GMR Genetics and Molecular Research
Online Journal
ISSN:1676-5680

Microsatellite marker-based identification and genetic relationships of olive cultivars from the Extremadura region of Spain

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Genet. Mol. Res. 11 (2): 918-932 (2012)
Received August 2, 2011
Accepted September 7, 2011
Published April 10, 2012
DOI http://dx.doi.org/10.4238/2012.April.10.7

ABSTRACT. Seventy-seven olive accessions corresponding to 25 cultivars from the Extremadura region of Spain were studied using four microsatellite or SSR markers in order to fingerprint them, and evaluate genetic similarity and relationships between local and introduced olive cultivars. The number of alleles per locus ranged from 4 to 8, with a mean of 6.25 alleles per primer pair (a total of 25 alleles). The observed heterozygosity ranged from 0.58 to 0.95, while the expected heterozygosity varied between 0.68 and 0.83. The polymorphism information content values ranged from 0.63 to 0.79. The mean polymorphism information content value of 0.70 for the SSR loci provided sufficient discriminating ability to evaluate the genetic diversity among the cultivars. The SSR data allowed unequivocal identification of all the cultivars; a combination of three SSR markers was sufficient to discriminate all 25 olive cultivars. A dendrogram was prepared, using the unweighted pair-group method with arithmetic mean clustering algorithm; it depicted the pattern of

relationships between the cultivars. Most of the local cultivars grouped according to their geographic origin. No clear clustering trends were observed when the morphological traits of fruit endocarps or fruit use of cultivars were employed as analysis criteria. We conclude that there is a high level of variability among local olive cultivars from the Extremadura region at both the morphological and molecular levels; these data should be useful for identifying and distinguishing local germplasm.

Key words: DNA fingerprinting; Fruit-endocarp or fruit-pit characters; Genetic diversity; *Olea europaea*; SSR

INTRODUCTION

The olive (*Olea europaea* L.), one of the most economically important fruit trees in the Mediterranean basin, includes the cultivated type (var. *sativa*) as well as the wild type (var. *oleaster*). Because of the long life span of this species, it has undergone relatively little selection, and thus the cultivated gene pool is presumably quite close to that of the ancestor genotype (Rugini et al., 2011). Cultivated olives, which grow in the same habitats as the wild type, bear certain morphological particularities, such as generally larger fruit size and higher oil content in the mesocarp (Rugini et al., 2011). The cultivated olive is an evergreen, out-crossing, vegetatively propagated tree that adapts quite easily to many and varied environmental conditions with high genetic variability due to both plant longevity as well as to the scarcity of genotype turnover over centuries of cultivation (Bracci et al., 2011). Although more than 2600 distinct olive cultivars have been described, this may be an underestimation, given of the lack of data on minor local cultivars in many olive-growing areas. This broad genetic diversity has been characterized in different eastern and western Mediterranean countries based both on morphological and on molecular characters (Cipriani et al., 2002; Owen et al., 2005; Rallo et al., 2005; Sarri et al., 2006; Cordeiro et al., 2008; Ipeck et al., 2009; Fendri et al., 2010; Roubos et al., 2010; Sesli and Yeğenoğlu, 2010). Molecular methods for olive cultivar fingerprinting have been demonstrated to be effective, but microsatellite or simple sequence repeat (SSR) analysis is becoming the preferred choice for its high discriminatory power and simpler interpretation (Bracci et al., 2011). In an effort to trace the provenance of olive cultivars, recent reports have provided a list of recommended SSR markers and procedures for genotyping olive (Doveri et al., 2008; Baldoni et al., 2009). Also, the local germplasm for limited or small cultivation areas has recently been characterized by SSR markers, suggesting high levels of genetic diversity as well as notable variability of wild and cultivated types at the regional scale (Poljuha et al., 2008; Bracci et al., 2009; Belaj et al., 2010).

In Spain, olive germplasm is estimated to include about 272 cultivars (Barranco and Rallo, 2000; Rallo et al., 2005), most of them from uncertain origin and probably selected for rainfed cultivation. The real number of cultivars may well be higher because of the lack of information on many local minor cultivars scattered throughout the country. Knowledge of these less-known cultivars would be useful as they may have traits, which had not previ-

ously been deemed important but which might offer advantages in current olive cultivation. The main areas of olive cultivation in Spain are located in the Mediterranean regions of Andalusia (southern), Castilla-Mancha (central), Extremadura (central-western), Valencia (central-eastern), and Catalonia (northeastern) (Rallo et al., 2005). Olive germplasm richness has been confirmed in Spain by molecular methods, particularly in main cultivars (Besnard et al., 2001; Diaz et al., 2006; Sarri et al., 2006). Recently, SSR markers have been used to characterize the wild trees from the main olive growing areas (Andalusia, Valencia and Catalonia) together with their genetic diversity and relationships with olive cultivars (Belaj et al., 2007, 2010).

In the Extremadura region alone, olive cultivars (both for table consumption as well as for oil), occupy some 265,000 ha, with 80% of them being main cultivars and the rest being a mixture of minor cultivars (Delgado-Martinez, 2006). The main cultivars from Extremadura as well as from other Spanish regions have been identified by morphological description (Barranco and Rallo, 2000; Rallo et al., 2005) as well as using SSR markers (Diaz et al., 2006; Sarri et al., 2006). Information is limited, however, on minor olive cultivars of this region.

In the present report, four microsatellite or SSR markers were used to evaluate the genetic diversity of 25 olive cultivars grown in Extremadura, a small area of traditional olive cultivation. The goal is to use SSR markers to identify, characterize, and establish relationships between local olive cultivars that are geographically related.

MATERIAL AND METHODS

Plant material, DNA extraction, and morphological description of fruit-endocarp

This study used a panel of 77 accessions belonging to 25 olive cultivars, localized in the Extremadura region (central-western Spain) and collected from their natural orchards (Table 1), including: a wild type of *Olea europaea sylvestris*, 15 cultivars that are recognized as native or local to Extremadura (5 main and 10 minor local cultivars), and 9 introduced or foreign cultivars from Portugal ('Galega', 'Carrasqueña' and 'Redondil'), from southern Spain ('Gordal Sevillana', 'Manzanilla Sevillana', 'Ocal', and 'Picual'), from northeastern Spain ('Arbequina') and from central Spain ('Cornicabra'). For the collecting areas, the precise geographical location is available on request. When available, 10 different trees were sampled for each site in order to represent the maximum genetic diversity occurring in an accession.

Total DNA was extracted from young leaves from these cultivars as described in Belaj et al. (2001). For the morphological description of fruit-endocarp or fruit-pit, 11 characters were selected (Table 2), from the pomological pattern widely used for olive cultivar characterization (Rallo et al., 2005).

SSR analysis

Four microsatellite (SSR) markers were used in this study. Two markers (DCA9, DCA18) described in Sefc et al. (2000), and two markers (EMO2, EMO3) by De La Rosa

et al. (2002) were selected for their high polymorphism among olive cultivars, their easily scored patterns and their small-scale stuttering (Table 3). The 20-µL reactions contained 50 ng template DNA, 1.5 mM $MgCl_2$, 0.3 mM dNTP, 10 pmol of each primer, and 1.5 U Taq DNA polymerase (Gibco-BRL) in 1X PCR buffer. The cycling regime consisted of 94°C for 4 min, followed by 34 rounds of 94°C for 30 s; 50-60°C (primer pair dependent; Sefc et al., 2000; De La Rosa et al., 2002) for 45 s and 72°C for 60 s, with a final step of 72°C for 10 min (Table 3).

Data analysis

Genetic relationships between olive genotypes were studied on the basis of a similarity matrix using the proportion of alleles (Nei and Li, 1979). A phenogram was drawn based on the unweighted pair-group method with arithmetic mean algorithm (UPGMA) using the NTSYS-pc ver. 2.11a program (Rohlf, 2000). The SSR data were analyzed using several genetic parameters such as: number of alleles per locus; observed heterozygosity (H_O, calculated as the number of heterozygotes per locus divided by the number of individuals typed); expected heterozygosity (H_E) or gene diversity (Nei, 1987), and the polymorphism information content (PIC) calculated for each locus (Botstein et al., 1980).

RESULTS AND DISCUSSION

SSR polymorphism

The olive accessions listed in Table 1 were chosen after three years of morphological observations to represent olive cultivars grown in the Extremadura region of Spain. Three accessions of the one *sylvestris* cultivar from Extremadura, 29 accessions of introduced cultivars (9 foreign cultivars), currently predominant in new plantations in the region and originating from other regions of Spain and Portugal (‘Picual’, ‘Arbequina’, ‘Manzanilla Sevillana’, ‘Gordal Sevillana’, ‘Cornicabra’, ‘Galega’, ‘Carrasqueña’, ‘Ocal’, and ‘Redondil’), and 45 accessions belonging to 15 local cultivars, including 5 main and 10 minor local cultivars, from the Extremadura region were included in the study. The analysis of the endocarp distinctive characters revealed high variability among Extremadura cultivars, especially for weight, form, and symmetry of the position ‘A’, while the position of the maximum transversal diameter, and the number and distribution of vascular bundles over the endocarp surface showed little phenotypic variation (Table 2). Therefore, this analysis has identified the endocarp characters that were most variable and useful in discriminating local cultivars from the Extremadura region. Prior reports using morphological and agronomical traits (Barranco and Rallo, 2000; Rallo et al., 2005) as well as molecular analysis (Diaz et al., 2006; Sarri et al., 2006; Belaj et al., 2010) have confirmed the diversity of Spanish olive germplasm. Nevertheless, the most of the genotypes determined in our study correspond to olive material present in only one sampling area from Extremadura or local cultivars (15 local cultivars among the 25 determined in this study). Also, given that only 9 cultivars (main cultivars) coincided with those of previous studies, a comparison of results was not feasible.

Table 1. Description of olive cultivars used in this study with code numbers, geographical origin and diffusion area in Extremadura (C = center; N = north, S = south) and use (O = oil; T = table olive; O/T = oil and table olive) of fruits.

Code	Cultivar name	Accession No.	Origin/diffusion area/typology	Cultivation area in Extremadura (%)	Use
2	'Carrasqueña'[b]	4	Portugal/Extremadura (Badajoz), C-S area/foreign cultivar	16	O
4	'Oliva'[a,c]	3	Spain/Extremadura (Badajoz), S area/local minor cultivar	<1	O
5	'Morisca'[a]	4	Spain/Extremadura (Badajoz), C-S area/local main cultivar	12	O
6	'Pico Limon'[a]	4	Spain/Extremadura (Badajoz), S area/local cultivar	4	O
9	'Pico Real'[a,c]	2	Spain/Extremadura (Badajoz), S area/local minor cultivar	<1	O
11	'Azulejo'[a,c]	3	Spain/Extremadura (Badajoz), S area/local minor cultivar	<1	O
12	'Perito'[a,c]	2	Spain/Extremadura (Badajoz), S area/local minor cultivar	<1	O
13	'Manzanilla Real'[a,c]	3	Spain/Extremadura (Badajoz), C area/local minor cultivar	<1	O/T
19	'Redondil'[b]	4	Portugal/Extremadura (Badajoz), C-S area/foreign cultivar	<1	O/T
24	'Ocal'[b]	2	Spain/Extremadura (Badajoz), C-S area/foreign cultivar	<1	O
25	'Colora'[a,c]	2	Spain/Extremadura (Badajoz), C area/local minor cultivar	<1	O
32	'Gordal Sevillana'[b]	4	Spain/Extremadura (Badajoz), S area/foreign cultivar	<1	T
41	'Galega'[b]	3	Portugal/Extremadura (Badajoz), C area/foreign cultivar	<1	O/T
48	'Cornicheˈ[a,c]	2	Spain/Extremadura (Badajoz), C-S area/local minor cultivar	<1	O
52	'Azulito'[a,c]	2	Spain/Extremadura (Badajoz), S area/local minor cultivar	<1	O
58	'Verdial de Badajoz'[a]	4	Spain/Extremadura (Badajoz), C-S area/local cultivar	14	O
84	'Manzanilla Cacereña'[a]	4	Spain/Extremadura (Caceres), N area/local main cultivar	28	O/T
92	'Manzanilla Sevillana'[b]	4	Spain/Extremadura (Badajoz), S area/foreign cultivar	3	T
93	'Cornicabra'[b]	4	Spain/Extremadura (Caceres and Badajoz), N-C area/foreign cultivar	15	O
95	'Redondillo'[a,c]	3	Spain/Extremadura (Badajoz)/C-S area/local minor cultivar	<1	O
98	'Cuerno Real'[a,c]	3	Spain/Extremadura (Badajoz)-S area/local minor cultivar	<1	O
100	'WT'	3	Spain/Extremadura (Badajoz), C area/wild trees	<1	O
104	'Picual'[b]	3	Spain/Extremadura (Badajoz), C area/foreign cultivar	1	O
105	'Arbequina'[b]	3	Spain/Extremadura (Badajoz), C area/foreign cultivar	<1	O
114	'Original'[a,c]	2	Spain/Extremadura (Badajoz), C area/local minor cultivar	<1	T

Cultivars marked with (a) are considered to be autochthonous or native or local, those marked with (b) are considered to be foreign, since they were introduced into Extremadura, and those marked with (c) are considered to be minor cultivars with a cultivation area of <1% of the total area of olive cultivation in Extremadura.

Table 2. Endocarp morphological characters of 25 olive genotypes analyzed.

Cultivar	Weight	Form	Symmetry of position A	Symmetry of position B	Position of the maximum transversal diameter	Form of the apex	Form of the base	Roughness of the surface	Number of vascular bundles over the surface	Distribution of vascular bundles over the surface	Presence of mucro
'Carrasqueña'	H	O	S	S	C	R	R	R	M	R	P
'Oliva'	H	EL	A	SA	C	P	P	R	M	R	P
'Morisca'	H	EL	A	S	C	P	P	R	H	R	P
'Pico Limon'	VH	EL	SA	SA	C	P	R	R	M	R	P
'Pico Real'	H	EP	S	S	B	P	T	W	L	G	P
'Azulejo'	H	EL	SA	S	C	P	P	R	M	R	P
'Perito'	H	EP	SA	S	C	P	R	R	M	R	P
'Manzanilla Real'	H	O	S	S	C	R	T	W	M	G	P
'Redondil'	H	EP	S	S	A	R	P	R	M	R	P
'Ocal'	H	EL	SA	SA	C	P	R	R	M	R	P
'Colora'	H	O	SA	SA	B	R	R	R	M	R	P
'Gordal'	H	EL	SA	S	C	P	P	W	M	G	P
'Galega'	M	EP	SA	S	C	P	P	R	M	R	p
'Corniche'	H	EL	A	SA	C	P	P	R	M	R	P
'Azulito'	H	EP	A	S	C	R	P	R	M	R	P
'Verdial de Badajoz'	VH	EP	A	S	A	R	P	R	M	R	P
'Manzanilla Cacereña'	H	EP	SA	S	C	P	P	R	M	R	P
'Manzanilla Sevillana'	H	O	SA	S	A	R	P	R	M	R	P
'Cornicabra'	H	EL	A	SA	C	P	P	R	M	R	A
'Redondillo'	VH	EP	SA	S	C	P	R	W	M	G	A
'Cuerno real'	VH	EL	A	S	C	P	P	W	H	R	P
'Acebuche'	L	EP	SA	S	C	R	R	S	M	G	P
'Picual'	H	EP	A	SA	C	P	R	W	M	R	A
'Arbequina'	L	O	S	S	C	R	R	R	M	R	P
'Original'	VH	EP	S	S	C	P	P	R	H	G	A

Weight: L = low (less than 0.3 g); M = medium (0.3 to 0.45 g); H = high (0.45 to 0.7 g); VH = very high (greater than 0.7 g).
Form: S = spherical (length/width less than 1.4); O = ovoid (length/width 1.4 to 1.8); EP = elliptic (length/width 1.8 to 2.2); EL = elongated (length/width greater than 2.2).
Symmetry of position A: S = symmetric; SA = slightly asymmetric; A = asymmetric.
Symmetry of position B: S = symmetric; SA = slightly asymmetric.
Position of the maximum transversal diameter: B = toward the base; C = centered; A = toward the apex.
Form of the apex: P = pointed; R = rounded.
Form of the base: P = pointed; T = truncated; R = rounded.
Roughness of the surface: S = smooth; R = rough; W = wrinkled.
Number of vascular bundles over the surface: L = low (less than 7); M = medium (7 to 10); H = high (greater than 10).
Distribution of vascular bundles over the surface: R = regular; G = grouped together in the suture.
Presence of mucro: P = present; A = absent.

Seventy-seven accessions belonging to 25 olive cultivars from Extremadura were genotyped at 4 SSR loci (Table 1). The SSR loci used in this study were carefully selected among sets of primer pairs developed for olive (Sefc et al., 2000; De La Rosa et al., 2002). SSR locus characteristics are presented in Table 3. All 4 SSR markers were polymorphic in all 25 analyzed cultivars. No intracultivar variability was detected. A total of 25 alleles were generated by the 4 SSR loci, ranging from 4 at locus EMO2 to 8 at locus DCA18, with an average number of 6.25 alleles per locus and an average of 3.12 effective alleles per locus (Table 3). A number of reports have indicated the high variability in the average number of alleles per locus in olive cultivars (Carriero et al., 2002; De La Rosa et al., 2002; Diaz et al., 2006; Sarri et al., 2006; Belaj et al., 2010). This diversity may be associated with the variation in the loci as well as in the number of genotypes and their location. The number of alleles per locus detected for DCA9 and DCA18 markers among Extremadura olive cultivars proved to be lower than those found in cultivars from different areas of the Mediterranean basin (Sefc et al., 2000; Sarri et al., 2006; Baldoni et al., 2009). In contrast, locus DCA18 presented a higher number of alleles (8) than those found at the regional level in Croatia (Poljuha et al., 2008), Iran (Noormohammadi et al., 2007) or Italy (Bracci et al., 2009; Muzzaluppo et al., 2009).

Table 3. Description of the 4 microsatellite loci used in this study.

Locus	Sequence (5'-3')	Ta (ºC)	Repeat motif	Size range (bp)	N_A	N_E	H_O	H_E	PIC
EMO2[a]	F: CTCGCACTTTAAATTCATATGGGTAGGT R: GCGTGCTTGGGTGCTTGTTTG	60	$(AG)_5$-G-$(GA)_{10}$	213 (201-243)	4	2.74	0.71	0.68	0.63
EMO3[a]	F: GGTGTAGCCCAAGCCCTTAT R: TGCATGACCGTGGTGTAAGT	60	$(CA)_7$	214 (205-215)	6	4.79	0.89	0.83	0.79
DCA9[b]	F: AATCAAAGTCTTCCTTCTCATTTCG R: GATCCTTCCAAAAGTATAACCTCTC	55	$(GA)_{12}$	191 (161-205)	7	2.90	0.58	0.78	0.65
DCA18[b]	F: AAGAAAGAAAAAGGCAGAATTAAGC R: GTTTTCGTCTCTCTACATAAGTGAC	50	$(CA)_4(CT)_1(CA)_3(GA)_{19}$	178 (168-184)	8	4.26	0.95	0.80	0.76

[a]De La Rosa et al. (2002). [b]Sefc et al. (2000). Ta = annealing temperature; N_A = number of alleles; N_E = effective number of alleles; H_O = observed heterozygosity; H_E = expected heterozygosity; PIC = polymorphic information content.

The H_O for the 25 olive cultivars ranged from 0.58 at DCA9 to 0.95 at DCA18 with a mean value of 0.78 (Table 3). Among the 4 loci, the EMO3 locus (H_E = 0.83) showed the highest value of genetic diversity while the lowest diversity value was found using primer EMO2 (H_E = 0.68), with a mean value of 0.75. In accord with prior findings (Diaz et al., 2006), our high heterozygosity values were similar to those of several studies that used SSR markers on olive cultivars in Italian regions as such Sicily (La Mantia et al., 2005) and Emilia (Ganino et al., 2007), of the Istria region in Croatia (Poljuha et al., 2008) as well as in the southern Marmara region in Turkey (Ipek et al., 2009). Also, the mean observed heterozygosity values (H_O = 0.78) of the present study were higher than expected (H_E = 0.75) and in general higher than previous studies using cultivars from different areas of the Mediterranean basin (Sarri et al., 2006). On the contrary, the loci EMO3, DCA9, and DCA18 had lower numbers of alleles and heterozygosity levels than reported by Belaj et al. (2010) for wild and cultivated olive from other Spanish regions, most probably due to the less diverse genotypes assessed and the lower number of samples analyzed.

The sizes and frequencies of alleles are presented in Table 4. Allele size ranged from 162 bp in DCA9 to 225 bp for one allele of EMO2. Allele frequencies were low, particularly at

loci with a high number of alleles. The lowest allele frequency (0.01) was recorded for alleles 165 bp of DCA18 in 'Arbequina' (foreign cultivar), 173 bp of DCA18 in 'Azulito' (local cultivar) and 184 bp at DCA18 in 'Ocal' (foreign cultivar). These 3 alleles were observed in only one cultivar (unique alleles) within the whole set analyzed. The most common allele, with an allele frequency of 0.46, was the allele of 201 bp at the locus EMO2 (Table 4).

Table 4. Allele size (bp) and frequency (in parentheses) for each simple sequence repeat locus in 25 olive genotypes.

Locus	No. of alleles	Allele size							
EMO2	4	201	210	220	225				
frequency		(0.46)	(0.37)	(0.12)	(0.05)				
EMO3	6	205	207	209	211	213	215		
frequency		(0.14)	(0.21)	(0.04)	(0.28)	(0.05)	(0.25)		
DCA09	7	162	170	185	190	193	195	197	
frequency		(0.41)	(0.03)	(0.41)	(0.03)	(0.01)	(0.05)	(0.06)	
DCA18	8	165	167	169	173	175	177	179	184
frequency		(0.01)	(0.12)	(0.34)	(0.01)	(0.29)	(0.13)	(0.06)	(0.01)

Discrimination and identification of Extremadura olive cultivars

PIC values estimate the discriminatory power of markers. The average PIC values for the 4 SSR loci was 0.70, and the PIC value ranged from 0.63 for EMO2 to 0.79 for EMO3 among the 25 olive cultivars (Table 3). All microsatellite loci displayed high PIC values, enabling the identification of all the individuals analyzed. Calculated PIC values classified 2 loci (EMO2 and DCA9) as informative markers (PIC > 0.5) and 2 loci (EMO3 and DCA18) as suitable for genetic mapping (PIC > 0.7). Therefore, the high PIC and heterozygosity levels of most loci in our analyses indicate that this combination of SSRs is a reliable tool for discrimination of cultivars originating from small cultivation areas of Extremadura.

The polymorphism level as well as the associated information and reproducibility constitute vital criteria for selecting a given set of SSR loci (Baldoni et al., 2009). SSR polymorphism, however, also depends on the sample size and diversity of accessions analyzed. In the present study, the combination of allelic patterns found with the four SSRs (DCA9, DCA18, EMO2, and EMO3) was able to identify all accessions, since all cultivars were uniquely characterized. The SSR profile for each cultivar analyzed is listed in Table 5. Five of 25 (20%) olive cultivars were identified by only one allele, 16 (72%) cultivars were identified by a combination of two alleles, and 2 (8%) cultivars by a combination of three alleles (Table 5). Most of the unique banding profiles useful for cultivar identification were found at locus DCA18. A minimum number of three SSR markers (DCA9, DCA18 and EMO3) were chosen for identification of all 25 olive cultivars. This was due to the high proportion of unique alleles (5 of 25 in those three SSR). As expected, these three SSR were among the four with the highest genetic variation and discrimination power. Therefore, in our study, a low number of polymorphic SSR markers differentiated a large number of olive accessions, in agreement with prior findings (Baldoni et al., 2009). A high number of alleles discriminated were cultivated from wild olive, with 96% (24 of 25) detected exclusively in cultivated accessions, while 4% appeared exclusively in wild accessions (allele of 170 bp at locus DCA9). Most of the alleles in cultivated accessions were common between local or foreign cultivars (16 and 8% of alleles

were only detected in local and foreign cultivars, respectively). In the present study, allele identification detected evidence of the survival of indigenous wild oleasters, in agreement with previous research using allozymes (Lumaret et al., 2004) and molecular markers (Belaj et al., 2007; Erre et al., 2010). Nevertheless, as opposed to allele comparison, cultivated olive registered higher heterozygosity levels. Comparable findings have been reported for other restricted areas, such as the Mediterranean island of Sardinia, and the Spanish regions of Andalusia, Catalonia and Valencia (Erre et al., 2010; Belaj et al., 2010). Successive crossing among these individuals as well as with introduced cultivars, followed by genotype selection for enhanced agronomic performance could have resulted in greater heterozygosity than maintained by vegetative propagation (Besnard et al., 2001; Erre et al., 2010; Belaj et al., 2010).

Table 5. SSR fingerprints for the 25 olive cultivars analyzed.

Code	Cultivar	EMO2	EMO3	DCA9	DCA18
2	'Carrasqueña'	201	**207-211**	162	**169-177**
4	'Oliva'	201-210	211-215	162-185	169-175
5	'Morisca'	201	211-215	162-185	169-175
6	'Pico Limon'	201-210	**211**	**185-195**	169-175
9	'Pico Real'	201-210	**211-215**	**185-195**	169-175
11	'Azulejo'	201-210	**205-215**	185	**169-179**
12	'Perito'	201-210	**209-213**	162-185	169-175
13	'Manzanilla Real'	201-210	**209-211**	162-185	169-175
19	'Redondil'	201-225	211	162	**175-179**
24	'Ocal'	201-220	213	197	**169-184**[a]
25	'Colora'	201-220	207-211	**190**[a]	169-175
32	'Gordal'	201	**207-209**	**162-195**	169-177
41	'Galega'	201-210	**205-207**	**162-195**	169-177
48	'Corniche'	201-210	207-215	185	169-175
52	'Azulito'	210-225	205-215	162	**169-173**[a]
58	'Verdial de Badajoz'	201-210	**211-215**	162-185	**167-175**
84	'Manzanilla Cacereña'	**210-225**	**207-211**	162-185	169-175
92	'Manzanilla Sevillana'	201-210	**207-211**	162-185	**169-179**
93	'Cornicabra'	201-210	205-207	**162-185**	**169-177**
95	'Redondillo'	201	207-215	162-185	169-175
98	'Cuerno Real'	201-210	**205-215**	162-185	**167-175**
100	'WT'	210	205	**170**[a]	167-179
104	'Picual'	201-210	207-215	182-190	167-175
105	'Arbequina'	210-220	211	185	**165-175**[a]
114	'Original'	201	**205-213**	**162-197**	177

[a]Unique alleles; unique allelic patterns are shown in bold.

Genetic relationships among olive cultivars

The SSR marker genotypes were used to evaluate the relatedness of the studied accessions by hierarchical clustering using UPGMA (Figure 1). This analysis clearly separated all the cultivars, with similarity coefficients between all possible pairs of genotypes ranging from 0.10 to 0.87. The dendrogram showed a clear separation between the wild and cultivated olive trees at a similarity value of 0.10. 'WT', which corresponds to the *sylvestris* cultivar from Extremadura, did not group with any other cultivar, suggesting a high phylogenetic distance between this wild tree and the other studied olive trees. The remaining olive cultivars were separated gradually, with no well-differentiated groups with the exception of two major groups (I and II), at a genetic similarity of 0.30. A high level of genetic diversity was observed between local Extremadura and foreign cultivated trees. There was evidence of a cultivar

relationship according to their geographic origin (local or foreign cultivar) (Figure 1). In contrast, there was no clear clustering of cultivars in relation to their growing area, end use or morphological traits based on endocarp traits. The foreign cultivar 'Ocal' was not included in any of the groups, probably because it has an independent origin. This cultivar can be found outside Extremadura and is also cultivated in the region of Andalusia (southern Spain). 'Ocal' had a very similar endocarp to that of 'Pico Limon', a local cultivar with an elongated shape and the same symmetries (Table 2), but with fruits of different shape (Parra-Lobato MC, Delgado-Martinez FJ and Gomez-Jimenez MC, unpublished results). On the other hand, the local cultivar 'Azulito' also clustered separately as one independent branch. 'Azulito' are the most distinct among local cultivars, and are locally cultivated in southern-central Extremadura (a minor cultivar used for oil). The separation of this cultivar from the others may be the result of its adaptation to the environmental and cultivation practices in the south of the region. 'Azulito' is morphologically similar to another local cultivar 'Verdial de Badajoz' (the main cultivar used for oil), with an ellipsoidal endocarp that differed only in the position of the maximum transversal endocarp diameter (Table 2).

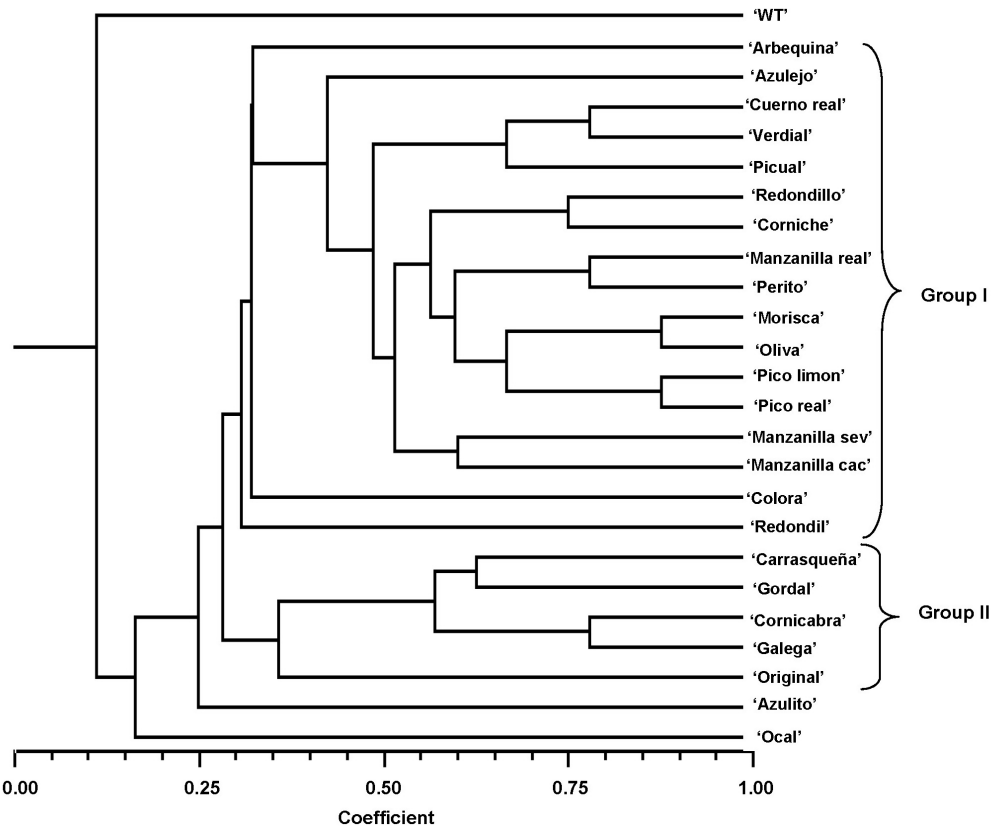

Figure 1. Dendrogram of the identified olive cultivars based on Jaccard coefficient and unweighted pair group method with arithmetic mean (UPGMA) cluster analysis. WT = wild type.

Group I included all the cultivars originating from Extremadura (local cultivars), with the exception of 'Original', sharing similarity values between 0.32 and 0.87. In fact, 'Original', cultivated in the central zones of Extremadura, showed morphological and agronomic characteristics different from the other local Extremadura cultivars. The cultivar 'Original' is used as a table olive and it was the local cultivar with the highest endocarp weight (1.44 g) and fruit weight (9.34 g) as compared to the other local cultivars. From the analysis of group I it is possible to distinguish two subgroups, which clustered at a similarity value of 0.49, with the rest of cultivars forming independent branches. The first subgroup included two local cultivars 'Cuerno Real' and 'Verdial de Badajoz' (0.77 of similarity coefficient), as well as the foreign cultivar 'Picual' (0.65 of similarity). The cultivar 'Picual' is widespread among the new plantations in Extremadura region (C-S areas), and cultivated in other regions of the center and south of Spain for olive-oil production. 'Picual' was the most distinct cultivar in the first subgroup. The second subgroup was separated into two main clusters (0.51 of similarity). The first cluster of this subgroup included most of the local cultivars grown in Extremadura: 'Redondillo', 'Corniche', 'Manzanilla Real', 'Perito, 'Morisca', 'Oliva', 'Pico Limon', and 'Pico Real'. All these local cultivars are minor old cultivars and characteristic of a restricted area of cultivation that possesses a special climate and soil. The highest similarity coefficients were found between 'Oliva' and 'Morisca', as well as 'Pico Limon' and 'Pico Real' (0.87 of similarity in both cases). These four cultivars, growing in the south of the region, have many common features, including their use for oil production. Despite evident morphological differences, based on endocarp criteria, between 'Pico Limon' and 'Pico Real' (Table 2), the SSR analysis revealed that they differed in only 1 allele, suggesting a close relationship and probably a common ancestry. The same result was observed in the case of 'Morisca' and 'Oliva', but these cultivars exhibit very similar morphological characteristics, with elongated and large-sized endocarps (Table 2). Two main cultivars, 'Manzanilla Cacereña' (the main local cultivar) and 'Manzanilla Sevillana' (the main foreign cultivar from southern Spain), constituted the second cluster at 0.60 of similarity. These cultivars are two of the most important cultivars in the canned-olive fruit industry, and widely cultivated in the north and south of the region, respectively. The distinct position of these cultivars was previously reported with SSR (Diaz et al., 2006) and could be explained by their geographical distribution and morphological differences. Two foreign, 'Redondil' and 'Arbequina', and two local cultivars, 'Colora' and 'Azulejo', were the most distinct in group I, and clustered separately into 4 independent branches at similarity values of 0.32, 0.35, 0.35, and 0.43, respectively. A number of main cultivars of Extremadura, including 'Verdial de Badajoz', 'Morisca' and 'Manzanilla Cacereña' were grouped with the main cultivars ('Picual' and 'Manzanilla Sevillana') of southern Spain (Andalusia) (Figure 1). This could be due to geographical and climatic similarities between the two regions. By contrast, 'Arbequina', a main cultivar from northeastern Spain, did not cluster together. Similar results have also been revealed by SSR analysis of main cultivars from Spain (Belaj et al., 2010).

The second group (II) contained the largest number of foreign cultivars, which are morphologically very different and of heterogeneous origin: 'Galega', 'Cornicabra', 'Gordal Sevillana', and 'Carrasqueña'. They were separated from the local cultivar 'Original' at a similarity value of 0.35 (Figure 1). 'Original' was the most distinct cultivar in the group II. Gordal Sevillana' and 'Original', two cultivars included in this group II, have large fruit-endocarps and are used as table olives. 'Cornicabra' and 'Carrasqueña' are used for oil pro-

duction, while 'Galega' is typically a dual-use cultivar. Group II comprises a heterogeneous cluster of cultivars with very different origins, where some cultivars from other regions of Spain ('Cornicabra' and 'Gordal Sevillana') and Portugal ('Galega' and 'Carrasqueña') are included. 'Cornicabra' has been reported to be related to 'Picual' using RAPD markers (Belaj et al., 2001) and with 'Picual' and 'Morisca' when SSR markers were used (Diaz et al., 2006). However, in our study, 'Cornicabra', 'Picual' and 'Morisca' did not cluster together.

Prior studies using molecular techniques have shown a clustering of olive cultivars based on their morphological traits and fruit use (Besnard et al., 2001; Rotondi et al., 2003; Baldoni et al., 2006; Cordeiro et al., 2008; Fendri et al., 2010). Those genetic relationships may reflect the selection pressure for agronomic and fruit quality characters. However, our results do not show agreement between morphological and molecular analyses.

The possibility of distinguishing the area of origin of each cultivar and the geographical distribution of the high variability in the cultivated olive is still being investigation. This information may contribute to the conservation of relevant local cultivars and might be useful for tracing the genotypes best suited to particular environmental conditions. Besnard et al. (2001) found no grouping of olive cultivars from different countries, implying ongoing exchange among growers in olive-growing countries throughout history. Similarly, other studies have reported no apparent clustering of olive cultivars by geographic origin, whereas olive genotypes from different origins clustered closely together according to molecular markers (Owen et al., 2005). However, other researchers have reported cultivar clustering by geographical cultivation area (Claros et al., 2000; Besnard et al., 2001). Using RAPD markers, cultivars from restricted areas were grouped according to geographical origin, but without evident clustering based on fruit size or other morphological traits (Sanz-Cortez et al., 2001). In the Mediterranean basin, RAPD profiles were correlated with the use of fruits and the country of origin, suggesting a selection scheme from different genetic pools in different areas (Besnard et al., 2001; Cordeiro et al., 2008; Rugini et al., 2011). Sarri et al. (2006), using SSR markers, found limited grouping according to geographic origin and grouped olive cultivars as eastern, central and western Mediterranean populations. However, it is not generally observed, even using powerful genetic markers like SSRs, except in those regions where the genetic exchange has been very limited. Although the SSR technique has been used to compare the main Spanish cultivars with other Mediterranean ones (Diaz et al., 2006; Doveri et al., 2008; Bracci et al., 2009), no specific clustering among the main Spanish genotypes has been found. Most of the genetic diversity was found to be attributable to differences among genotypes within a country. Within Italy, cultivars from limited or small areas have been grouped, using SSR, on the basis of geographical origin (Carriero et al., 2002; Muzzalupo et al., 2009). In Spain, 17 main cultivars are grown depending on the region (Barranco, 1995). The level of genetic differentiation and relationships between wild olives and local cultivars from the regions of Andalusia (southern Spain), Catalonia and Valencia (eastern Spain), and the variability of wild olive trees from Spain were recently investigated by means of SSR markers (Belaj et al., 2010). These studies have shown that the current diversity found in Spanish olive cultivars may be regionally differentiated, and it supported the hypothesis of origin of both, autochthonous and allochthonous. Although no clear separation was discerned between local and foreign cultivars of Extremadura, local cultivars were assigned mainly to group I. Of the two distinct groups, I and II, of olive cultivars, group I is linked to a geographically defined area. In fact, 12 (80%) of the 15 local or native cultivars of Extremadura were cluster in group I

(Figure 1): 2 cultivars in the first subgroup, 9 cultivars in the second subgroup, and 1 clustered independently. Therefore, this analysis structured the variability relative to the geographic origin of Extremadura olive cultivars, while there was no apparent clustering according to endocarp morphological characters. These findings imply that olive cultivars from this region were independently selected, as reported previously between Andalusian and Catalonian cultivars (Belaj et al., 2010), and they agree with the hypothesis of autochthonous origin of most olive cultivars as well as their limited diffusion from the areas of origin (Besnard et al., 2001).

Extremadura is among the main olive growing regions of Spain. A previous study, based on morphological description, suggested the existence of 21 different local cultivars (Delgado-Martinez, 2006) from Extremadura. In the present study, SSR analysis unambiguously discriminated 15 local or native cultivars (5 main and 10 minor local cultivars). Moreover, high genetic diversity was detected between cultivars from Extremadura. In general, our results corroborate the usefulness of SSR markers for identification and genetic diversity analysis of local olive cultivars. Although Spanish genotypes have been fingerprinted, further research is necessary to clarify the variation and genetic structure of local olive as a measure to preserve the olive germplasm from restricted or small areas. Such information will aid the selection of cultivars for germplasm collections, providing information of rare or diverse genetic backgrounds in native and local accessions while monitoring the trade of plant material. The morphological and molecular identification of 10 novel genotypes among the minor local cultivars reflected cultivated genotypes not previously reported for the region of Extremadura. In addition, our findings contribute to an overall understanding of regional olive germplasm in Spain.

In conclusion, the local cultivars of Extremadura (central-western Spain) here investigated represent distinctive olive genotypes at the molecular level. Evidence of relationships for most local cultivars according to their geographic origin was established, but these local cultivars do not appear to form a distinct genetic group, when compared to other olive cultivars introduced from southern Spain. These findings underline the need for reassessing sampling methods, considering geographic origins of the material sampled, including well-characterized cultivars from bordering regions.

ACKNOWLEDGMENTS

Research supported by grants from the ‘Ministerio de Ciencia e Innovacion’, Spain (#BFU2007-62566 and #BFU2010-18116). F.J. Delgado-Martinez was the recipient of a fellowship from the University of Extremadura (Spain).

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