Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA)
Available online at www.inia.es/sjar
http://dx.doi.org/10.5424/sjar/2012103-501-11

Spanish Journal of Agricultural Research 2012 10(3), 731-740
ISSN: 1695-971-X
eISSN: 2171-9292

Sexual compatibility of the olive cultivar 'Kalamata' assessed by paternity analysis

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Abstract

Paternity analysis was used to assess the self-incompatibility of the olive (*Olea europaea* L.) cultivar 'Kalamata' and to identify some compatible pollenisers under a Mediterranean-type climate. Eight microsatellite markers were used for genotyping three 'Kalamata' mother trees, 120 embryos, and all potential pollen donors. The identified alleles were analysed using FaMoz software and showed that 'Kalamata' was highly self-incompatible. Only three 'Kalamata' embryos were assigned to 'Kalamata' self-fertilisation, even though it was the most available pollen donor. The alleles were also analysed using NTSYS-pc (version 2.02 k) software and identified 54 potential pollen donors in the study site; however, not all of them were located within the effective pollination distance of the mother trees (30 m in olive). According to the results of this study, 'Kalamata' (as a host) was compatible with 'Barnea', 'Benito', and 'Katsourela' (six 'Kalamata' embryos assigned in each) but incompatible with 'Arbequina', 'Azapa', and 'Picual' (zero 'Kalamata' embryos assigned in each). The olive growers could use some of these compatible pollenisers with 'Kalamata' to guarantee good fruit set.

Additional key words: *Olea europaea* L.; polleniser; self-compatible; self-incompatibility.

Resumen

Compatibilidad sexual del cultivar 'Kalamata' de olivo evaluada mediante un análisis de paternidad

Se utilizó un análisis de paternidad para evaluar la auto-incompatibilidad del olivo (*Olea europaea* L.) 'Kalamata' e identificar, en un clima de tipo mediterráneo, algunos polinizadores compatibles. Se utilizaron ocho marcadores microsatélites para el genotipado de tres árboles madre 'Kalamata', 120 embriones y todos los potenciales donantes de polen. Los alelos identificados fueron analizados mediante el software de FaMoz, que mostró que 'Kalamata' fue altamente auto-incompatible. Sólo tres embriones de 'Kalamata' fueron asignados a la autofecundación de 'Kalamata', a pesar de que fue el donante de polen más disponible. Los alelos también fueron analizados mediante NTSYS-pc, que identificó 54 posibles donantes de polen en el sitio de estudio; sin embargo, no todos ellos se encontraban dentro de la distancia de polinización efectiva de los árboles madre (30 m en olivo). De acuerdo con los resultados de este estudio, 'Kalamata' (como huésped) fue compatible con 'Barnea', 'Benito', y 'Katsourela (seis embriones 'Kalamata' asignados a cada uno), pero incompatible con 'Arbequina', 'Azapa' y 'Picual' (cero embriones 'Kalamata' asignados a cada uno). Los olivareros podrían utilizar algunos de estos polinizadores compatibles con 'Kalamata' para garantizar una buena fructificación.

Palabras clave adicionales: autocompatible; autoincompatibilidad; *Olea europaea* L.; polinizador.

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Received: 01-10-11. Accepted: 20-06-12

Abbreviations used: A_E (effective number of alleles); A_O (observed number of alleles); EP (exclusion probability); EPD (effective pollination distance); FAM (a fluorescent dye for labelling oligonucleotides); H_E (expected heterozygosity); HEX (a fluorescent dye for labelling oligonucleotides); H_O (observed heterozygosity); IP (identity probability); ISI (index of self-incompatibility); LOD (log of the odds ratio); NED (a fluorescent dye for labelling oligonucleotides); NOVA (National Olive Variety Assessment); NP (null allele probability); PCR (polymerase chain reaction); PD (power of discrimination); SI (self-incompatibility).

Introduction

Self-incompatibility (SI) is a mechanism to prevent self-fertilisation in plants. Most olive (*Olea europaea* L.) cultivars are self-incompatible or show some level of SI and need to be fertilised by other cultivars for successful fruit set (Fabbri *et al.,* 2004; Conner & Fereres, 2005). As a result, SI obliges olive growers to plant more than one cultivar in their orchards to ensure sufficient cross-pollination (Martin *et al.,* 2005; Mookerjee *et al.,* 2005). Climatic conditions, especially air temperature, have a significant effect on the degree of SI; thus, it can change from environment to environment and from year to year (Androulakis & Loupassaki, 1990; Lavee *et al.,* 2002). On the other hand, the olive pollen grain can be carried by wind as far as 12 km (Fabbri *et al.,* 2004); however the effective pollination distance (EPD) has been reported to be 30 m in normal conditions (Ayerza & Coates, 2004; Fabbri *et al.,* 2004).

Different methods have been used to study the SI of olive: measurement of fruit set (Cuevas *et al.,* 2001) and pollen tube observation (Cuevas *et al.,* 2001; Wu *et al.,* 2002) after controlled crossing, *in vitro* pollen germination and pollen tube growth in a culture medium sometimes containing pistil extracts of other cultivars (Lavee & Datt, 1978; Fernandez-Escobar *et al.,* 1983), and paternity analysis (de la Rosa *et al.,* 2004; Mookerjee *et al.,* 2005; Diaz *et al.,* 2006). In paternity analysis, the genotype of the mother plant is compared to the genotype of offspring to distinguish the father. Microsatellite markers are codominant and highly polymorphic, two characteristics that make them especially useful for paternity analysis (Queller *et al.,* 1993).

The aim of this study was to assess the SI of 'Kalamata' and the cross-incompatibility between 'Kalamata' (as a host) and other cultivars using eight microsatellite markers in order to select good pollenisers for a Mediterranean-type climate. 'Kalamata' is one of the most popular table olives grown worldwide and in Australia (Kailis & Davies, 2004) and can also be used for oil extraction.

Material and methods

Plant materials

The study was conducted in 2004 on three flowering trees of 'Kalamata' (trees 30IIA, 30IIB, and 30IIIB, as mother trees 1, 2, and 3, respectively) at the National Olive Variety Assessment (NOVA) collection (Fig. 1). The NOVA collection, located at the Roseworthy Campus, University of Adelaide, South Australia, Australia, at an elevation of 68 m above sea level, latitude 34.52 S, and longitude 138.68 E, was established in 1998. Roseworthy has a Mediterranean-type climate with an average annual rainfall of 440.3 mm with 328.8 mm (about 75%) falling in winter between April and October (Australian Bureau of Meteorology). The collection consisted of three replicates of two tree plots of 100 accessions (600 trees). Tree spacing was 6 m within rows by 7 m between rows. Irrigation was applied by in-line drippers with a 3.6 L h^{-1} flow rate. The irrigation schedule was based on soil moisture monitoring using EnviroSCAN® probes and was applied before crop water stress occurred. Annual leaf tissue tests in January monitored tree nutrient levels, and appropriate fertilisers were applied. Weeds were controlled along the tree rows using contact and residual herbicides. Rye corn was sown between the tree rows each winter as a cover crop and slashed in November to control weeds and increase soil organic matter.

All trees were in good physiological condition, and their genetic identities had been confirmed by DNA fingerprinting using RAPDs (Guerin *et al.,* 2002). Leaf samples were collected from the 95 cultivars present at the NOVA collection for genotyping the mother trees and potential pollen donors. The samples were transferred on ice to the laboratory and kept at 4 °C until use. The other five named cultivars at the collection were excluded, because they were genetic repeats of other cultivars (Guerin *et al.,* 2002). Fifteen mature fruits from each cardinal side of the mother trees (north, south, east, and west) were collected for genotyping the embryos. The samples were transferred on ice to the laboratory and kept at 4 °C until use. Forty embryos per mother tree were separated for DNA extraction (10 embryos from each side of the tree). To do this, the fruit flesh was removed, the stones were cracked open using a vice, and the embryos were separated from the endosperm using a pair of forceps.

DNA extraction

DNA was extracted from the leaf samples of all the 95 cultivars using a modified method (Mekuria *et al.,* 1999) of Doyle & Doyle (1990). In this method, 100 mg of leaf tissue was added to a 2 mL microcentrifuge tube and ground with liquid nitrogen using a small pestle. The absorbance of DNA samples was determined, and

the quality was calculated by the ratio of absorbance at 260 and 280 nm. DNA samples with absorbance ratios more than 1.8 were used for further analysis and stored at –20 °C.

DNA was extracted from the all 120 embryos collected from the 'Kalamata' mother trees individually. The embryos were ground in a 2 mL microcentrifuge tube with 500 mL of grinding buffer [100 mM Tris, pH 8.0, 20 mM EDTA, pH 8.0, 4 mg mL^{-1} diethyl dithio carbamic acid (added just before use), 100 µg mL^{-1} DNase-free RNase A (added just before use)] and incubated at 65 °C for 10 min. After adding 500 mL of lysis buffer [100 mM Tris, pH 8.0, 20 mM EDTA, pH 8.0, 1 M NaCl, 2% (w/v) SDS, 1% (w/v) sodium metabisulphite (added just before use)], the samples were incubated for an extra 30 min at 65 °C. One millilitre of phenol-chloroform-isoamylalcohol (25:24:1 v/v/v) was added, mixed, and centrifuged at 13,200 rpm for

→ N		Replication I Rows										Replication II Rows										Replication III Rows										
		1	2	3	4	5	6	7	8	9	10	1	2	3	4	5	6	7	8	9	10	1	2	3	4	5	6	7	8	9	10	
Tree	8	1	1	2	2	3	3	4	4	5	5	1	1	2	2	3	3	4	4	5	5	1	1	2	2	3	3	4	4	5	5	8
B	5	88	20	98	36	77	42	72	46	41	82	64	**30**	93	59	18	42	10	90	99	6	40	98	56	44	81	100	10	55	80	14	1
A	5	88	20	98	36	77	42	72	46	41	82	64	**30**	93	59	18	42	10	90	99	6	40	98	56	44	81	100	10	55	80	14	1
B	4	86	52	83	27	28	10	84	85	58	22	31	78	63	35	51	82	22	5	49	80	3	91	75	22	93	66	7	9	26	41	2
A	4	86	52	83	27	28	10	84	85	58	22	31	78	63	35	51	82	22	5	49	80	3	91	75	22	93	66	7	9	26	41	2
B	3	70	9	24	44	12	30	87	45	8	78	15	24	17	54	2	98	83	94	91	73	45	34	51	76	13	59	88	27	1	15	3
A	3	70	9	24	44	12	30	87	45	8	78	15	24	17	54	2	98	83	94	91	73	45	34	51	76	13	59	88	27	1	15	3
B	2	74	39	2	3	60	18	100	50	53	51	32	9	69	21	39	20	52	29	40	34	4	17	46	58	29	48	**30**	89	49	74	4
A	2	74	39	2	3	60	18	100	50	53	51	32	9	69	21	39	20	52	29	40	34	4	17	46	58	29	48	30	89	49	74	4
B	1	67	21	54	61	48	59	26	57	14	35	65	8	95	14	53	41	58	1	96	62	70	47	82	69	19	83	99	60	57	62	5
A	1	67	21	54	61	48	59	26	57	14	35	65	8	95	14	53	41	58	1	96	62	70	47	82	69	19	83	99	60	57	62	5
B	5	75	29	94	47	55	90	97	16	1	5	19	87	76	26	100	72	84	97	71	89	16	54	6	20	43	86	53	31	11	87	1
A	5	75	29	94	47	55	90	97	16	1	5	19	87	76	26	100	72	84	97	71	89	16	54	6	20	43	86	53	31	11	87	1
B	4	92	32	15	4	7	64	19	56	65	31	56	45	38	57	50	46	85	16	25	66	64	2	8	28	5	23	38	36	21	71	2
A	4	92	32	15	4	7	64	19	56	65	31	56	45	38	57	50	46	85	16	25	66	64	2	8	28	5	23	38	36	21	71	2
B	3	33	40	91	11	13	99	71	25	96	49	4	44	68	61	3	36	27	47	11	79	94	95	78	77	84	18	25	32	79	67	3
A	3	33	40	91	11	13	99	71	25	96	49	4	44	68	61	3	36	27	47	11	79	94	95	78	77	84	18	25	32	79	67	3
B	2	81	69	17	68	43	93	76	38	95	63	92	70	81	67	74	88	86	75	33	23	73	50	33	97	68	42	65	35	52	12	4
A	2	81	69	17	68	43	93	76	38	95	63	92	70	81	67	74	88	86	75	33	23	73	50	33	97	68	42	65	35	52	12	4
B	1	23	34	73	79	37	6	89	66	62	80	7	12	43	48	60	77	28	55	13	37	37	90	72	92	63	39	24	61	85	96	5
A	1	23	34	73	79	37	6	89	66	62	80	7	12	43	48	60	77	28	55	13	37	37	90	72	92	63	39	24	61	85	96	5
	8	5	5	4	4	3	3	2	2	1	1	5	5	4	4	3	3	2	2	1	1	5	5	4	4	3	3	2	2	1	1	8

Figure 1. Field map of the NOVA collection showing the trees used in the study. Cultivars were as follow: *1* Frantoio; *2* Picual; *3* Barnea; *4* Manzanillo; *5* Arbequina; *6* Leccino; *7* Pendolino; *8* Hojiblanca; *9* Coratina; *10* Mission (WA); *11* 177; *12* Picual; *13* Frantoio; *14* Manaiki; *15* Barouni; *16* Manzanillo; *17* Verdale; *18* Sevillano; *19* Sevillano; *20* UC13A6; *21* Verdale; *22* Azapa; *23* Benito; *24* Verdale; *25* Jumbo Kalamata; *26* Frantoio; *27* Queen of Spain; *28* Koroneiki; *29* Frantoio; *30* Kalamata; *31* Katsourela; *32* Koroneiki; *33* Souri; *34* Amelon; *35* Areccuzo; *36* Ascolana; *37* Atro Rubens; *38* Atroviolacea Brun Ribier; *39* Mission; *40* Frantoio; *41* Arbequina; *42* Black Italian; *43* Group 4; *44* Blanquette – Early; *45* Group 2; *46* Frantoio; *47* Group 5; *48* Group 2; *49* Frantoio; *50* Buchine; *51* Columella; *52* Frantoio; *53* Sevillano; *54* Group 7; *55* Dr Fiasci; *56* Group 6; *57* Frantoio; *58* FS17; *59* Group 5; *60* Gros Reddeneau; *61* Verdale Aglandau; *62* Institute; *63* Group 3; *64* Group 3; *65* Large Pickling; *66* Frantoio; *67* Group 5; *68* Frantoio; *69* Verdale Aglandau; *70* Group 1; *71* Mission; *72* Frantoio; *73* Group 5; *74* Nevadillo Blanco; *75* Group 7; *76* Oblitza; *77* Hoji Blanca; *78* Group 6; *79* Group 3; *80* Group 1; *81* Frantoio; *82* Group 5; *83* Pigale; *84* Group 1; *85* Praecox; *86* Frantoio; *87* Regalise de Languedoc; *88* Rouget; *89* Group 4; *90* Verdale Aglandau; *91* Verdale Aglandau; *92* Group 2; *93* Verdale (Blackwood); *94* Volos; *95* Frantoio; *96* Picual; *97* Barnea; *98* Manzanillo; *99* Arbequina; *100* Hojiblanca. Shaded border cells show the cultivars planted as barrier row (including cultivars 1-5 and 8). A and B indicate two trees that were planted for each cultivar in each replication.

10 min. The supernatant was removed to a fresh tube, and DNA was precipitated by adding 500 µL of isopropanol, mixing, incubating on ice for 15 min, and centrifugation at 13,200 rpm for 5 min. The supernatant was decanted, and DNA was washed with 1 mL of wash buffer [76% (v/v) ethanol, 10 mM ammonium acetate], spun on a daisy wheel for 10 min, and centrifuged at 13,200 rpm for 5 min. The supernatant was decanted, and the pellet was dried, dissolved in 50 µL TE buffer, and stored at –20 °C.

Genotyping

Eight microsatellite primers were used for genotyping the mother trees, embryos, and potential pollen donors (Table 1). FAM- and HEX-labelled primers were obtained from GeneWorks Pty Ltd, Adelaide, SA, Australia, and NED-labelled primers were obtained from Applied Biosystems, USA. The primers were used for amplification in three groups: 1) UDO8, EMO2, and DCA9, 2) UDO24, DCA4, and DCA14, and 3) UDO6 and DCA3. The segregation of the microsatellite primers used had been previously tested by Mookerjee *et al.* (2005). The only new marker was DCA9, which was tested on the progeny of a cross between 'Frantoio' and 'Kalamata', and the segregation of the amplification products fitted the expected 1:1:1:1 (χ^2 = 7.000, df 3).

Polymerase chain reaction (PCR) was performed in a volume of 6 µL containing 60 ng DNA of parents or 0.1 µL DNA of embryos (measurement not performed due to the small quantity of the DNA extracted for each embryo), 0.5 mM of each dNTP, 0.15 U of Immolase™ DNA Polymerase (Bioline), 1 × ImmoBuffer [16 mM $(NH_4)_2$ SO_4, 670 mM Tris-HCl pH 8.3, 0.1% Tween-20], 2 mM $MgCl_2$, and 0.5 µM of each forward and reverse primer using a MJ Research Tetrad thermal cycler (MJ Research). The PCR program included an initial denaturation at 95 °C for 7 min, 35 cycles of 45 s at 95 °C, 45 s at 55 °C, 45 s at 72 °C, and a final extension at 72 °C for 20 min. The PCR products were diluted 1:100 and 3 µL was separated on an ABI Prism 3730 DNA Analyser (Applied Biosystems) using LIZ 500 standard. The alleles were scored using GeneMapper version 3.7 (Applied Biosystems).

Data analysis

For each locus allele, frequency and the following genetic parameters in the parent population were calculated:

— A_O: the observed number of alleles.

— A_E: the effective number of alleles, which is a measure of diversity, was calculated according to the

Table 1. Microsatellite loci used for genotyping and paternity analysis

Locus	Full name (origin)	Fluorescently labelled primer sequences (5'-3')	Annealing temperature (°C)	Alleles scored (bp)
UDO6	UDO99-006 (Cipriani *et al.,* 2002)	F: FAM-TCAGTTTGTTGCCTTTAGTGGA R: TTGTAATATGCCATGTAACTCGAT	57	148, 160, 168, 170, 174, 178, 182
UDO8	UDO99-008 (Cipriani *et al.,* 2002)	F: HEX-AAAAACACAACCCGTGCAAT R: AAATTCCTCCAAGCCGATCT	57	156, 162, 164, 166, 172, 178
UDO24	UDO99-024 (Cipriani *et al.,* 2002)	F: HEX-GATTTATTAAAAGCAAAACATACAAA R: CAATAACAAATGAGCATGATAAGACA	57	166, 172, 179, 181, 186, 188, 192, 202
EMO2	EMO2AJ416320 (de La Rosa *et al.,* 2002)	F: NED-CTCGCACTTTAAATTCATATGGGTAGGT R: GCGTGCTTGGGTGCTTGTTTG	57	202, 208, 212, 216
DCA3	ssrOeUA-DCA3 AJ279854 (Sefc *et al.,* 2000)	F: HEX-CCCAAGCGGAGGTGTATATTGTTAC R: TGCTTTTGTCGTGTTTGAGATGTTG	50	230, 235, 237, 241, 243, 247, 251
DCA4	ssrOeUA-DCA4 AJ279855 (Sefc *et al.,* 2000)	F: NED-CTTAACTTTGTGCTTCTCCATATCC R: AGTGACAAAAGCAAAAGACTAAAGC	55	130, 136, 140, 156, 162
DCA9	ssrOeUA-DCA9 AJ279859 (Sefc *et al.,* 2000)	F: FAM-AATCAAAGTCTTCCTTCTCATTTCG R: GATCCTTCCAAAAGTATAACCTCTC	55	162, 166, 172, 183, 192, 197, 207
DCA14	ssrOeUA-DCA14 AJ279863 (Sefc *et al.,* 2000)	F: FAM-AATTTTTTAATGCACTATAATTTAC R: TTGAGGTCTCTATATCTCCCAGGGG	50	172, 176, 178, 180, 184, 188

formula (Morgante *et al.,* 1994): $A_E = 1/\sum p_i^2$, where p_i is the frequency of the i^{th} allele.

— H_O: the observed heterozygosity was calculated as the proportion of heterozygotes over genotypes for each locus.

— H_E: the expected heterozygosity or gene diversity reflects the level of polymorphism and was estimated using the formula (Nei, 1973): $H_E = 1 - \sum p_i^2$, where p_i is the frequency of the i^{th} allele.

— PD: The power of discrimination was calculated using PowerStats (version 12) software (Promega Corporation) as (Kloosterman *et al.,* 1993): $PD = 1 - \sum p_i^2$, where *pi* is the frequency of the i^{th} genotype.

— IP: Identity probability represents the probability that two individuals drawn from a population will have the same genotype (Jamieson & Taylor, 1997; Waits *et al.,* 2001). It was computed by FaMoz software (Gerber *et al.,* 2003) to show the probability of wrongly assigning a genotype as the pollen donor.

— EP: Exclusion probability was computed by FaMoz software (Gerber *et al.,* 2003) for paternity and shows the capability of the marker system to exclude any given relationship (Jamieson & Taylor, 1997) and in this experiment any unlikely pollen donor.

— NP: Null allele probability was estimated according to the formula (Brookfield, 1996):

$$NP = \left(H_E - H_O\right) / \left(1 + H_E\right).$$

Genotyping data were used for paternity analysis using FaMoz (http://www.pierroton.inra.fr/genetics/labo/Software/Famoz/index.html) a software generated by Gerber *et al.* (2003). FaMoz uses the genotypes of offspring, mother and potential pollen donors to calculate the log of the odds ratio (LOD) scores for any potential parentage relationship. The genotype with the highest LOD score is considered as the most likely pollen donor (Gerber *et al.,* 2003). To determine the threshold value of the LOD score to choose a genotype as a true pollen donor, simulation was done using 1,000 generated offspring from the genotyped parents. Possible genotyping error rate for both simulation and LOD score calculation was considered 0.01 (Gerber *et al.,* 2003). Genotyping errors include scoring errors, false homozygotes owing to null alleles or weak amplifications, and mishandled samples (Blouin, 2003), and it is normally in the range of 0.25% to 2% for microsatellites (Ewen *et al.,* 2000). The r × c Fisher exact test, two-tailed (also called Fisher-Freeman-Halton test) was conducted using StatsDirect statistical software (version 2.5.7) to analyse the difference among the pollen donors with widespread use in Australia.

The index of SI (ISI) (Zapata & Arroyo, 1978), which is the ratio of fruit or seed set after self-pollination to fruit or seed set after open-pollination, as a potential compatible cross, was calculated to assess the level of SI. A ratio equal to or lower than 0.2 indicates an incompatible cross, between 0.2 and 1 a partially compatible cross, and equal to or higher than 1 a compatible cross.

Results

Genetic parameters of the eight microsatellite loci used are reported in Table 2. The number of alleles per locus (A_O) ranged from four (EMO2) to eight (UDO24), with a mean of 6.3. Expected heterozygosity (H_E), also called gene diversity, varied from 0.643 (UDO24) to

Table 2. Genetic parameters of eight microsatellite loci in parental population

Locus	A_O	A_E	H_O	H_E	PD	IP	EP	NP[1]
UDO6	7	4.7	0.481	0.785	0.892	0.0714	0.5768	0.170
UDO8	6	2.9	0.481	0.653	0.795	0.1424	0.4306	0.104
UDO24	8	2.8	0.426	0.643	0.808	0.1257	0.4601	0.132
EMO2	4	3.5	0.222	0.714	0.755	0.2271	0.3191	0.287
DCA3	7	6.2	0.852	0.838	0.933	0.0396	0.6730	**–0.008**
DCA4	5	4.0	0.222	0.748	0.806	0.1199	0.4708	0.301
DCA9	7	5.3	0.833	0.811	0.909	0.0597	0.6079	**–0.012**
DCA14	6	3.2	0.685	0.691	0.858	0.1171	0.4733	**0.003**
All[2]	6.3	4.1	0.525	0.735	0.844	0.0000	0.9968	NA

A_O: Observed number of alleles. A_E: Effective number of alleles. H_O: Observed heterozygosity. H_E: Expected heterozygosity or gene diversity. PD: Power of discrimination. IP: Identity probability. EP: Exclusion probability. NP: Null allele probability. [1] The values in bold mean that the test was significant. NA: Not applicable. [2] The values are cumulative for IP and EP and mean for the other parameters.

0.838 (DCA3). The cumulative identity probability (IP < 0.00001) showed that the probability of assigning a wrong genotype as the pollen donor was very low. The high cumulative exclusion probability (EP = 0.9968) showed that the marker system was able to exclude almost all (99.68%) unlikely pollen donors for any given offspring. Null allele probability (NP) showed that expected heterozygosity (H_E) in DCA3, DCA9, and DCA14 was not different from observed heterozygosity (H_O) statistically; thus, homozygosity was used instead of null heterozygosity.

The LOD scores for the most likely pollen donors ranged from 2.08 to 4.77. There were some embryos that had LOD scores lower than the threshold calculated in simulation (2.00) (unassigned embryos). There were also some assigned embryos, which had more than one possible pollen donor with the same LOD score. The pollen donors with the same LOD score were those with close genetic distance like 'Frantoio' and 'Mission (WA)', 'Picual' and 'Azapa', and 'Verdale' and 'Benito'. Such embryos were not used to select the compatible pollenisers. Table 3 shows the number of unassigned and assigned embryos. In 'Kalamata' 1, for example, 10 out of 40 embryos did not assign to any pollen donor, 6 assigned embryos had more than one possible pollen donor (with the same LOD score), and 24 assigned embryos were used to select the compatible pollenisers.

NTSYS-pc was used to generate a dendrogram including all 95 genotypes of the NOVA collection and showed the presence of 54 different cultivars (Fig. 2) in the study site (the NOVA collection). Since only some of them are grown commercially in Australia, they were classified into two groups. One group includes 22 cultivars with widespread use in Australia, and the other group includes the other 32 cultivars, with limited use in Australia (Table 4). The Fisher exact test was performed for the pollen donors with widespread use in Australia and showed a highly significant difference among them ($p < 0.001$).

The ISI was calculated to assess the level of SI in Kalamata. The number of 'Kalamata' embryos assigned to 'Kalamata' itself (3) was used as the fruit set after self-pollination, and the number of embryos assigned to all other pollen donors (69) used as the fruit set after open-pollination. The ISI of 0.04 showed that 'Kalamata' is a self-incompatible cultivar.

Table 5 shows the selected good and poor pollenisers. 'Barnea' was a good polleniser for 'Kalamata', and is widely planted in Australia. 'Mission (WA)', 'Benito', and 'Katsourela' were three good pollenisers but have limited use in Australia. The number of embryos assigned to 'Mission (WA)' and 'Benito' were 14 and 6, even though one and none of the mother trees were in the 'Kalamata' EPD, respectively. A pollen donor was considered as a poor polleniser only when all of the mother trees were in the EPD. 'Frantoio', the most widespread cultivar at the study site, was a poor polleniser for 'Kalamata', even though 'Frantoio' trees were abundantly located around all of the mother trees, some very close to them (< 10 m).

Discussion

In this study, the sexual compatibility of some olive cultivars with 'Kalamata' (as a host) was assessed using eight microsatellite markers. Previous studies used four (de la Rosa *et al.,* 2004; Diaz *et al.,* 2006) and eight microsatellite markers (Mookerjee *et al.,* 2005) for paternity analysis in olive and four (Robledo-Arnuncio & Gil, 2005), five (Isagi *et al.,*, 2004), and six microsatellite markers (Oddou-Muratorio *et al.,* 2003) for paternity analysis in other tree species. The high EP (0.9968) showed that the applied markers were able to exclude almost all (99.68%) unlikely pollen donors for any given offspring.

Null alleles are alleles with no detectable PCR product after electrophoresis. The identification of null

Table 3. Number of embryos unassigned, assigned, and used to select pollenisers

Kalamata mother trees	Number of embryos			
	Total	Unassigned	Assigned with more than one possible pollen donor (same LOD score)	Assigned/Used to select pollenisers
1	40	10	6	24
2	40	15	1	24
3	40	15	1	24

Table 4. Number of embryos assigned to putative pollen donors in Kalamata

Pollen donors with widespread use in Australia	Mother trees				Pollen donors with limited use in Australia	Mother trees			
	1	2	3	Total		1	2	3	Total
N	24	24	24	72	N	24	24	24	72
Arbequina	**0**	**0**	**0**	0	Amelon	0	0	0	0
Ascolana	0	0	0	0	Areccuzo	**0**	**0**	0	0
Azapa	**0**	**0**	**0**	0	Atro Rubens	0	0	0	0
Barnea	**2**	**2**	**2**	6	Atroviolacea Brun Ribier	0	0	0	0
Barouni	**0**	**0**	**0**	0	Benito	0	3	3	6
Coratina	**1**	1	**1**	3	Black Italian	**0**	**0**	0	0
Frantoio	**0**	**0**	**0**	0	Blanquette-Early	0	1	1	2
Sevillano	**1**	**0**	**0**	1	Buchine	0	0	0	0
Hojiblanca	**0**	**0**	**0**	0	Columella	**1**	**0**	**0**	1
Jumbo Kalamata	2	0	0	2	Dr Fiasci	0	1	**1**	2
Kalamata	**1**	**1**	**1**	3	FS17	1	1	**1**	3
Koroneiki	**2**	0	0	2	Group 1	**0**	1	**1**	2
Leccino	0	0	0	0	Group 2	**0**	**0**	**0**	0
Manzanillo	**0**	**0**	**0**	0	Group 3	**0**	**0**	0	0
Mission	0	0	0	0	Group 4	1	1	**1**	3
Nevadillo Blanco	1	0	**0**	1	Group 5	**1**	**1**	**1**	3
Pendolino	0	0	**0**	0	Group 6	**0**	**2**	2	4
Picual	**0**	**0**	**0**	0	Group 7	**0**	**0**	**0**	0
Souri	0	0	0	0	Institute	0	0	**0**	0
UC13A6	0	0	**0**	0	Katsourela	**2**	**2**	**2**	6
Verdale	**0**	**0**	0	0	Large Pickling	1	0	0	1
Verdale Aglandau[1]	**0**	0	**0**	0	Manaiki	2	0	0	2
					Mission (WA)	2	6	**6**	14
					Oblitza	0	0	**0**	0
					Pigale	0	0	**0**	0
					Praecox	**1**	**0**	0	1
					Queen of Spain	1	0	**0**	1
					Regalise de Languedoc	1	1	**1**	3
					Rouget	0	0	**0**	0
					Verdale (Blackwood)	**0**	**0**	**0**	0
					Volos	0	0	0	0
					177	0	0	**0**	0

Boldface shows that the mother trees were within the effective pollination distance (EPD) of the pollen donor (30 m). [1] Also called 'Hardy's Mammoth' in Australia.

alleles is important in paternity analysis. If non-null homozygotes are scored incorrectly as null heterozygotes, false pollen donors can be assigned as likely fathers. For example, if an embryo has the genotype A/A, it will be scored as A/null and may be assigned to false fathers such as B/null and C/null. The NP test (Brookfield, 1996) did not show a significant difference between H_E and H_O in DCA3, DCA9, and DCA14; thus, homozygosity was used instead of null heterozygosity.

Little information is available about the level of SI in 'Kalamata'. It has been reported to have a high level of SI in Crete between 1979 and 1983 (ISI = 0.14, 0.14, 0.16, 0.13, and 0.12, respectively) (Androulakis & Loupassaki, 1990) and at Roseworthy, SA, Australia (Wu *et al.,* 2002). 'Kalamata' was also completely self-incompatible at the much cooler Gumeracha site in SA, Australia in a study using paternity analysis (Mookerjee *et al.,* 2005). The results presented here confirmed the presence of a high level of SI in 'Kalamata' at Roseworthy, SA, Australia (ISI = 0.04). Only three 'Kalamata' embryos were assigned to 'Kalamata' itself (as a pollen donor) from 74 embryos assigned and used for the analysis, and it is low considering the amount

of 'Kalamata' pollen that must have been in contact with the stigmas on the mother trees. Under the conditions of this study the only good polleniser with widespread use in Australia was 'Barnea' (six assigned embryos). Good pollenisers with limited use in Australia were 'Mission (WA)', 'Benito', and 'Katsourela' (14, 6, and 6 assigned embryos, respectively).

Olive pollen grains may be carried as far as 12 km (Fabbri *et al.,* 2004), but a polleniser cannot be effective from that distance. The EPD in olive has been reported to be 30 m (Ayerza & Coates, 2004; Fabbri *et al.,* 2004). All good and poor pollenisers in this study were within the EPD of all three 'Kalamata' mother trees, except for 'Mission (WA)' and 'Benito' that show high cross-compatibility with 'Kalamata' (as a host).

Wind is the primary agent of olive pollination, though insects often visit olive flowers to collect pollen (Martin *et al.,* 2005). Long-term averages of climatic data from the Roseworthy Agricultural College Weather Station (34.51 S, 138.68 E) (Australian Bureau of Meteorology) shows that at the NOVA collection there are some air currents at 9 am during November (olive flowering time) in all directions and at 3 pm especially towards south west (calm days were less than 2%).

Among the poor pollenisers, 'Arbequina', 'Azapa', and 'Picual' may be considered cross-incompatible with 'Kalamata', because they had flowers and overlapping anthesis in 2003 (Sweeny, 2005), were close to the mother trees (less than 15 m in at least two cases), and none of them was male sterile in 2003 (J. Guerin, pers. comm. 2006). The other poor pollenisers did not share some of these features and might have had no chance to reach, pollinate, and fertilise the host flowers. 'Verdale (Blackwood)', for example, did not have overlapping anthesis with 'Kalamata'; therefore it was considered as poor polliniser for Kalamata but not cross-incompatible with it.

Table 5. Good and poor pollenisers selected for Kalamata

	Pollenisers (number of embryos assigned)	
	With widespread use in Australia	**With limited use in Australia**
Good	Barnea (6)	Mission (WA) (14) Benito (6) Katsourela (6)
Poor[1]	Arbequina (0) Azapa (0) Barouni (0) Frantoio (0) Hojiblanca (0) Manzanillo (0) Picual (0)	Group 2 (0) Group 7 (0) Verdale (Blackwood) (0)

[1] A pollen donor was considered as a poor polleniser when all of the mother trees were within the effective pollination distance (EPD) of the pollen donor but without any fertilisation.

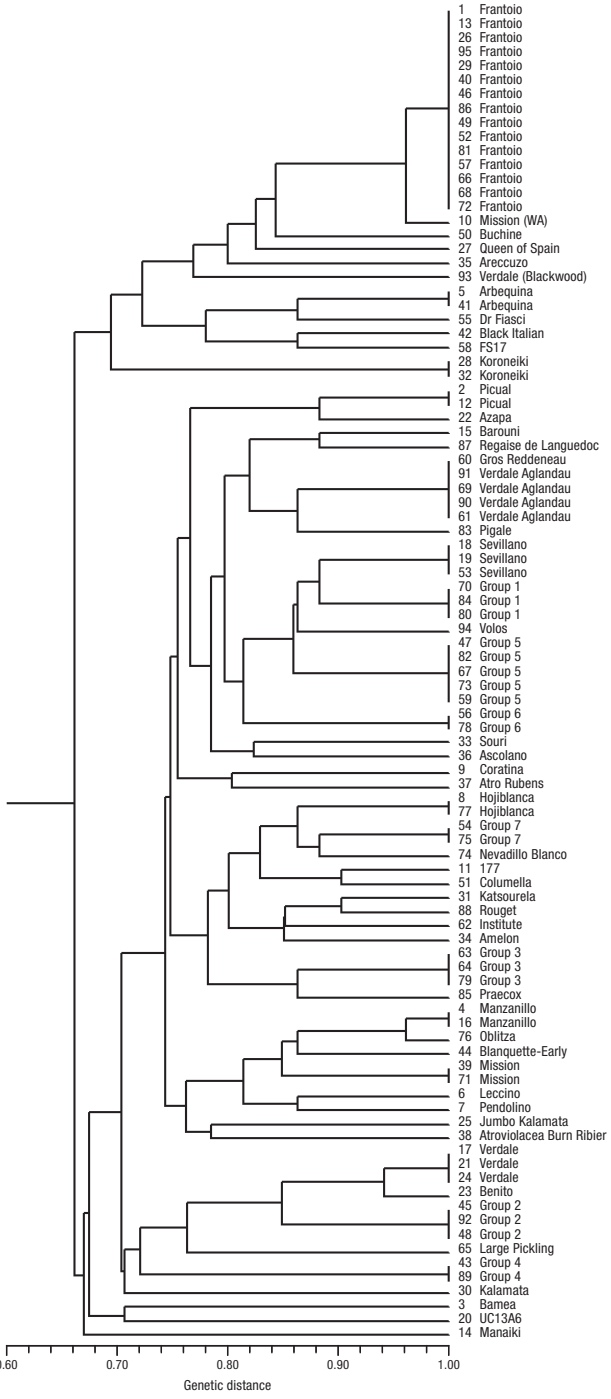

Figure 2. Genetic distance of the 95 genotypes present in the study site.

'Frantoio' was cross-compatible with 'Kalamata' in previous studies in South Australia (Wu *et al.,* 2002; Mookerjee *et al.,* 2005). This is in contrast with the findings of the present study, in which no embryo was assigned to 'Frantoio'. This may be due to differences in air temperature between the study sites at the time of flowering. Pollen-incompatibility is influenced by temperature and varies from environment to environment and from year to year (Androulakis & Loupassaki, 1990; Lavee *et al.,* 2002). High temperature during anthesis decreases self-fertilisation by inhibiting pollen tube growth in the style, while cross-fertilisation is considerably less affected (Lavee *et al.,* 2002). Another probable reason is the close genetic distance of 'Frantoio' and 'Mission (WA)' (Guerin *et al.,* 2002). FaMoz software showed more than one likely pollen donor (with same LOD score) for eight 'Kalamata' embryos ['Frantoio' and 'Mission (WA)' in five of them]. Fourteen embryos were assigned to 'Mission (WA)', and 'Frantoio' was the second likely pollen donor in all of them. The mean difference between the LOD scores of 'Mission (WA)' and 'Frantoio' in these 14 embryos was 0.09, much lower than the mean difference between the LOD scores of the first and second likely pollen donor in other assigned embryos (0.43). It seems that it was difficult for the marker system and FaMoz to distinguish between 'Frantoio' and 'Mission (WA)' as pollen donors due to their close genetic relationship, and this may be the reason for the high number of embryos assigned to 'Mission (WA)' (14 embryos), while only one of the 'Mission (WA)' trees was within the EPD of the 'Kalamata' mother trees. Although, eight microsatellite markers were enough for NTSYS-pc to discriminate 'Frantoio' and 'Mission (WA)', they were not enough for FaMoz probably because FaMoz uses only half of the alleles to recognise the most likely pollen donor. More microsatellite markers are suggested to prevent such problems.

In this study, 'Picual' was cross-incompatible with 'Kalamata', which confirms the results of a previous study in the NOVA collection conducted by pollen tube observation after controlled crossing (Wu *et al.,* 2002). 'Mission', 'Manzanillo', 'Pendolino', 'Leccino', 'Sevillano', and 'UC13A6' fertilised either no or only a small number of 'Kalamata' embryos in the present study, as was also found by Mookerjee *et al.* (2005) (less than 1 and 3 embryos, respectively). At both study sites, the pollen donors were located beyond the EPD of the 'Kalamata' mother trees. 'Mission' (Mookerjee *et al.,* 2005), 'Manzanillo', and 'Pendolino' (Wu *et al.,* 2002) have been reported to be cross-incompatible with 'Kalamata' in South Australia. 'Verdale', 'Verdale Aglandau', 'Group 2' (three genotypes that did not match with international standards but did with each other), and 'Group 3' (three genotypes that did not match with international standards but did with each other) did not fertilize any embryos, and only two cases of each were within the EPD of the 'Kalamata' mother trees. 'Verdale' fertilized only 1/160 'Kalamata' embryos in the previous study (Mookerjee *et al.,* 2005).

The results presented here suggest that 'Kalamata' was self-incompatible under the Mediterranean-type climatic conditions of Roseworthy, SA, Australia. It was cross-compatible with 'Barnea', 'Benito', and 'Katsourela' but cross-incompatible with 'Arbequina', 'Azapa', and 'Picual'. The olive growers of this and similar regions could use some of these compatible pollenisers with 'Kalamata'. More studies are needed to investigate the sexual compatibility relationships between 'Kalamata' and 'Frantoio'. The results obtained suggest that a larger microsatellite marker system may be beneficial for assessing the paternity analysis of olive when multiple cultivars are present, as in the NOVA collection.

Acknowledgments

Thanks to Susan Sweeney from Primary Industries and Resources South Australia for making the NOVA site available, the staff of Australian Genome Research Facility, especially Nicole Burtt, for help in DNA genotyping, and Dr Sophie Gerber for advice on using FaMoz software. Esmaeil Seifi was supported by a Ministry of Science, Research, and Technology, Iran Scholarship.

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