ISSN : 1812-5379 (Print)
ISSN : 1812-5417 (Online)
http://ansijournals.com/ja

JOURNAL OF AGRONOMY

ANSInet
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308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Journal of Agronomy 7 (2): 176-181, 2008
ISSN 1812-5379

SSR Marker Based DNA Fingerprinting of Tunisian Olive (*Olea europaea* L.) Varieties

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Abstract: This study aimed to test the efficiency of SSR markers to identify and to differentiate a set of 26 Tunisian olive varieties maintained at four collections. SSR analysis from 10 primer pairs revealed a total of 86 polymorphic alleles with 4 to 14 alleles per locus that allowed unique genotyping of the examined varieties. The Polymorphism Information Content (PIC) values ranged from 0.548 to 0.796 (average 0.719). The efficiency was found very high with average discriminating power overall 10 loci higher than 0.9. A combination of three SSR markers (ssrOeUA-DCA9, ssrOeUA-DCA13 and ssrOeUA-DCA16) was proposed for rapid identification of analysed cultivars. Three cultivars (Gerboui, Ain Jarbouaa and Regregui) thought to be identical resulted in absolutely different SSR profiles. This research showed that SSR is a suitable and effective tool to characterise olive varieties in Tunisian germplasm collections. The outcome of this study could be useful for varietal survey and the construction of a database of all olive varieties in Tunisia.

Key words: *Olea europaea*, olive, SSR, DNA fingerprinting

INTRODUCTION

The olive (*Olea europaea* L.) tree is an important oil crop that has been traditionally cultivated throughout the Mediterranean basin and the oil obtained from its drupes is an increasingly important product due to its nutritional and healthy features (Visioli and Galli, 1998) compared to other vegetable oils. Since the beginning of its domestication, olive has been propagated vegetatively to exploit the best combination of traits which arose by random crosses or mutations. As a result, a great number of varieties are present in all the countries where this species is cultivated, raising several problems for germplasm management and preservation (Carriero *et al*., 2002). Correct varietal identification is therefore crucial, since identification of olive cultivars is complicated by the large number of varietal synonyms and homonyms, the intensive exchange of plant material, the presence of varietal clones and problems of varietal certification in nurseries.

Traditionally, morphological and agronomic traits are used to identify olive cultivars; however, this is a slow process due to the long juvenile period of the trees and it is subjected to environmental influences. Molecular analysis offers data complementary to morphological characters for plant germplasm classification. Recently, molecular marker techniques have been used to characterize olive cultivars and clones. Microsatellite or Simple Sequence Repeat (SSR) markers are currently becoming the markers of choice for the molecular characterisation of different plant species because of their abundance, high polymorphism content, codominance, ease of detection and transferability across studies. A number of microsatellite markers have already been developed in olive and their primer sequences have been published (Rallo *et al*., 2000; Sefc *et al*., 2000; Carriero *et al*., 2002; Cipriani *et al*., 2002). The usefulness of these markers has been mainly assessed in samples of olive varieties cultivated in the northern part of the Mediterranean area.

Our interest was focused on the Tunisian olives. This Mediterranean country occupies the fourth place on world scale in terms of olive production (IOOC, 2004). Tunisian olive plantations are spread all over the country in different edapho-climatic conditions and count about 67 million trees covering 1.6 million of hectares and estimated to include more than 50 cultivars. After hundreds of years of uncontrolled propagation the result is that more than 70 different names are used (Trigui *et al*., 2002), same genotypes are known with different names,

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phenotypically different trees are identically called and moreover several names are supposed only to represent phenotypical variation of the same cultivar. Therefore, genotype information of Tunisian olive cultivars is particularly important for cultivar identification and to enhance the classification of germplasm collections. The results of microsatellite analysis of twenty-six Tunisian olive varieties maintained at four collections were presented here. We show that microsatellites can be used for rapid and reliable identification of olive varieties.

MATERIALS AND METHODS

Plant materials and DNA extraction: Molecular characterisation was performed on 34 olive (*Olea europaea* L.) trees: 33 trees belonging to 25 cultivars and one tree without denomination (Table 1). These were obtained from three collections maintained in experimental orchards at the Institut de l Olivier (IO, Chott Meriem, Sousse), the Centre de Biotechnologie (Technopole de Borj Cedria, Hammam-Lif) and Sbitla (Kasserine). Three cultivars introduced from Italy (Coratina), Spain (Manzanilla de Sevilla) and France ('Picholine'), were added for reference to allow comparison of SSR-based profiles produced by different laboratories. Frozen leaves (400-500 mg) were ground to a fine powder in a reciprocal grinding apparatus (Mixer Mill MM 300, Retsch, Haan, Germany). Genomic DNA was extracted from the ground tissue according to Geuna *et al.* (2003). DNA yield and quality were assessed by gel electrophoresis using standards.

SSR markers: Ten developed primer pairs for olive microsatellite loci (Sefc *et al.*, 2000) were used. The loci amplified by these primer pairs were designated as: ssrOeUA-DCA1, ssrOeUA-DCA3, ssrOeUA-DCA4, ssrOeUA-DCA7, ssrOeUA-DCA9, ssrOeUA-DCA11 ssrOeUA-DCA13, ssrOeUA-DCA15, ssrOeUA-DCA16 and ssrOeUADCA18.

Polymerase chain reaction and electrophoresis: PCR reactions were performed in a total volume of 20 μL containing 100 mM Tris-HCl (pH 9.0), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM each dNTP, 0.125 μM each primer, 0.2 Units of Taq DNA polymerase (Platinum Taq DNA Polymerase; Invitrogen) and 10 ng template DNA. To enable the detection and sizing of PCR fragments, the forward primer was labelled with 33P-ATP. Amplification reactions were performed in a PTC-100 thermal cycler (MJ Research Inc., Watertown, MA, USA), which was programmed to follow the conditions reported by Sefc *et al.* (2000). PCR products were resolved in 6% denaturating polyacrylamide gels and autoradiographed on X-ray film using standard procedures.

Table 1: List of olive accessions studied

Cultivars	Use	Collection
Meski	Table	Chott Meriem, Borj Cedria
Gerboui	Oil and Table	Chott Meriem, Tunis
Marsaline	Oil and Table	Chott Meriem, Tunis
Chétoui	Oil	Chott Meriem, Borj Cedria
Sayali	Oil and Table	Chott Meriem
Zarrazi	Oil and Table	Chott Meriem, Tunis
Chemlali	Oil	Chott Meriem, Tunis, Sbeitla
Neb Djemel	Oil	Chott Meriem
Rakhami	Oil	Chott Meriem
Chemchali	Oil and Table	Chott Meriem
El Hor	Oil	Sbeitla
Oueslati	Oil	Sbeitla, Tunis
Mongar Ragma	Oil and Table	Sbeitla
Swabaa Algia	Table	Sbeitla
Semni	Oil	Sbeitla
Kbiret Louzir	Oil	Borj Cedria
Jdallou	Oil	Borj Cedria
Kchiunet Sig	Oil	Borj Cedria
Injassi	Table	Borj Cedria
Dhokkar	Oil	Borj Cedria
Toffahi	Oil and Table	Borj Cedria
Jemri Dhokkar	Oil	Borj Cedria
Regregui	Oil and Table	Borj Cedria
Ain Jarbouaa	Oil and Table	Borj Cedria
Unknown	ND	Borj Cedria

ND: Non Determined

Data analysis: Polymorphism Information Content (PIC), a measure of the allelic diversity at a locus, was estimated for each of the SSR loci assayed using the following equation:

$$PIC = 1 - \sum_{i=1}^{l} p_i^2 - 2\sum_{i=j+1}^{l}\sum_{j=1}^{l-1} p_i^2 p_j^2$$

with p_i and p_j as the frequencies of the i^{th} and j^{th} alleles at a locus with *l* alleles in a population, respectively (Botstein *et al.*, 1980). Expected heterozygosity (He) was calculated using the formula developed by Nei (1978):

$$He = 1 - \sum_{i=1}^{l} p_i^2$$

with p_i as the frequency of the i^{th} allele at the studied locus. Observed heterozygosity (H_o) was obtained as the ratio among heterozygous individuals and the total number of genotypes per locus. The discriminating power (Dj) of the j^{th} assay unit was calculated according to Tessier *et al.* (1999):

$$Dj = 1 - Cj = 1 - \sum_{i=1}^{l} p_i \frac{(Np_i - 1)}{N - 1}.$$

PIC, H_e and H_o were estimated using the CERVUS software, version 2 (Marshall *et al.*, 1998).

RESULTS

Microsatellites were successfully amplified in all analysed varieties with the ten primer pairs used. The patterns generated by primer pair ssrOeUA-DCA18 in the olive accessions studied are shown in Fig. 1. At locus

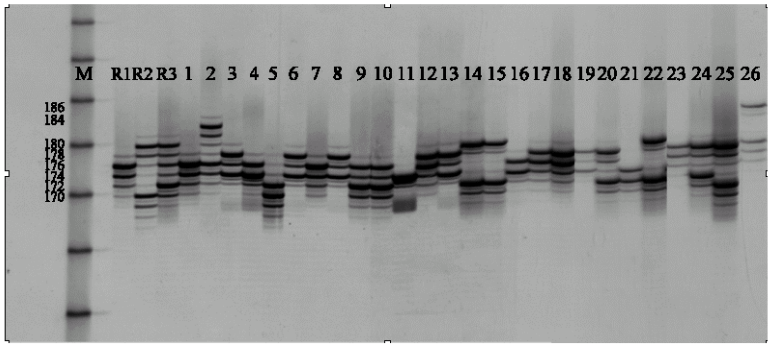

Fig. 1: Autoradiogram of a polyacrylamide-gel separation of the ^{33}P-labelled PCR-amplified microsatellite ssrOeUA-DCA18 in autochthonous (Meski (1), Sayali (2), Chemlali (3), Gerboui (4), Marsaline (5), Chétoui (6), Besbassi (7), Kchinet Sig (8), Neb Djemel (9), Dhokkar (10), Oueslati (11), Mongar Ragma (12), Swabaa Algia (13), Semni (14), Kbiret Louzir (15), Jdallou (16), Injassi (17), Chemchali (18), Rakhami (19), Toffahi (20), Zarrazi (21), El Hor (22), Ain Jarbouaa (23), Jemri Dhokkar (24), Unknown (25) et Regregui (26)) and introduced olive accessions (Coratina (R1), Picholine (R2) and Manzanilla de Sevilla (R3)). Fragment size of PCR/SSR products in base pairs (bp) are reported on the left. (M) 10 bp DNA marker (Invitrogen, Carlsbad, CA, USA)

Table 2: Product size range, allele number, genotype number, He, H_o, D and PIC of the 10 SSR loci studied

Locus	Range of sizes (bp)	No. of alleles	No. of genotypes	H_e	H_o	D	PIC
ssrOeUA-DCA1	208-240	9.0	11.0	0.716	0.731	0.914	0.664
ssrOeUA-DCA3	232-252	8.0	14.0	0.794	0.846	0.920	0.745
ssrOeUA-DCA4	132-188	14.0	17.0	0.810	0.769	0.954	0.775
ssrOeUA-DCA7	129-169	9.0	14.0	0.815	0.480	0.928	0.773
ssrOeUA-DCA9	163-209	11.0	16.0	0.817	0.846	0.948	0.776
ssrOeUA-DCA11	129-163	8.0	11.0	0.717	0.577	0.886	0.655
ssrOeUA-DCA13	120-140	5.0	11.0	0.732	0.577	0.809	0.680
ssrOeUA-DCA15	244-268	4.0	6.0	0.616	0.731	0.797	0.548
ssrOeUA-DCA16	124-182	10.0	15.0	0.834	0.923	0.939	0.796
ssrOeUA-DCA18	170-186	8.0	13.0	0.825	0.962	0.911	0.782
Average		8.6	12.8	0.768	0.744	0.901	0.719

ssrOeUA-DCA4, a third band was detected in all varieties in addition to the other 14 alleles. At locus ssrOeUA-DCA1, we observed a weak amplification of long alleles in aid to the short ones which was overcome by prolonging the gel/film exposure time.

All ten microsatellite markers were polymorphic across the screened genotypes, revealing a total of 86 alleles. The number of alleles for each locus varied from four at locus ssrOeUA-DCA15 to fourteen at locus ssrOeUA-DCA4, with an average of 8.6 (Table 2). Allele sizes were found to vary among the ten loci; the differences between the longest and shortest alleles ranging from 16 to 58 base pairs (bp). Allele frequencies were low, particularly at loci with a high number of alleles (Table 3). The frequency of each allele at the loci was generally low. The frequency of 86% of the polymorphic alleles ranged between 0.019 and 0.25 and that of the remaining was higher than 0.25, whereas one allele of the least polymorphic locus (ssrOeUA-DCA15) showed a frequency higher than 0.5. The number of observed genotypes per locus ranged from 6 (ssrOeUA-DCA15) to 17 (ssrOeUA-DCA4) with a total of 128 different genotypes, revealing 28.5% of all possible genotypes (Table 2). PIC values ranged from 0.548 for ssrOeUA-DCA15 to 0.796 for ssrOeUA-DCA16 with an average of 0.719 and classified all loci as informative markers (PIC>0.5) and six loci as suitable for mapping (PIC>0.7). The expected heterozygosity (H_e) equalled 0.768 and varied from 0.616 to 0.834. Observed heterozygosity (H_o) ranged from 0.480 to 0.962 with an average of 0.744.

Among the 86 polymorphic alleles, 28 were specific to eighteen olive varieties. One specific allele was detected in varieties Meski, Neb Djemel, Oueslati, Jemri Dhokkar, Jdallou and Besbassi, two in varieties Unknown, Gerboui, Kchinet Sig, Semni, Swabaa Algia and Kbiret Louzir. Three specific alleles were characteristic of Regregui and Sayali and the highest number of variety-specific alleles was found in Dhokkar (five). The unknown genotype was not identical to any other accession included in this study.

The allelic polymorphisms found allowed the discrimination of all analysed accessions. No differences were found among the amplification profiles obtained from different individuals (trees) of the same accession.

Table 3: Allele size (bp) and allele frequencies given (*in italics*), in 26 olive genotypes at ten microsatellite loci

	Alleles													
Locus	1	2	3	4	5	6	7	8	9	10	11	12	13	14
ssrOeUA-DCA1	208	216	218	220	224	226	228	230	240					
	0.462	*0.269*	*0.039*	*0.039*	*0.039*	*0.019*	*0.039*	*0.077*	*0.019*					
ssrOeUA-DCA3	232	236	238	240	242	244	248	252						
	0.289	*0.019*	*0.25*	*0.019*	*0.25*	*0.019*	*0.077*	*0.077*						
ssrOeUA-DCA4	132	134	138	140	142	148	156	158	162	164	166	176	178	188
	0.019	*0.385*	*0.019*	*0.039*	*0.019*	*0.019*	*0.058*	*0.019*	*0.096*	*0.039*	*0.019*	*0.039*	*0.039*	*0.192*
ssrOeUA-DCA7	129	131	133	145	147	149	151	167	169					
	0.02	*0.04*	*0.08*	*0.08*	*0.1*	*0.08*	*0.32*	*0.26*	*0.02*					
ssrOeUA-DCA9	163	173	177	183	185	187	195	199	205	207	209			
	0.077	*0.231*	*0.019*	*0.019*	*0.019*	*0.077*	*0.327*	*0.019*	*0.154*	*0.039*	*0.019*			
ssrOeUA-DCA11	129	135	139	143	149	153	155	163						
	0.039	*0.019*	*0.019*	*0.385*	*0.365*	*0.019*	*0.096*	*0.058*						
ssrOeUA-DCA13	120	122	124	132	140									
	0.115	*0.173*	*0.442*	*0.077*	*0.192*									
ssrOeUA-DCA15	244	256	266	268										
	0.558	*0.039*	*0.231*	*0.173*										
ssrOeUA-DCA16	124	126	144	146	150	158	162	174	178	182				
	0.308	*0.039*	*0.019*	*0.135*	*0.173*	*0.058*	*0.058*	*0.173*	*0.019*	*0.019*				
ssrOeUA-DCA18	170	172	174	176	178	180	184	186						
	0.039	*0.173*	*0.231*	*0.192*	*0.25*	*0.077*	*0.019*	*0.019*						

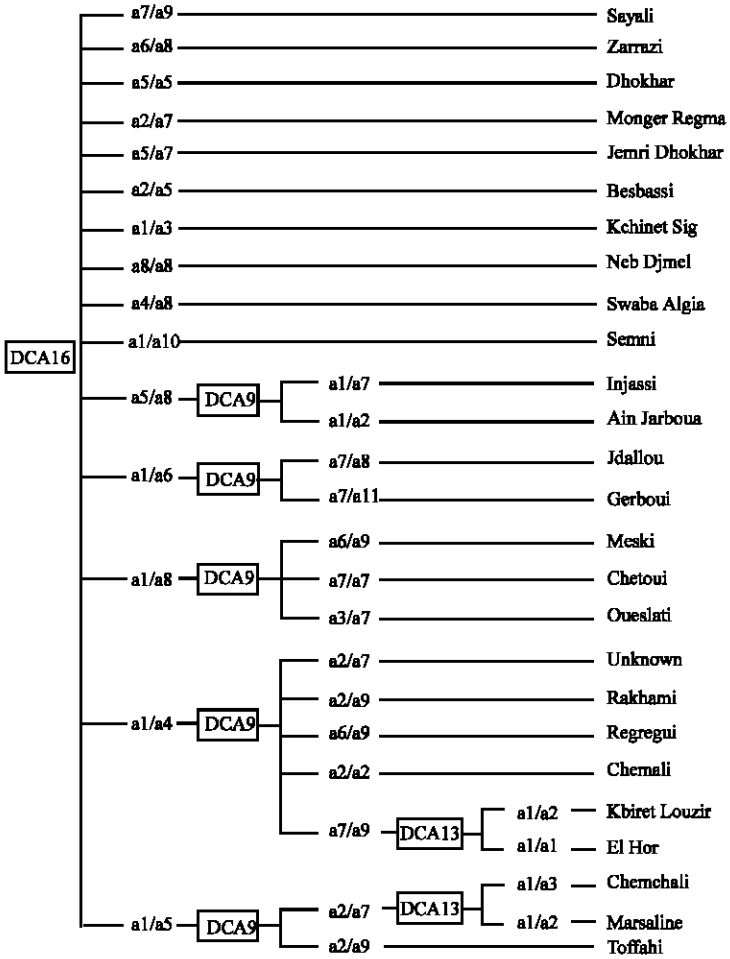

Fig. 2: Microsatellite identification key of the 26 olive genotypes based on fingerprints at three loci

Before the establishment of variety identification key, we have calculated the discriminating power for each locus (Table 2). The latter varied among loci in a range from 0.954 (ssrOeUA-DCA4) to 0.797 (ssrOeUA-DCA15) and the average discriminating power over all loci was 0.901, indicating a very low probability of identical genotypes. The characteristics of some markers were discriminatory for reliable use for variety identification. ssrOeUA-DCA4 showed the highest discriminating power but it generated complex banding patterns while weak amplification of long alleles was observed at locus ssrOeUA-DCA1. A minimum set of markers was therefore chosen for rapid identification of 26 olive varieties. This included ssrOeUA-DCA9 and ssrOeUA-DCA16 with the highest discriminating power and ssrOeUA-DCA13 which has the highest discriminating capacity among locus that differentiated Kbiret Louzir and El Hor varieties. The identification key for the 26 olive accessions is shown in Fig. 2. Specific allele profiles at locus ssrOeUA-DCA16 were first assigned to 10 varieties (Zarrazi, Neb Jmel, Sayali, Besbassi, Mongar Ragma, Swabaa Algia, Semni, Kchinet Sig, Dhokkar et Jemri Dhokkar). The varieties (Meski, Gerboui, Regregui, Injassi, Chemlali, Jdallou, Oueslati, Toffahi, Unknown, Chétoui, Ain Jarbouaa, et Rakhami) were differentiated by ssrOeUA-DCA9. Finally, the accessions Marsaline, Chemchali, Kbiret Louzir and El Hor were differentiated by ssrOeUA-DCA13

DISCUSSION

For the management of *ex situ* plant germplasm, two important goals have to be reached. First, all accessions should be characterised in order to eliminate cases of mislabelling and redundancies and to create a complete data base. Second, to keep a minimum of accessions which should represent a maximum of variability constituting a core collection (Khadari *et al*., 2003). The results present in this study showed that SSR markers can be successfully used to genotype olive collections such as some Tunisian ones. In the set of 26 accessions, 86 alleles were detected which multiplied into a large number of observed genotypes at each locus, giving high discrimination value for varietal identification. Using as few as three SSR markers (ssrOeUA-DCA9, ssrOeUA-DCA13 and ssrOeUA-DCA16) we were able to differentiate all olive varieties. We have also clarified a case of synonymy; previous assumptions made by morphological markers (Trigui *et al*., 2002) had defined as putatively synonyms the cultivars Gerboui, Ain Jarbouaa and Regregui. This possibility can be excluded because of the differences shown at 9 of the 10 polymorphic loci considered. Some accessions are planted in more than one collection however, differences were not found among the amplification profiles from different individuals of the same accession. Lack of polymorphism among different trees of the same accession from different field trials indicates the low probability of mislabelling and planting errors when a careful management procedure is followed in the collections schedule. As for other fruits, names of Tunisian olive cultivars refer to some particular traits of the fruit (Injassi = pear like fruit, Swabaa Algia = fingers of the nice women, Toffahi = apple like fruit); some names are toponyms (Oueslati = from Oueslatia; Besbassi = from Besbassia) and others refer to the practical utility of the varieties (Dhokkar = Pollinator), we found that generic names of Tunisian olive cultivars include different genotypes.

High polymorphism was detected in the analysis of 26 olive varieties with ten microsatellite markers. The average number of alleles per locus reported herein (8.6) was close to the average of 8.7 found by Sefc *et al*. (2000) for the same ten SSRs using 9 Italian and 38 Iberian olive cultivars. The average He value in their study (0.732) as well as that (0.775) reported by Khadari *et al*. (2003), were close to our value (0.768) revealing high polymorphism rates for olive microsatellites. High variability of olive microsatellites was also shown in the studies by Carriero *et al*. (2002) with an average of 5.7 alleles over ten loci in 20 varieties and Rallo *et al*. (2000), who analysed a set of 46 olive cultivars from the olive Germplasm Bank at Cordoba (Spain) using 5 SSR markers and reported an average of 5.2 alleles per locus. However, marker polymorphism also varied according to size and origin of germplasm sampled. Co-ancestry of cultivars may reduce the genetic variability of a cultivar group dramatically. High levels of polymorphism in olive were also observed with RAPD markers (Belaj *et al*., 2001; Nikoloudakis *et al*., 2003) as well as with isozymes (Trujillo and Rallo, 1995). As in other fruit tree species (Hormaza *et al*., 1994; Nicese *et al*., 1998; Oraguzie *et al*., 2001) the high level of polymorphism may reflect the outcrossing nature of olive species.

The results of microsatellite based genotyping of Tunisian olive accessions presented here should be confirmed by using other DNA-based fingerprinting techniques such as AFLP. A better understanding of the effectiveness of the different molecular markers is considered an essential step toward olive germplasm characterisation and classification.

CONCLUSION

This research showed that SSR is a suitable and effective tool to characterise olive varieties in some

Tunisian germplasm collections. The outcome of this study could be useful for varietal survey and the construction of a database of all olive varieties in Tunisia.

ACKNOWLEDGMENT

We gratefully acknowledge Dr. Dalenda Boujnah (Institut de l'Olivier, Sousse), Prof. Mohamed Boussaid (Institut National des Sciences Appliquées et de la Technologie) for the plant material supplied.

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