

# Self-incompatibility of ‘Arbequina’ and ‘Picual’ Olive Assessed by SSR Markers

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**ABSTRACT.** We studied the self-incompatibility of two main Spanish olive (*Olea europaea* L.) cultivars, ‘Picual’ and ‘Arbequina’, by testing the selfing of the seeds with microsatellites. For this purpose, we used a rapid single-seed DNA extraction method and four highly polymorphic microsatellites. We analyzed seeds produced in branches bagged for selfing from mono- and multi-cultivar orchards in 2002 and 2003. We did not find any seed coming from selfing in the bagged branches, for either cultivar, in the two types of orchards. Additionally, we tested seeds coming from free pollination in mono-cultivar orchards from different locations. In the case of ‘Picual’ olive, only three seeds out of the 70 collected were the product of selfing, although they came from mono-cultivar orchards located in areas where the cultivar used as the female parent was predominant. From the 20 seeds of ‘Arbequina’ olive harvested in the middle of two high-density plantations, not one was a product of selfing. According to this, olive would behave as an allogamous species in mono-cultivar growing conditions and the pollen coming from long distances would be able to produce a normal bearing. Therefore, there is strong evidence to support the idea that the cultivars studied could be self-incompatible. Future experiments in self-compatibility should include a paternity check of the possible self seeds obtained.

Olive is cultivated along the Mediterranean Basin in mono-cultivar orchards with variable extensions (Barranco, 1997). Many authors have recommended the presence of trees of other cultivars to act as pollinators to ensure a good fruit set (Lavee and Datt, 1978) and others have reported that cross-pollination enhances the fruit set compared to selfing (Cuevas et al., 2001; Fernández-Escobar and Gómez-Valledor, 1985). In ‘Gordal Sevillana’ olive trees, cross-pollination has reduced the number of small parthenocarpic fruit (“shotberries”) in comparison to self-pollinated trees (Fernández-Escobar and Gómez-Valledor, 1985). However, the different evaluations made to test the self-compatibility of olive cultivars show a great variability between years and locations (Cuevas et al., 2001; Griggs et al., 1975; Lavee et al., 2002). Furthermore, there are considerable differences between studies made on the same cultivars, as cited by Bradley and Griggs (1963) and Wu et al. (2002). To explain this variability, the existence of a “pseudo-compatibility” mechanism, influenced by environmental conditions, has been proposed (Bradley and Griggs, 1963). In other fruit tree species, however, self-incompatibility is known to be under predominant genetic control with a low environmental influence (Sedgley, 1994).

In olive, two methodologies are commonly used to measure the degree of self-compatibility: first, by comparing the fruit set from bag-isolated flowering branches with those free or cross-pollinated, and, second, by studying the pollen tube growth in the pistil of self- and free- or cross-pollinated flowers. The possibility of the contamination of bag-isolated branches with foreign pollen has never been considered as a source of variability in the degree of self-incompatibility, although olive is a wind-pollinated species with a small pollen grain (Pacini and Juniper, 1979).

In other species, scientists have used morphological characteristics for distinguishing self- from cross-pollinations (Ibarra et al., 1997). More recently, randomly amplified polymorphic DNA (RAPD) and simple sequence repeat (SSR) markers have proven to be useful for this purpose (Barrell et al., 1997; Streiff et al., 1999). The codominant nature of SSRs makes them especially suitable for differentiating between self- and cross-seedlings. De la Rosa et al. (2004) have recently applied this methodology to paternity testing of olive breeding progenies.

In the present work, we have used SSR markers in order to investigate the self-compatibility of ‘Picual’ and ‘Arbequina’ olive. Additionally, we have evaluated the influence of environmental conditions on the outcrossing rate under normal mono-cultivar growing conditions.

## Materials and Methods

**POLLINATION TESTS.** We tested for selfing fruit coming from self- and free pollinations made on ‘Picual’ and ‘Arbequina’ olive in 2002 and 2003. We compared two different situations

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according to the composition of the airborne pollen: 1) we used trees located in a multi-cultivar field [Germplasm Bank of the Centro de Investigación y Formación Agraria (CIFA), Alameda del Obispo, Córdoba, Spain] as an ideal situation for outcrossing; 2) we considered ‘Picual’ and ‘Arbequina’ olive trees inside mono-cultivar orchards as a favorable situation for selfing. To test the occurrence of selfing in both situations, we isolated flowering branches of trees used as the female parents when buds were in DII phenological stage (Tous and Romero, 1993), as done in previous experiments (De la Rosa et al., 2004). For this purpose, we employed double plastic perforated and Tyvek bags (Del Río and Caballero, 1999; Smith and Mehlenbacher, 1994), previously used in pollination experiments in olive and hazelnut (*Corylus americana* Walt.), respectively. To check for spontaneous self-pollination, we did not add pollen at all. We considered four bagged and two free-pollinated branches of two trees per cultivar and location. We tested for selfing all fruit coming from bagged branches and a minimum of nine from free-pollinated ones using microsatellite markers. Furthermore, we compared the fruit set of self- and free-pollinated branches.

In order to evaluate the percentage of outcrossing of ‘Arbequina’ and ‘Picual’ olive in different environments, we undertook a more extensive survey of free-pollinated fruit on mono-cultivar fields in 2003. Specifically, we collected fruit from ‘Picual’ olive from seven locations in Andalusia, southern Spain, as shown in Fig. 1, where the climatic conditions at flowering were different but in which this cultivar is the most abundant. In the case of ‘Arbequina’ olive fruit, we collected them at two different high-density plantations (2000 trees/ha), located in Hornachuelos and Pedro Abad, as shown in Fig. 1. In all cases, we sampled the fruit from trees located in the middle of mono-cultivar fields with an extension of at least 5 ha. In such situations, it is likely that most of the airborne pollen around the sampled trees belongs to the cultivar used as female parent. Finally, we tested the selfing origin of five and 10 fruit from two trees of ‘Picual’ and ‘Arbequina’ per location, respectively.



Fig. 1. Locations where fruits were harvested in order to test the percentage of outcrossing. Fruits from the cultivar Arbequina were sampled in (1) Hornachuelos and (2) Pedro Abad, and fruits from the cultivar Picual were collected in (3) Alcaudete, (4) Baena, (5) Córdoba, (6) El Carpio, (7) Iznalloz, (8) Mancha Real, and (9) Martos. These locations belong to the provinces of Córdoba, Jaén, and Granada as indicated. On the small map (top left), the location of Andalusia within Spain is highlighted.

**DNA ISOLATION FROM OLIVE SEEDS AND MICROSATELLITE AMPLIFICATION.** We tested the selfing origin on ungerminated seeds directly. We harvested the fruit in November and we extracted the seeds. We removed the seedcoat due to its inhibitory effect on the subsequent polymerase chain reaction (PCR). After optimizing the protocol, we performed the DNA extractions employing the DNeasy Plant Mini kit (Qiagen, Valencia, Calif.) and following the supplier’s instructions.

For paternity tests, we selected a set of four highly polymorphic microsatellites, *ssrOeUA-DCA3*, *ssrOeUA-DCA9*, *ssrOeUA-DCA18* (Sefc et al., 2000), and *EMO3* (De la Rosa et al., 2002), from those developed in olive. We performed the amplifications in 15- $\mu$ L volume solutions containing 15 ng of DNA, 15 mM Tris-HCl pH 8.0, 50 mM KCl, 2.5 mM MgCl<sub>2</sub>, 0.25 mM of each dNTP, 0.2  $\mu$ M of forward (fluorescently labeled) and reverse primers, and 1 U of *AmpliTag*-Gold polymerase (Applied Biosystems, Foster City, Calif.). We carried out the PCRs on a Gene Amp PCR system 9600 (Applied Biosystems) programmed with a denaturation at 94 °C for 11 min, 35 cycles of 94 °C for 30 s, the annealing temperature reported by the aforementioned authors for 45 s and 72 °C for 2 min, plus a final elongation at 72 °C for 7 min. Finally, we analyzed the samples on an automatic sequencer ABI 310 (Applied Biosystems).

**DATA ANALYSIS.** According to previous works (De la Rosa et al., 2004), we considered one seed as being a product of self-pollination if we only found maternal alleles at all four microsatellite loci analyzed.

Additionally, we employed the data available for the four microsatellites used in a wide range of cultivars, including ‘Picual’ and ‘Arbequina’ (De la Rosa et al., 2002; Rallo, 2001), to estimate the paternity of seeds not resulting from selfing. We estimated the ability of the above cited microsatellites for parentage analysis by the calculation of the parentage exclusion (PE) and combined exclusion (CPE) (Garber and Morris, 1983; Jamieson and Taylor, 1997) probabilities when one parental genotype is unavailable (Table 1):

$$PE = 1 - 4 \sum_{i=1}^n p_i^2 + 2 \left( \sum_{i=1}^n p_i^2 \right)^2 + 4 \sum_{i=1}^n p_i^3 - 3 \sum_{i=1}^n p_i^4$$

where  $p_i$  are the allelic frequencies.

$$CPE = 1 - (1 - P_1)(1 - P_2)(1 - P_3) \dots (1 - P_k)$$

where  $k$  is the number of loci considered.

In the case of free-pollination assays, we used the genotype data obtained with the four microsatellites to estimate the multi-locus outcrossing rate ( $t_m$ ) in the two cultivars considered as the percentage of seeds with a cross-fecundation origin.

Table 1. Microsatellites employed for parentage analysis, number of olive cultivars used for allelic frequency estimation (De la Rosa et al., 2002; Rallo, 2001), parentage exclusion [PE (capability of a particular marker to exclude the parentage relationship between two individuals)] and combined parentage exclusion [CPE (capability of a set of markers to exclude the parentage relationship between two individuals)] values.

Microsatellite	Olive cultivars (no.)	PE	CPE
<i>ssrOeUA-DCA3</i>	48	0.45	
<i>ssrOeUA-DCA9</i>	24	0.54	0.92
<i>ssrOeUA-DCA18</i>	57	0.51	
<i>EMO3</i>	24	0.40	

## Results and Discussion

**DNA ISOLATION FROM OLIVE SEEDS AND MICROSATELLITE AMPLIFICATION.** The rapid single-seed DNA extraction method employed here has proved to be very useful for genotyping large amounts of samples. The quantity and quality of DNA obtained are enough for performing PCRs. This DNA isolation protocol averts the need for seed germination and growing of seedlings and the consequent risk of losing samples. Additionally, labor, costs, and infrastructure requirements are drastically reduced if this methodology is routinely applied in incompatibility studies.

The codominant inheritance, polymorphism (7–12 alleles per locus), and high combined parentage exclusion probability value (Table 1) of the four microsatellites employed in this study, allowed the distinction of self- and outcross-pollination events and, in most cases, the identification of a single matching pollen donor from the available data. In all the seeds analyzed, we found at least one of the maternal alleles in the four microsatellite loci used. The 28.2% of the seeds analyzed had four non-maternal alleles (one per microsatellite loci), 38.4% had three, 23.7% had two, and only 8.2% were classified as outcrossed seedlings on the basis of a single non-maternal allele (corresponding to one of the four microsatellite loci used). The remaining 1.6% had only maternal alleles and, supposedly, they may represent self-fecundated products.

**SELF-POLLINATION STUDIES.** None of the seeds coming from bag-isolated branches (with both plastic perforated and Tyvek bags) of ‘Picual’ and ‘Arbequina’ female trees, analyzed during 2002 and 2003, were really products of self-fecundations (Table 2). In such cases, we only found a small amount of fruit coming from outcrossing, jointly with shotberries (parthenocarpic or seedless fruit). Shotberries are typical of other cultivars (Fernández-Escobar and Gómez-Valledor, 1985; Sibbett et al., 1992), but are not normally observed in ‘Picual’ and ‘Arbequina’ olive. This

is especially striking in the case of assays performed in mono-cultivar fields. In those cases, we selected the female trees from the center of plantations, which were surrounded by other trees belonging to the same cultivar. In that situation, it is probable that most of the airborne pollen belonged to the cultivar employed as the female parent, this being the ideal situation for selfing. Therefore, the two cultivars used as female parents seem to show a marked self-incompatibility. The finding of fruit from outcross-pollination inside bag-isolated branches suggests that pollination bags used for selfing are not totally pollen-proof. However, they reduce the amount of foreign pollen around bagged flowers and produce a fruit set reduction (Table 2). The differences in fruit sets between self- and free-pollination assays were significant ( $P < 0.01$ ) in all the trees studied. Another possible explanation for this fact is the presence of foreign pollen settling in tree branches before they were bagged. Similarly, most of the fruit analyzed from free pollination from both ‘Picual’ and ‘Arbequina’ olive were also products of cross-pollination.

In the light of these results, future studies dealing with the comparison of the pollen tube growth in pistils in self- or cross-pollination events, as reported in the literature (Cuevas, 2005), should be followed or complemented by the verification of the progeny paternity. Also, the comparison between self- and free-pollination fruit sets employed in previous works does not seem to be a suitable method for testing self-incompatibility if it is not followed by a paternity check of the fruit obtained.

**FREE-POLLINATION STUDIES.** To test the percentage of outcrossing and whether the mentioned self-incompatibility was stable in different environments, we collected fruit from the free-pollination of ‘Picual’ olive trees in seven different typical olive-growing locations in Andalusia (Spain), in which the climatic conditions were diverse, but where the cultivar used as the female parent was highly predominant (Table 3). We also collected fruit from the free-pollination of ‘Arbequina’ olive trees from high-density

Table 2. Occurrence of selfing of ‘Picual’ and ‘Arbequina’ olive seeds resulting from self- and free-pollinations carried out in 2002 and 2003. The type of orchard, the cultivar (cv.), the number of non-self seeds vs. the total number of seeds analyzed (non-self seeds ratio), and the most probable father of every seed are indicated. In seeds coming from self-bagged branches no differences were found between the types of bags used, so data have been pooled.

Orchard	Cv. <sup>z</sup>	Year	Self-bagged			Free-pollinated		
			Fruit set (no. fruit/inflorescence)	Non-self seeds ratio	Putative father (no. of seeds) <sup>z</sup>	Fruit set (no. fruit/inflorescence)	Non-self seeds ratio	Putative father (no. of seeds) <sup>z</sup>
Mono-cultivar	P	2002	0.03	2:2	LS(1), U(1)	0.20	9:9	H(1), LS(1), MS(2), U(5)
		2003	0.01	1:1	U(1)	0.09	9:10 <sup>y</sup>	GS(1), H(5), LS(1), <b>P(1)</b> , U(2)
	A	2002	0.40	20:20	H(0-1), LS(0-1), MS(2-3), P(12-13), U(4)	1.12	20:20	GS(1), H(0-1), LS(1-2), MS(2), P(13-15), U(1)
		2003	0.12	28:28	LS(1-2), P(24-25), U(1)	0.52	10:10 <sup>y</sup>	P(10)
Multi-cultivar	P	2002	0.02	4:4	A(3), MS(1)	0.31	11:12	GS(1-2), H(5-6), MS(1), <b>P(1)</b> , U(2)
	A	2002	0.14	10:10	H(1), MS(2), P(6), U(1)	0.49	10:10	MS(5), P(3), U(2)

<sup>z</sup>A = ‘Arbequina’; GS = ‘Gordal Sevillana’; H = ‘Hojiblanca’; LS = ‘Lechín de Sevilla’; MS = ‘Manzanilla de Sevilla’; P = ‘Picual’; U = Unknown. Seeds coming from self-fecundation events are shown in bold type.

<sup>y</sup>Seeds harvested from ‘Arbequina’ and ‘Picual’ olive trees in mono-cultivar orchards during 2003 are those collected in Pedro Abad and Martos, respectively, reported in Table 3.

Table 3. Occurrence of selfing of seeds collected from 'Arbequina' and 'Picual' olive trees (free-pollination) in 2003. The location and Spanish province, abundance of the cultivar employed as female parent in each province, number of non-self seeds vs. the total number of seeds analyzed (non-self seeds ratio), and the most probable father of every seed are shown. For each location, average mean (Mean T), maximum (Max T), and minimum temperature (Min T); precipitation, and relative humidity (RH) of flowering months (April and May) in 2003 are indicated.

Female cultivar	Province	Location	Female cultivar abundance (%) <sup>z</sup>	Tree	Non-self seeds ratio	Putative father (no. of seeds) <sup>y</sup>	Climatic conditions <sup>x</sup>				
							Max T (°C)	Mean T (°C)	Min T (°C)	Precipitation (mm)	RH (%)
Arbequina	Córdoba	Hornachuelos	26.35	1	10:10	P(9), U(1)	25.34	18.94	12.54	0.89	56.74
				2	10:10	P(8), MS(1), U(1)					
Arbequina	Córdoba	Pedro Abad	11.09	1	5:5	P(5)	25.46	17.71	9.95	0.77	61.45
				2	5:5	P(5)					
Picual	Jaén	Alcaudete	95.72	1	5:5	GS(0-3), H(2-5)	22.51	16.60	10.70	0.72	55.37
				2	5:5	GS(1-2), H(3-4)					
Picual	Córdoba	Baena	72.25	1	5:5	H(5)	25.33	18.07	10.81	0.66	56.64
				2	5:5	GS(0-1), H(2-4), LS(0-1), MS(0-1)					
Picual	Córdoba	Córdoba	72.38	1	5:5	A(2), GS(0-2), H(0-1), MS(0-1), U(1)	25.54	18.55	11.55	0.63	59.66
				2	5:5	A(2), H(1), U(1), m.d.(1)					
Picual	Córdoba	El Carpio	98.25	1	5:5	A(1), H(1), LS(1), U(2)	25.46	17.71	9.95	0.77	61.45
				2	4:5	H(1), <b>P(1)</b> , U(3)					
Picual	Granada	Iznalloz	96.66	1	5:5	H(1), U(4)	21.22	15.00	8.79	0.85	57.33
				2	5:5	H(2), MS(1), U(2)					
Picual	Jaén	Mancha Real	97.98	1	4:5	A(1), <b>P(1)</b> , U(3)	24.30	17.99	11.68	0.55	53.05
				2	5:5	H(1-2), GS(1), LS(0-1), U(2)					
Picual	Jaén	Martos	99.53	1	4:5	H(1), GS(1), LS(1), <b>P(1)</b> , U(1)	25.04	17.00	8.95	0.43	56.99
				2	5:5	H(4), U(1)					

<sup>z</sup>Data kindly supplied by Servicio de Estudios y Estadísticas, Consejería de Agricultura y Pesca, Junta de Andalucía. Olive oil farm model developed by Unidad de Prospectiva, Desarrollo Agrario y Pesquero.

<sup>y</sup>A = 'Arbequina'; GS = 'Gordal Sevillana'; H = 'Hojiblanca'; LS = 'Lechín de Sevilla'; MS = 'Manzanilla de Sevilla'; P = 'Picual'; U = Unknown; m.d. = missing data. Seeds coming from self-fecundation events are shown in bold.

<sup>x</sup>Data kindly supplied by the Red de Información Agroclimática de Andalucía, Investigación y Formación Agraria, Pesquera y Alimentaria (IFAPA), Spain.

orchards at two different locations. This ensured that most of the airborne pollen belonged to the cultivar employed as the female parent. Again, only three seeds out of 70 collected from 'Picual' olive trees and none of the 'Arbequina' olive seeds turned out to be the product of self-pollination. This indicates that both cultivars behave as almost strictly allogamous, even in the case of a very strong presence of maternal airborne pollen, such as in the normal growing conditions considered. Particularly remarkable is the case of fruit collected in three locations of Jaén (Table 3), where 'Picual' accounts for 98.6% of the total olive trees grown, and which is one of the provinces with the highest olive oil production in the world (Junta de Andalucía, 2002).

Olive intravarietal diversity at molecular level has been reported (Belaj et al., 2004; Cipriani et al., 2002; Gemas et al., 2004; Khadari et al., 2003). However, sometimes, the somatic or gametic origin of the variability found was not clear (Belaj

et al., 2004) and, in other cases, putative intracultivar variation may be really due to homonyms. Furthermore, it has been demonstrated using morphological (Barranco, 1997; Barranco and Rallo, 1984) and DNA markers (Belaj et al., 2001, 2004; Mekuria et al., 1999; Wiesman et al., 1998) that generic names such as 'Gordal', 'Lechín', 'Manzanilla', 'Verdial', etc., include different cultivars. For this reason, we think that it is unlikely for intravarietal diversity to be the cause of the scarcity of self-pollinated fruit found in this study.

To estimate the multilocus outcrossing rate ( $t_m$ ) in the two cultivars considered, we used the genotype data of the seeds coming from free-pollination (Tables 2 and 3), obtained with the four microsatellites. In the case of 'Arbequina' olive, the  $t_m$  value was always 100% in both years and for the two types of fields considered. The  $t_m$  values derived from the free-pollination of 'Picual' were 100% and 91.67% in mono- and multi-cultivar

fields, respectively, in 2002, and 95.71% in mono-cultivar orchards in 2003 (only data from mono-cultivar fields are available in 2003 for both cultivars). We found no significant differences for either cultivar between years or between types of field. These high values of  $t_m$  are similar to those reported in other trees such as eucalyptus (*Eucalyptus grandis* Hill ex Maiden) (Chaix et al., 2003), no neighboring effects being detected since the pollen donors revealed by the paternity tests were not the trees closest to those used as female parents (especially in trees located in the mono-cultivar orchards).

Fecundation with pollen coming from long distances seems to be a frequent phenomenon in anemophilous trees, as has been previously verified with the use of genetic markers (Caron and Leblanc, 1992; Dow and Ashley, 1998; Friedman and Adams, 1985). The ability of olive pollen to be spread by the wind at long distances has also been reported (González Minero and Candau, 1997). So it seems possible that, even in locations where only one cultivar such as 'Picual' is planted, a sufficient amount of pollen coming from other cultivars of other locations could produce a normal fruit set. It should be noted that, in olive, 4% fruit set is enough to yield a normal bearing (Lavee et al., 1999). Therefore, although 'Picual' and 'Arbequina' olive seem to be self-incompatible, it is not clear that the presence of pollen donors in mono-cultivar orchards is needed under the conditions of southern Spain, since a normal bearing is observed.

These results contradict previous experiments on self-compatibility in olive, where a certain degree of self-compatibility has always been reported for many olive cultivars, including 'Picual' and 'Arbequina' olive (Cuevas, 2005). However, in previous works the selfing origin of fruit from bag-isolated flowers has never been checked due to the non-existence of an efficient method to do so. And when the degree of self-incompatibility was measured in different years or locations, the values found were quite variable (Cuevas et al., 2001; Griggs et al., 1975; Lavee et al., 2002; Morettini et al., 1972). As we have observed here, it is possible that in previous experiments foreign pollen could have entered the self-pollination bags, producing additional fruit set and distorting the evaluation of self-compatibility. This could be the cause of the "pseudo-compatibility" described by Bradley and Griggs (1963). Olive pollination tests have always been performed in a way similar to those for insect-pollinated species. However, wind-pollinated species should be treated differently, as described for *Quercus macrocarpa* Michx. (Dow and Ashley, 1998), and *Q. robur* L. and *Q. petraea* (Matt.) Liebl. (Streiff et al., 1999). Although we have studied 'Picual' and 'Arbequina' olive in the most favorable conditions for selfing that can be found in olive growing areas, as previously mentioned, almost all the fruit sampled were products of outcrossing.

We estimated the paternity of the seeds not coming from selfing by evaluating the allele not corresponding to the female parent for the four microsatellite loci amplified. Although the alleles of the analyzed samples and the assigned fathers matched in all cases, the Probability of Paternity value was considerably lower than 99.9% in almost all cases (data not shown) due to the high frequency of the alleles shared between the alleged father and the descendant.

Fruit coming from 'Arbequina' olive mono-cultivar orchards seem to have 'Picual' olive as the main pollen donor, as expected, considering that most of the airborne pollen belonged to this cultivar, as previously mentioned. In the case of 'Picual' olive, among the most likely parents are other main cultivars in Andalusia (Junta de Andalucía, 2002), like 'Hojiblanca', 'Lechín de Sevilla',

and 'Manzanilla de Sevilla' or cultivars commonly planted by farmers in the borders of the orchards for domestic consumption of table olives, such as the previously mentioned 'Manzanilla de Sevilla' and 'Gordal Sevillana'. These same cultivars appeared as the main pollen donors in experiments carried out in 2002 in both types of orchards (Table 2). This supports the above expounded hypothesis, since small amounts of non-maternal airborne pollen could be sufficient to produce a normal fruit set in 'Arbequina' and 'Picual' olive, even in the case of a high local density of the maternal cultivar.

Finally, the total absence and the low percentage of self-fruit in 'Arbequina' and 'Picual', respectively, in the putatively selfing conditions reported here, suggest the existence of a self-incompatibility system in olive, similar to other highly heterozygous fruit tree species (Sedgley, 1994), although additional research in the case of 'Picual' olive is needed to come to a definitive conclusion. In any case, it would be advisable for future studies on this topic to include a paternity check of the supposed self-fruit obtained.

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