves following the method descibed by Murray and Thompson (1980) with modifications (Belaj 1998).

SSR-enriched library construction and screening

A small insert enriched library for GA repeats was constructed according to Gianfranchesci et al. (1998) with modifications. ‘Arbequina’ cv DNA was digested with the restriction enzyme *Tsp*509I (New England Biolabs) and separated on a 2% agarose gel. Fragments ranging from 200 to 700 bp were recovered on a nitrocellulose membrane (NA-45 DEAE, Schleicher and Schuell) and ligated to adaptors at the *Tsp*509I restriction site. Fragments containing (GA) repeats were selected through hybridization with a biotinylated-tagged probe containing a $(CT)_{13}$ repeat bound to streptoavidin-coated beads (Dynal). The enriched fraction was recovered with the help of a magnetic rack, amplified and isolated from the PCR reactions (Qiaquick, Qiagen). Enriched DNA was digested with *Eco*RI (Amersham-Pharmacia-Biotech), ligated into the pUC 18 *Eco*RI/BAP (Amersham-Pharmacia-Biotech) vector and cloned in competent *Escherichia coli* cells (DH5) according to the manufacturer's instructions. Clones were grown on LB-agar media with 50 µg/ml of ampicillin. The first screening of recombinant clones was assessed trough the IPTG and X-Gal procedure. White colonies were transferred to 96-well microtiter plates and screened for GA repeats by blotting on nylon membranes (Hybond N, Amersham-Pharmacia-Biotech) and hybridization to ^{33}P-labeled poly (GA/CT) probes, as described by Sambrook et al. (1989). Positive clones containing SSRs were identified by autoradiography.

DNA was isolated from positive clones grown overnight in LB medium (QIAprep spin plasmid, Qiagen). Anchored PCR (Rafalski et al. 1995) was performed in order to confirm the presence of the microsatellite and its distance from the cloning site. Double-stranded DNA from the selected clones were sequenced on a ABI 373 stretch automated sequencer using a dye-terminator fluorescent kit (PE Biosystems).

Primer design and PCR amplification

SSR primer sequences were designed using the program Oligo 6.1.1/98 for Mac OS (Molecular Biology Insights) for lack of 3' hairpins or duplices, high and matched Tm (nearest neighbor algorithm), and predicted products of 100–200 bp length. Oligonucleotides were synthesized commercially (Amersham-Pharmacia-Biotech).

PCR reactions were performed in a 15-µl vol containing 10 ng of olive genomic DNA (DNA template), 15 mM Tris-HCl pH 8.0, 50 mM KCl, 2.50 mM $MgCl_2$, 0.25 mM of each dNTP, 0.20 µM of forward and reverse primers and 1 U of Ampli*Taq*-Gold polymerase (PE Biosystems). Microsatellites were amplified on a Gene Amp PCR system 9600 (PE Biosystems). PCR profiles consisted of one polymerase activation cycle at 94°C for 11 min; 35–40 amplification cycles of two temperatures (96°C for 30 s and 68/70-°C for 1 min) or three temperatures (96°C for 30 s; 60/64 for 1 min; 72°C for 1 min) depending on the SSR (Table 2); with a final elongation cycle of 5 min at 72°C.

For fluorescent allele-size determination, forward primers of IAS-oli11, IAS-oli12, IAS-oli17 and IAS-oli22 were labeled with a HEX, FAM or TET fluorescent tag (PE Biosystems) (Table 2) and PCR was performed as previously described.

Table 1 Olive cultivars studied and countries of origin

Cultivar	Country	Cultivar	Country
Alfafara	Spain	*Gerboui*	Tunisia
Arbequina	Spain	*Gordal Sevillana*	Spain
Ascolana Tenera	Italy	*Hojiblanca*	Spain
Ayvalik	Turkey	*Kaissy*	Syria
Bical	Spain	*Kalamon*	Greece
Blanqueta	Spain	*Konservolia*	Greece
Carolea	Italy	*Koroneiki*	Greece
Castellana	Spain	*Leccino*	Italy
Changlot Real	Spain	*Lechín de Granada*	Spain
Chemlali	Argelia	*Lechín de Sevilla*	Spain
Chetoui	Tunisia	*Manzanilla Cacereña*	Spain
Cakir	Greece	*Manzanilla de Sevilla*	Spain
Cobrançosa	Portugal	*Memeçik*	Turkey
Coratina	Italy	*Meski*	Tunisia
Cordovil de Serpa	Portugal	*Moraiolo*	Italy
Cornicabra	Spain	*Morisca*	Spain
Dolce Agogia	Italy	*Morrut*	Spain
Domat	Turkey	*Picholine Marrocaine*	Marrocco
Empeltre	Spain	*Picual*	Spain
Farga	Spain	*Picudo*	Spain
Frantoio	Italy	*Sevillenca*	Spain
Galega	Portugal	*Uslu*	Turkey
Gemlik	Turkey	*Verdial de Huevar*	Spain

Table 2 Microsatellite description: name, type of repeat, theoretical product length, number of alleles detected, amplification conditions and fluorescent tag

Name[a]	**Type of repeat**	**Expected length**	**Number of alleles**[b]	**Amplification profile (0 C)**[c]	**Fluorescent tag**
IAS-oli01	$G(A)_6G(A)_6G(A)_4(GA)_{19}$		–		
IAS-oli02	$(CT)_{16}AAA(CA)_2T(CT)_9$	107	m.p.	3 T(94/60/72)	
IAS-oli06	$(GA)_{17}$	227	3	2 T(96/68)	
IAS-oli07	$(GA)_{13}$	113	1	2 T(96/68)	
IAS-oli08	$(GA)_{30}$	163	m.p	3 T(94/60/72)	
IAS-oli09	$(CA)_{10}(TA)_4(GA)_{21}$	203	1	2 T(96/68)	
IAS-oli10	$(GA)_{15}GT(GA)_6$	179	m.p	2 T(96/68)	
IAS-oli11	$(GA)_{18}$	150	9	2 T(96/68)	FAM
IAS-oli12	$(GA)_{17}$	144	7	3 T(94/60/72)	HEX
IAS-oli17	AGGAG $(AGAGGG)_7$ AGGGAG	189	4	3 T(94/64/72)	TET
IAS-oli19	$(CT)_{10}TTC(CTT)_4(CT)_{19}$	194	m.p	2 T(96/70)	
IAS-oli21	$(GA)_{11}$	157	–		
IAS-oli22	$(GA)_6(GT)_5GACCT(GA)_5$	134	3	3 T(94/64/72)	TET

[a] Information about primer sequences can be obtained from the authors by request
[b] m.p. indicates multiple product
[c] 2 T: two temperature cycles; 3 T: three temperature cycles

Resolving PCR products

PCR products were run in 3.5% Metaphor agarose gels (FMC Bioproducts), in the presence of ethidium bromide (0.5 µg/ml), at 110 V for 3 h:30 min. Fluorescent amplification products were mixed with deionized formamide and an internal size standard (Genescan 350-TAMRA, Perkin Elmer) in the (1:12:0.5) volume ratio, denatured at 95°C for 5 min and analyzed on the automatic sequencer ABI 310 (PE Biosystems). The GeneScan software version 3.1 for Mac Os was used for sample analysis.

Heterozygosity

To assess the level of polymorphism of olive SSRs the expected heterozigosity (h) of each microsatellite was calculated according to the formula $h= 1- \sum (p_i)^2$ (Nei 1973) where h represents the probability that two alleles from the same locus would be different when chosen at random; and p the frequency of the ith allele at one locus.

Results

Isolation and characterization of microsatellites

To increase the frequency of microsatellites, an enriched genomic library for GA repeats was constructed. A total of 360 clones were obtained, from which around 54 (15%) gave a positive signal when hybridized with a ^{33}P-radioactive (CT) probe, and showing different intensity levels. Finally, 43 of these clones were sequenced and only 24, the ones with a strong signal, really contained a microsatellite sequence four of them being redundant (Table 3).

Fifty five per cent of the microsatellites found were perfect dinucleotide repeats, 30% imperfect and 15% compound. Since the library was enriched for (GA) repeats, this was the most common repeated motif present in almost all the microsatellite sequences. Nevertheless, (CA) and (TA) tandems, as well as (GAA) repeats, were also observed asociated to (GA) repeats in compound sequences. A hexamer repeat $(AGAGGG)_7$ was also found in one of the clones. The number of repeats ranged from 3 to 30–35 in single and compound motif repeats, respectively. A high percentage of sequences had less than five repeats or were too close to the insertion point within the vector, therefore being unsuitable for SSR marker development.

Table 3 Enrichment results

Total sequencing reactions	**43**
Lacking inserts	3
Lacking microsatellites	16
Redundant sequences	4
Microsatellite sequences	**20**
Type of repeat	
Perfect	11
Imperfect	6
Compound	3
Too small or too close to insertion	7

Microsatellite polymorphism

Specific primers were finally designed for 13 microsatellite sequences and their level of polymorphism was investigated in 46 olive cultivars. Only five of them (IAS-oli06, IAS-oli11, IAS-oli12, IAS-oli17 and IAS-oli22) amplified polymorphic products of the expected size range (Fig. 1). The remaining primers either failed amplification (IAS-oli01 and IAS-oli21), amplified multiple products (IAS-oli02, IAS-oli10, IAS-oli8 and IAS-oli19), or were monomorphic (IAS-oli07, IAS-oli09).

Twenty six alleles were detected in Metaphor agarose for the five polymorphic and specific loci. The number

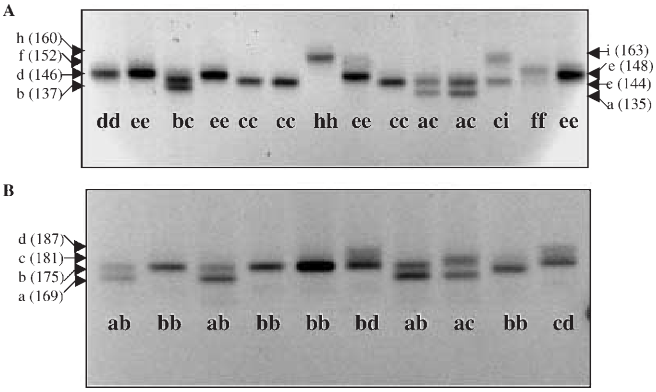

Fig. 1A, B Microsatellite polymorphism in 3.5% metaphor agarose gel: **A** Alleles detected at loci IAS-oli11 for 14 olive cultivars; **B** alleles detected at locus IAS-oli17 for ten olive cultivars

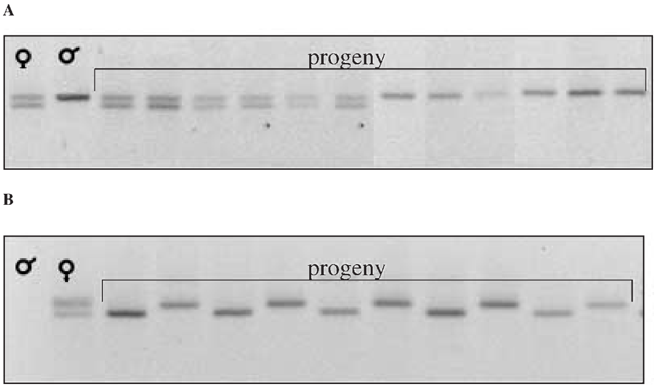

Fig. 2A,B Microsatelite inheritance in the ‘Leccino’ × ‘Dolce Agogia’ population. **A** Mendelian segregation at locus IAS-oli 17. **B** Null allele segregation at locus IAS-oli 12

Table 4 Allele characterization: locus name, heterozygosity (*h*), allele size and frequencies

Locus	*h*	Allele (size)[a]	Frequency
IAS-oli06	0.46	a* (227)	0.682
		b* (240)	0.057
		c* (250)	0.261
IAS-oli11	0.71	a** (135)	0.0217
		b** (137)	0.0109
		c** (144)	0.4565
		d** (146)	0.0326
		e** (148)	0.2283
		f** (152)	0.0435
		g** (154)	0.0109
		h** (160)	0.0435
		i** (161)	0.1522
IAS-oli12	0.71	a** (120)	0.0698
		b** (126)	0.4302
		c** (128)	0.0349
		d** (132)	0.0233
		e** (134)	0.2674
		f** (138)	0.0116
		g** (143)	0.1628
IAS-oli17	0.65	a** (169)	0.0426
		b** (175)	0.1702
		c** (181)	0.468
		d** (187)	0.319
IAS-oli22	0.61	a** (128)	0.1944
		b** (130)	0.514
		c** (132)	0.292

[a] * (allele size estimated in agarose gel), ** (fluorescent allele sizing on ABI 310)

of alleles per locus ranged from three to nine. The expected heterozygosity for the 46 cultivars tested ranged from 0.46 in locus IAS-oli06 to 0.71 in the loci IAS-oli11 and IAS-oli12 (Table 4). The lowest allelic frequency (0.0109) was observed in two alleles of the most-polymorphic locus (IAS-oli11), each of them present in just one cultivar, whereas one allele of the less-polymorphic locus (IAS-oli06) showed the highest frequency (0.682).

Forty four different genotype profiles were obtained with this combination of five loci, being able to identify 95% of the cultivars analyzed with unique profiles. Only two cultivar pairs (‘Morisca’-‘Picudo’ and ‘Castellana’-‘Manzanilla Cacereña’) remainded undistinguishable from one another. Three cultivars (‘Ascolana Tenera’, ‘Kalamon’ and ‘Picholine Marrocaine’) could be identified by the presence of a unique allele in loci IAS-oli11 and IAS-oli12 respectively, as well as eight other cultivars with a unique allele combination at a single locus.

Mendelian segregation was confirmed in the ‘Leccino’ × ‘Dolce Agogia’ population for those loci were the parents were polymorphic (Fig. 2a). The presence of at least one null allele was detected in locus IAS-oli 12 since, systematically, PCR failed in amplifying this SSR or else showed a very weak amplification from some cultivars. Moreover, the inheritance of this allele was tested analyzing the former cross where ‘Leccino’ amplifies two alleles for this locus and ‘Dolce Agogia’ none. All the progeny plants analyzed carried the null allele and, therefore, showed just one of the ‘Leccino’ alleles in a 1:1 ratio (Fig. 2b).

Stutter bands are common in dinucleotide microsatellites and are produced by the slippage of the polymerase during amplification (Luty et al 1990) These bands can complicate the pattern and lead to a very complex microsatellite morphology. Although the detection of this kind of band is reduced in Metaphor agarose, they are visible in automatic sequencers. Due to this phenomenon IAS-oli11 showed a very complex morphology when analyzed on the ABI 310.

Discussion

Microsatellites are desirable markers both in mapping initiatives or marker-assisted selection in breeding programs since they are very reliable transferable codominant markers, PCR based and highly polymorphic. Nevertheless, their development is rather laborious and expensive. We have followed an ‘‘enrichment’’ strategy for the development of SSRs. Library enrichment is particullary interesting for a species like olive, with no available genomic libraries to screen. Actually, it has proved to yield a 100—300-fold increase of positive clones with respect to standard libraries in other species. In our study, although 15% of the clones have hybridized with the (GA) probe, only 24 out of 43 did actually contain the expected microsatellite sequence. This high frequency of false positive signals has been reported before (Chavarriaga-Aguirre et al. 1998; Hicks et al. 1998) and may be reduced by increasing the stringency of the hybridization conditions. The rate of enrichment success obtained (6.6% of the clones), although lower than mean values obtained for other tree crops like apple (Guilford et al. 1997), *Cocos* (Rivera et al. 1999), *Prunus* (Cipriani et al. 1999) and *Eucalyptus* (Brondani et al. 1998), falls within the range observed for similar enriched libraries developed for *Pinus* spp. (Fisher et al. 1998) and is clearly higher than for un-enriched strategies (Liu et al. 1995; Hicks et al. 1998). We have also found a high rate of redundant clones (4 of 24), suggesting the existence of multiple copies of some microsatellites along the genome. This bias could also be explained by the fact that during the enrichment procedure (adaptor ligation, amplification of single-strand enriched DNA, bacterial growth before plating, etc) some fragments were arbitrarily selected over the rest. This tendency for redundancy in enriched libraries has been also pointed out by Brondani et al. (1998), Fisher et al. (1998) and Rossetto et al. (1999) when developing SSRs in *Pinus*, *Eucalyptus* and tea tree, respectively.

Although enrichment was performed for (GA/CT) repeats, the presence of other repeated motives (always associated with GA tandems in compound microsatellites) indicate that they may be quite common within the olive genome. Thus, it may be interesting to enrich for their presence. The GT/CA tandem seems to be especially frequent since it was present in three out of four compound microsatellites. These observations agree with those made by Sefc et al. (1999) for *Vitis* spp. and Liu et al. (1995) for *Paspalum* spp.

We have detected a very interesting polymorphic hexamer repeat (IAS-oli17), with four different alleles in 46 genotypes. Even more, the heterozygosity level found for this locus (0.65) is quite high. To our knowledge, this is the first time that an SSR marker based on an hexamer repeat has been described in a plant species. Fluorescent sizing revealed that differences in the number of bases between alleles of this locus were based on multiples of six suggesting that, for IAS-oli17, polymorphism is exclusively based on the number of repeats. Nevertheless, microsatellite polymorphism may not only be based in repeats number but may also be found in regions flanking the microsatellite, as shown by non-correlative allele sizes in IAS-oli11. Deletion/insertion of a single base or even long DNA fragments in the flanking regions have been reported before as a source of variation in SSRs (Gianfranceschi et al. 1998; Buteler et al. 1999; Sefc et al. 1999). Additionally, polymorphism can affect the priming site, giving rise to the appearance of null alleles like the one observed in IAS-oli12 (Fig. 2b). The detection of null alleles in microsatellite loci is quite common (Fisher et al. 1998; Gianfranceschi et al. 1998; Sefc et al. 1999; Tanaka et al. 1999), especially in highly outbred heterozygous species. Their presence is indicated by an excess of homozygotes but should be confirmed by segregation analysis. Caution should be exercised when scoring these loci, since heterozygosity is understimated and segregation distorted. These alleles may be overcome by re-designing primers at different locations when possible.

The level of polymorphism of an SSR is thought to be related to the number of repeats, as observed by Fisher et al. (1998). Nevertheless, in our study we have found that one of the more polymorphic SSRs (IAS-oli12) was not the longest one but had a medium number of repeats. Furthermore, the longest microsatellite, IAS-oli09, with 35 repeats (21+4+10), amplified two monomorphic products in all cultivars tested. Therefore, our results show no clear relationship between length and heterozygosity, and agree with observations made by Dayanandan et al. (1998) in poplars and Kangfu et al. (1999) in bean.

We have observed a high frequency of microsatellites amplifying multiple unspecific products. Multiple products are the result of multiple priming sites along the genome. This phenomenon is quite common since microsatellite sequences may be associated with highly repetitive DNA (Smith and Devey 1994). Multiple-locus amplification is particularily common in species with an allopolyploid origin, and may be related to genome fusion and chromosome duplication events during evolution, as pointed out by Buteler et al. (1999). If products from different loci could be distinguished, they would be very interesting for multilocus genotyping. Yet, most of the time the patterns obtained are too complex, as are the ones observed in our study, due to competition and the overlapping of bands. Although one perfect microsatellite (IAS oli08) showed this pattern, multiple products seem to be associated with compound microsatellites, since four out of five compound sequences gave rise to these products.

The extraordinary discriminatory capacity of microsatellite markers observed in other species have been confirmed in our study. Forty two out of forty six cultivars have been fully identified by the five microsatellites described. Around 88% of the cultivars could be identified with just as few as three SSRs. The presence of easily scorable, unique alleles and/or allele combinations, make them an ideal system for cultivar identification. A high level of polymorphism has also been found for the same cultivars, both with isozymes using pollen extracts (Trujillo et al. 1995) and with RAPDs (Belaj 1998). However, the number of isozymes systems is limited and RAPDs are hampered by difficulties in reproducibility and their dominant character, which reduces their usefullness for mapping purposes. Futhermore, the information content per locus is much higher in SSRs than in RAPDs, as pointed out by Dayanandan et al. (1998). The same olive cultivars were fully identified by the use of four RAPD primers, each of them implying 4–5 bands

(Belaj 1998). Assuming each band as a locus, a total of 18 RAPD loci were needed, whereas five SSR loci matched the same identification level.

In summary, in this paper we report the development of the first microsatellite markers in the olive tree and confirm the usefullness of this kind of marker as a powerful tool for cultivar identification. Their confirmed codominant character make them an ideal system for paternity analysis and linkage mapping.

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References

Angliolillo A, Mencuccini M, Baldoni L (1999) Olive genetic diversity assessed using amplified fragment length polymorphism. Theor Appl Genet 98: 411–421

Barranco D (1997) Variedades y patrones. In: Barranco D, Fernández-Escobar R, Rallo L (eds.). El cultivo del olivo 2nd edn. Mundi-Prensa, Madrid, pp 81–87

Barranco D, Rallo L (1985) Las variedades de olivo cultivadas en España. Olivae 9: 16–20

Belaj A (1998) Identificación y clasificación de variedades de olivo (*Olea europaea* L.) del Banco de Germoplasma de Córdoba con marcadores RAPDs. MSc, Universidad de Córdoba, Spain

Bogani P, Cavalieri D, Petruccelli R, Polsinelli L, Roselli G (1994) Identification of olive tree by using random amplified polymorphic DNA. Acta Hort 356: 98–101

Brondani RPV, Brondani C, Tarchini R, Grattapaglia D (1998) Development, characterization and mapping in *Eucalyptus grandis* and *E. urophylla*. Theor Appl Genet 97: 816–827

Buteler MI, Jarret RL, LaBonte DR (1999) Sequence characterization of microsatellites in diploid and polyploid *Ipomoea*. Theor Appl Genet 99: 123–132

Chavarriaga-Aguirre P, Maya MM, Bonierbale MW, Kresovich S, Fregene MA, Tohme J, Kochert G (1998) Microsatellites in Cassava (*Manihot esculenta* Crantz): discovery, inheritance and variability. Theor Appl Genet 97: 493–501

Cipriani G, Lot G, Huang WG, Marrazzo MT, Peterlunger E, Testolin R (1999) AC/GT and AG/CT microsatellite repeats in peach [*Prunus persica* (L) Batsch]: isolation, characterisation and cross-species amplification in *Prunus*. Theor Appl Genet 99: 65–72

Dayanandan S, Rajora OP, Bawa KS (1998). Isolation and characterization of microsatellites in trembling aspen (*Populus tremuloides*). Theor Appl Genet 96: 950–956

Fabbri A, Hormaza JI, Polito VS (1995) Random amplified polymorphic DNA analysis of olive (*Olea europaea* L.) cultivars. J Am Soc Hort Sci 120: 538–542

Fisher PJ, Richardson TE, Gardner RC (1998) Characteristics of single- and multi-copy microsatellites from *Pinus radiata*. Theor Appl Genet 96: 969–979

Gianfranceschi L, Seglias N, Tarchini R, Komjanc M, Gessler C (1998) Simple sequence repeats for the genetic analysis of apple. Theor Appl Genet 96: 1069–1076

Guerriero R, Loreti F, Bartolini S, Vitogliano C, Giorgelli F (1994) Clonal selection and self compatibility in the olive cultivar 'Leccino'. Technical Meeting of Working Groups 1 and 4, Córdoba, Spain, 14–16 Dec

Guilford P, Prakash S, Zhu JM, Rikkerink E, Gardiner S, Basset H, Forster R (1997) Microsatellites in *Malus* × *domestica* (apple): abundance, polymorphism and cultivar identification. Theor Appl Genet 94: 249–254

Hicks M, Adams D, O'Keefe S, Macdonald E, Hodgetts R (1998) The development of RAPD and microsatellite markers in lodgepole pine (*Pinus contorta* var. *latifolia*). Genome 41: 797–805

Kangfu Y, Soon JP, Poysa V (1999) Abundance and variation of microsatellite DNA sequences in beans (*Phaseolus* and *Vigna*). Genome 42: 27–34

Liu Z-W, Jarret RL, Kresovich S, Duncan RR (1995) Characterization and analysis of simple sequence repeat (SSR) loci in seashore paspalum (*Paspalum vaginatum* Swartz). Theor Appl Genet 91: 47–52

Luty JA, Guo Z, Willard HF, Ledbetter DH, Ledbetter S, Litt M (1990) Five polymorphic microsatellite VNTRs on the human X chromosome. Am J Hum Genet 46: 776–783

Murray MG, Thompson WF (1980) Rapid isolation of high-molecular-weight DNA. Nucleic Acids Res.8:4321–4325

Nei M (1973) Analysis of gene diversity in subdivided populations. Proc Natl Acad Sci USA 70: 3321–3323

Ouazzani N, Lumaret R, Villemur P, Guisto FD, Di GF (1993) Leaf allozyme variation in cultivated and wild olive trees (*Olea europaea* L.). J Hered 84:34–42

Panelli G, Famiani F, Rugini E, Bignani D, Natali S (1990) Preliminary characterization of olive somatic mutants from gamma irradiated ‘Frantoio’ and ‘Leccino’ plantlets. Acta Hort 286: 77–80

Rafalski JA, Morgante M, Vogel JM, Powell W, Tingey SV (1995) Generating and using DNA markers in plants. In: Birren B, Lai E (eds) Non-mammalian genome analysis: a practical guide. Academic Press, London New York, pp 75–134

Rivera R, Edwards KJ, Barker JHA, Arnold GM, Ayad G, Hodgkin T, Karp A (1999) Isolation and characterization of polymorphic microsatellites in *Cocos nucifera* L. Genome 42: 668–675

Rossetto M, McLauchlan A, Harriss FCL, Henry RJ, Baverstock PR, Lee LS, Maguire TL, Edwards KJ (1999) Abundance and polymorphism of microsatellite markers in the tea tree (*Melaleuca alternifolia*, Myrtaceae). Theor Appl Genet 98: 1091–1098

Rugini F, Panelli G (1994) In vivo and in vitro selection of polyploid mutants in olive cultivars ‘Frantoio’ and ‘Leccino’. XXIV Int Horti Congr, Kyoto, Japan, 21–22 August, Abstract-274

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual, 2nd edn. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York

Sefc KM, Regner F, Turetschek E, Glössl J, Steinkellner H (1999) Identification of microsatellite sequences in *Vitis riparia* and their applicability for genotyping of different *Vitis* species. Genome 42: 367–373

Smith DN, Devey ME (1994) Ocurrence and inheritance of microsatellites in *Pinus radiata*. Genome 37: 977–983

Tanaka K, Tsumura Y, Nakamura T (1999) Development and polymorphism of microsatellite markers for *Fagus crenata* and the closely related species *F. japonica*. Theor Appl Genet 99: 11–15

Tous J, Romero A, Plana J, Aranburu J (1998) Selección clonal de la variedad de olivo ‘Arbequina’. Presentación del clon IRTA-1–18. Phytoma 102: 15–28

Trujillo I, Rallo L, Arús P (1995) Identifying olive cultivars by isozyme analysis. J Am Soc Hort Sci 120: 318–324

Vergari G, Patumi M, Fontanazza G (1996) Use of RAPDs markers in the characterisation of olive germplasm. Olivae 60: 19–22