Selections of an Olive Breeding Program Identified by Microsatellite Markers

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ABSTRACT

This work reports on the use of 10 microsatellites for identifying and testing the paternity of the first 17 selections of an olive (*Olea europaea* L.) breeding program in Córdoba, Spain. The usefulness of the microsatellites was confirmed by the high discrimination power and polymorphism information content values and by the low probability of identity found in the 24 main Spanish cultivars. In the selections from the crosses 'Picual' × 'Arbequina' and 'Frantoio' × Picual, the putative male parent was the expected one. In the individuals coming from putative selfings of Arbequina and Picual and from the putative crosses Frantoio × Arbequina, Arbequina × Frantoio and Picual × Frantoio, the male paternity assigned a priori was wrong. Only three microsatellites were needed to discriminate all the selections and to differentiate them from the 24 main cultivars. The probability of having a seedling with a certain allele combination for the 10 microsatellites was 7.63e-06, in the case of Arbequina × Picual and the reciprocal cross, and 1.53e-05 for the cross Frantoio × Picual. A dendrogram generated using the Dice similarity coefficient and the unweighted pair group method with arithmetic mean (UPGMA) agglomeration method showed every selection grouped with one of their two parents.

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Abbreviations: D_j, discrimination power; PIC, polymorphism information content; $P_{(ID)}$, probability of identity; SSR, simple sequence repeat; UPGMA, unweighted pair group method with arithmetic mean.

The olive tree (*Olea europaea* L.) has been grown in the countries around the Mediterranean Basin for the last 4000 yr. A wide variability in the olive germplasm, at both the morphological (Barranco et al., 2000, p. 360) and agronomic (Barranco et al., 2005) level, has been generated during this time. Despite the enormous impact of this crop on the economy of Mediterranean countries, the most important cultivars are ancient and come from the empirical selection made by growers throughout the centuries (Rallo et al., 2005).

Nonetheless, the modern olive oil industry requires new and more competitive cultivars that are better adapted to the new trends in olive growing. Desirable characteristics of these new varieties include a high oil content and quality, low alternate bearing, suitability for mechanical harvesting, and resistance to pests and diseases. In the case of table olives, other features include shape, size, ripening time uniformity, or a high pulp:stone ratio (Lavee, 1994).

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Some new cultivars, such as 'Barnea' (Lavee, 1986) in Israel and 'FS 17' (Fontanazza et al., 1998) and 'Briscola' (Roselli and Donini, 1982) in Italy, have been obtained in the last decades as a result of breeding programs. Nevertheless, they are still not widely grown. In 1990 an olive breeding program was set up in Córdoba, Spain (Rallo, 1995). Three cultivars, Arbequina, Frantoio, and Picual, were used as parents in the initial crosses and were chosen for several criteria, including high oil content, high productivity, a certain resistance to *Spilocea oleagina* and a good aptitude for mechanical harvesting. First, the juvenile period was considerably shortened by a forced growth protocol optimized by Santos-Antunes et al. (2005). Second, an exhaustive agronomic evaluation of 748 descendants of the above-mentioned crosses was undertaken (León et al., 2004) and the best 17 individuals were selected.

To register these genotypes as new cultivars, it has to be shown that they are new, distinct by at least one characteristic from any other cultivar, uniform, and stable, and a cultivar denomination has to be assigned to them. Distinctness, uniformity, and stability should be tested in growing trials. For the olive, 6- to 7-yr-old trees are needed for a proper measurement of these characteristics. Because the olive is easily propagated vegetatively, early identification and protection of these selections and potential new cultivars is necessary. This identification should permit the differentiation of a particular selection from others of the same or different crosses, as well as demonstrating its uniqueness from the existing cultivars.

At present, the microsatellite or simple sequence repeat (SSR) technique provides a reliable tool to solve the problems of genetic characterization and cultivar identification in olive, due to its high polymorphism, codominant inheritance, ease of use, and reproducibility of the results obtained (Rallo et al., 2000; Cipriani et al., 2002; De la Rosa et al., 2002; Díaz et al., 2006a). It is more precise, less expensive, simpler, and faster than the classic morphological methods. Additionally, it allows one to check the paternity of the selections at any step of the vegetative or seed propagation procedure, which could be an important piece of information for successive generations of the breeding program. In fact, microsatellites have been successfully applied in olive identification of traditional cultivars (Bandelj et al., 2002; Khadari et al., 2003; Belaj et al., 2004; De la Rosa et al., 2004; Montemurro et al., 2005; Sarri et al., 2006) and to test the success of breeding crosses (De la Rosa et al., 2004; Mookerjee et al., 2005; Díaz et al., 2006b).

The objectives of this work were to check the paternity of the first 17 selections of an olive breeding program and to discriminate them from other seedlings of the same crosses and from the 24 main Spanish cultivars, those most widely propagated in Spanish nurseries.

MATERIALS AND METHODS

Plant Material and DNA Isolation

Young leaves from the first 17 selections of the olive breeding program in Córdoba, Spain, their parents, and the 24 main Spanish cultivars (Barranco and Rallo, 2000) were used for the analysis by means of 10 SSR markers (Tables 1 and 2). The DNA was extracted according to Murray and Thompson (1980) with slight modifications (De la Rosa et al., 2002). All the descendants came from the Córdoba breeding program and were supposed to be the result of different diallel crosses among Arbequina, Frantoio, and Picual olive trees.

Microsatellite Amplification

A set of 10 microsatellites (Table 1) was chosen among those available in the literature (Sefc et al., 2000; Cipriani et al., 2002; Díaz et al., 2006a) according to their level of polymorphism, reproducible amplification in olive genome and ability to discriminate the alleles coming from every alleged parent (parental genotypes as different as possible). Amplification reaction mixtures were made according to De la Rosa et al. (2002) on a Gene Amp PCR system 9600 (Applied Biosystems, Foster City, CA). The amplification program consisted of denaturation at 94°C for 11 min, 35 cycles of 94°C for 30 s, the annealing temperature reported by the authors (Table 1) for 45 s and 72°C for 2 min, plus a final elongation at 72°C for 7 min. Samples were analyzed on the ABI 310 and ABI 3100 Genetic Analyzers (Applied Biosystems). The Genescan and Genotyper version 3.7 softwares (Applied Biosystems) were used to analyze the results, with the peaks checked visually to detect possible errors in the assignment of alleles.

Potential of Microsatellites for Individual Identification

To evaluate the information potential of the selected microsatellites, their discrimination power (D_j) (Tessier et al., 1999) and the polymorphism information content (PIC) (Botstein et al., 1980) values were calculated in the cited set of 24 main Spanish olive cultivars using

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

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

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

where p_i and p_j are the frequencies of the ith and jth alleles. The number of different and unique genotypes per each microsatellite marker was also estimated.

The probability of matching for two individuals fortuitously at all the 10 microsatellite loci was estimated by calculating the observed probability of identity (P_{ID}), using the allelic frequencies derived from the 24 mentioned cultivars and following the formula reported by Paetkau and Strobeck (1994):

$$P_{\mathrm{ID}} = \sum p_i^4 + \sum\sum (2p_i p_j)^2$$

where p_i and p_j are the frequencies of the ith and jth alleles.

Table 1. Simple sequence repeat (SSR) markers used in the paternity tests and the corresponding allelic profiles for the three putative parents. The discrimination power (D_j), polymorphism information content (PIC), and probability of identity (P_{ID}) values, as well as the number of total and unique genotypes, were estimated for each marker from the genotypes of the 24 olive cultivars mainly grown in Spain.

Source	SSR marker	Allelic profiles			D_j	PIC	P_{ID}	No. genotypes	
		'Arbequina'	'Frantoio'	'Picual'				Total	Unique
Sefc et al. (2000)	ssrOeUA-DCA3	228/240	234/240	236/246	0.94	0.80	0.06	13	6
	ssrOeUA-DCA9	182/203	180/207	182/190	0.95	0.82	0.04	13	6
	ssrOeUA-DCA13	116/120	116/116	116/116	0.67	0.60	0.17	4	1
	ssrOeUA-DCA16	121/144	147/154	123/152	0.85	0.81	0.05	15	10
	ssrOeUA-DCA18	165/175	173/175	167/173	0.91	0.78	0.07	12	7
Cipriani et al. (2002)	UDO99-011	116/129	114/124	116/118	0.91	0.79	0.06	11	5
	UDO99-019	129/154	129/167	129/129	0.32	0.16	0.07	4	2
	UDO99-043	174/174	174/214	208/212	0.94	0.82	0.05	13	9
Díaz et al. (2006a)	IAS-oli23	219/233	229/233	217/229	0.94	0.77	0.07	14	9
	IAS-oli27	124/125	124/124	116/125	0.89	0.71	0.10	11	6

Parentage Analysis and Discrimination

The putative maternity and paternity of the selections were tested by the presence of one maternal and one paternal allele for each of the 10 primer pairs considered. In those cases where the paternity was different than that expected, the real male parent was estimated. For this purpose, microsatellite data from the main 24 Spanish cultivars, including those planted near the female trees, such as 'Gordal Sevillana', 'Hojiblanca', and 'Manzanilla de Sevilla', were used.

Once the correct paternity was established, the probabilities of finding a seedling with the same allele pattern as a given one was calculated for each cross using the allele data of the real parents (Table 2). Additionally, the minimum number of alleles distinguishing any 2 of the 17 selections considered was calculated for the whole set of selections and by cross. The minimum number of alleles distinguishing any of the selections from any of the 24 main Spanish olive cultivars was also calculated.

Genetic Relationships between Selections

To verify the relationships between the selections and the cultivars used as parents, a dendrogram was generated with the similarity matrices derived from their genotypes for the 10 microsatellites using the SAHN-clustering and TREE programs of the NTSYS-pc version 2.02j package (Exeter Software, Setauket, NY). The Dice similarity coefficient (Dice, 1945) and the unweighted pair group method with arithmetic mean (UPGMA) agglomeration system were used for calculations.

RESULTS AND DISCUSSION

Informativeness of Microsatellites for Individual Identification

Values of D_j and PIC were calculated for each marker from the genotypes of the 24 main Spanish cultivars to evaluate the usefulness of each locus for discrimination issues (Table 1). The D_j values ranged from 0.32 (UDO99-019) to 0.95 (ssrOeUA-DCA-09), and the PIC values ranged from 0.16 (UDO99-019) to 0.82 (ssrOeUA-DCA-09 and UDO99-043). In general, and in agreement with previous works (Cipriani et al., 2002; Bandelj et al., 2004; Belaj et al., 2004; Khadari et al., 2003; Díaz et al., 2006a), the D_j and PIC values were high. This indicates the potential of this set of markers for olive genotype discrimination. Microsatellites with the highest D_j (>0.85) and PIC (>0.77) values (Table 1) were found to be the most useful for application in cultivar and selection identification, although not exactly in the order dictated by these two parameters, since ssrOeUA-DCA16, UDO99-011 and IAS-oli23 contributed to that application to a greater extent.

The probability of finding two cultivars with the same genotype at the 10 loci at random (overall P_{ID}) was 1.70e-11 in the set of the 24 main Spanish olive cultivars. The low level of P_{ID} obtained using only 10 microsatellite

Table 2. Putative and observed paternity for 17 selections of the olive breeding program of Córdoba, Spain. Probabilities of obtaining each genotype by considering the true parents are shown.

Breeding line	Female parent†	Putative male parent†	Observed male parent†	P(genotype)
'UC-I 1-21'	A	A	P	7.63e-06
'UC-I 7-8'	A	A	P	7.63e-06
'UC-I 10-54'	A	A	P	7.63e-06
'UC-I 6-9'	A	F	P	7.63e-06
'UC-I 9-67'	A	F	P	7.63e-06
'UC-I 11-10'	A	F	P	7.63e-06
'UC-I 1-19'	F	A	P	1.53e-05
'UC-I 10-30'	F	A	P	1.53e-05
'UC-I 4-62'	F	F	P	1.53e-05
'UC-I 7-60'	F	P	P	1.53e-05
'UC-I 2-68'	P	A	A	7.63e-06
'UC-I 4-1'	P	A	A	7.63e-06
'UC-I 5-44'	P	A	A	7.63e-06
'UC-I 7-34'	P	A	A	7.63e-06
'UC-I 8-7'	P	A	A	7.63e-06
'UC-I 11-16'	P	A	A	7.63e-06
'UC-I 8-20'	P	P	A	7.63e-06

†A: 'Arbequina'; F: 'Frantoio'; P: 'Picual'.

makers (Table 1) is a result of the high values of heterozygosity extensively reported in olive (Rallo et al., 2000; Díaz et al., 2006a; Reale et al., 2006).

Our results demonstrate that the set of markers used in this study can be considered to be highly effective for olive cultivar discrimination.

Parentage Analysis

At least one of the two maternal alleles was always present in all of the selections for all the SSR loci analyzed (Table 1). In seven cases, the putative male parent was also correct. All of these were descendants of the crosses Picual × Arbequina and Frantoio × Picual. In the remaining 10 selections, that is, all the putative selfings (Arbequina × Arbequina, Frantoio × Frantoio, and Picual × Picual) and the putative crosses Frantoio × Arbequina, Arbequina × Frantoio, and Picual × Frantoio, the male paternity was wrong. In the cases in which Arbequina and Frantoio were used as the female parents, the true male parent was Picual. And interestingly, when Picual olive trees acted as the female parent, the true pollen donor was Arbequina. Allele data of the seedlings excluded any of the other main Spanish cultivars, including those planted near the crossing orchard (Gordal Sevillana, Hojiblanca, and Manzanilla de Sevillá), as possible male parents. These results are concordant with previous findings in paternity testing of olive seedlings (De la Rosa et al., 2004; Díaz et al., 2007) and self-incompatibility studies (Díaz et al., 2006b), where Picual was the main contaminant pollen source except when it was the female parent. In such cases, the pollen coming from Arbequina was the most effective. Actually, Picual is the predominant cultivar in the area in which the crosses were performed (Junta de Andalucía, 2002). Therefore, it seems that illicit pollinations caused by airborne pollen could explain the discrepancies found between the expected and observed male paternity. As in previous studies (Díaz et al., 2006b; Mookerjee et al., 2005), no real selfings were found. Two of the bags currently used in olive breeding programs, plastic and Tyvek (DuPont, Wilmington, DE) bags, have been used to carry out the crosses reported here. However, both have been recently revealed to be permeable to external pollen (Díaz et al., 2006b; Díaz et al., 2007). Even using these bags, it seems that when two cultivars are cross-compatible (i.e., Picual and Arbequina), no pollen contamination is observed (the pollen from outside can gain access to the inner but it cannot compete with that coming from the cultivar used as father). And, quite the opposite, most of the fruits are sired by foreign pollen when the cultivars chosen as parents are incompatible (i.e., self-pollinations of Arbequina, Frantoio, and Picual). These results support the self- and cross-compatibility relationships described in previous studies performed in olive using a similar methodology (Díaz et al., 2006b, 2007).

Seedling Discrimination

Complete identification of all the 17 selections and 24 cultivars included in the study was possible using only three microsatellite loci from ssrOeUA-DCA3, 9, 16, 18, UDO99-011, 43 and IAS-oli23 in up to seven different combinations. As expected, those markers had high D_j and PIC values (Table 1).

Under the hypothesis of independence between markers, the probability of having a seedling with a certain allele combination for the 10 SSR loci studied was 7.63e-06 in the case of Arbequina × Picual and the reciprocal cross, and 1.53e-05 in the case of Frantoio × Picual (Table 2). These low probabilities of having any seedling with the same allele combination as any of the 17 reported here are a consequence of the high discrimination power of the SSR markers used and the great genetic variability shown by the species (Rallo et al., 2000; Sefc et al., 2000; De la Rosa et al., 2002).

The minimum number of alleles distinguishing two selections of the same cross were 8 in the case of Picual × Arbequina, 11 in Arbequina × Picual and 12 in Frantoio × Picual, whereas two selections of different crosses were differentiated by at least 5 alleles between the crosses Picual × Arbequina-Arbequina × Picual, 8 alleles for the crosses Frantoio × Picual-Picual × Arbequina and 11 alleles in the case of the crosses Frantoio × Picual-Arbequina × Picual. And at least 9 alleles were found to be different when the selections of the cross Picual × Arbequina were compared with the 24 main Spanish cultivars, 11 alleles in the case of Arbequina × Picual, and 13 alleles in the cross Frantoio × Picual.

Genetic Relationships between the Selections

The dendrogram resulting from a microsatellite-based genetic distance grouped the 17 genetically related selections and their three parents at a similarity coefficient higher than 0.40 (Fig. 1). In this cluster, two groups can be distinguished, these being the selections associated with one of their two parents in each cluster. Two of the selections were clearly separate from the other groups but clustered together with one of their parents. When the genotype data were thoroughly examined, we observed that these two individuals had inherited from the male and/or the female parent the less-frequent alleles among the offspring analyzed. For instance, in the case of the cross Frantoio × Picual, all the descendants studied here received the alleles 246 (ssrOeUA-DCA3), 167 (ssrOeUA-DC18), and 123 (ssrOeUA-DC16) from Picual, and the allele 167 (UDO99-019) from Frantoio, except the selection that cluster in a different group (UC-I 4-62), being the only descendant that received the other possible allele from the parents. The same was detected in the case of the other selection (UC-I 10-54), which stands apart from the group of its half- and full-sibs.

The moderately high values of the similarity coefficient found between selections in the same cluster (full-sibs in cluster Frantoio × Picual and half-sibs in the remaining ones) are due to the hypervariable nature of microsatellite markers, as has been previously observed when data coming from different types of markers have been compared (Belaj et al., 2003; Bandelj et al., 2004).

In summary, the SSR markers used here have been found to be very useful for unequivocally identifying selections from a breeding program. Given the easy vegetative propagation of the olive, an early identification of the interesting genotypes in a breeding program is clearly needed to protect a new release. The codominant nature of the SSRs and their reproducibility would make it possible, with only one assay, to distinguish the selections from any others of the same cross and from any of the main existing cultivars. At the same time, the paternity of those selections could be tested. The paternity results from the selections could aid breeders in planning future crossing strategies with relatively little effort.

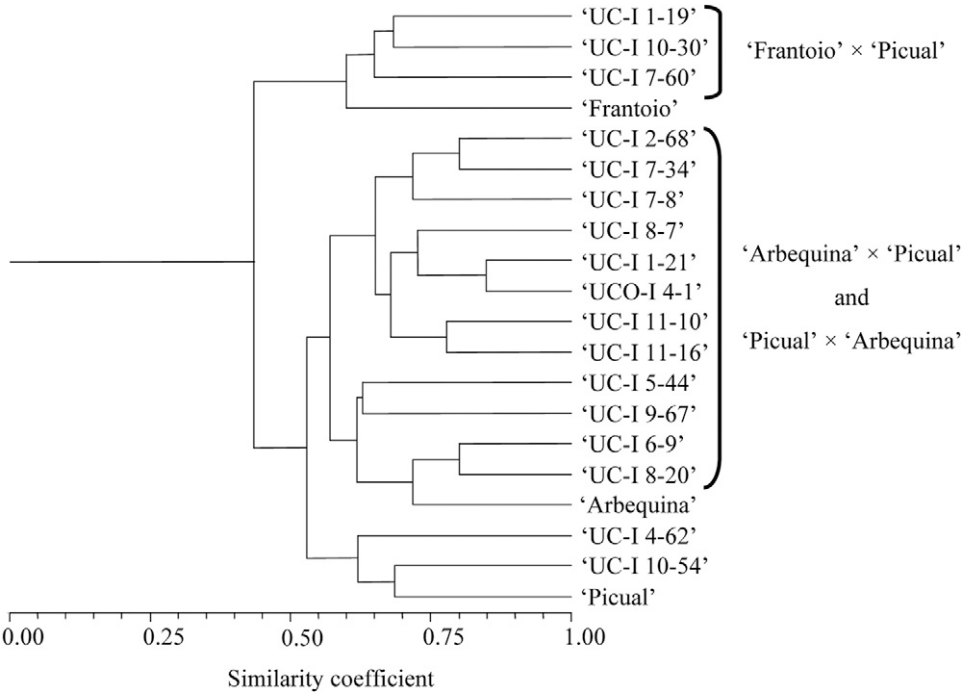

Figure 1. Associations between 17 selections from the breeding program in Córdoba, Spain, revealed by the unweighted pair group method with arithmetic mean (UPGMA) cluster analysis on the basis of simple sequence repeat genetic distance values using the Dice similarity coefficient.

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