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An investigation of the self- and inter-incompatibility of the olive cultivars ‘Arbequina’ and ‘Koroneiki’ in the Mediterranean climate of Sicily

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Abstract

In this investigation, the self-(in)compatibility of the Spanish cultivar Arbequina and the Greek cultivar Koroneiki was studied for the first time in Sicily, where these low vigour cultivars were recently introduced in super-intensive olive groves. Self- (S.P.) and open-pollination (O.P.) tests, observation of fruit set and paternity test of seeds with microsatellite (SSR) markers, were performed to ascertain whether these cultivars were self-fertile and/or inter-compatible. For S.P. tests, branches with flowers at the balloon stage were bagged. For the O.P. tests, flowers were left to pollinate under natural conditions. Fruits from S.P. and O.P. were collected in November and fruit set was calculated. Genomic DNA was extracted from seeds. None of the ‘Arbequina’ seeds studied in either the S.P. or O.P. tests originated from self-fertilization. In addition, none of these seeds had ‘Koroneiki’ as the pollen parent. In contrast, ‘Koroneiki’ was found to be predominantly self-compatible in self-bagged branches, with 70% of the seeds originating from self-fertilization. However, the incidence of self-fertilization was low (11%) in seeds from the O.P. test. Low levels of inter-compatibility were found between ‘Arbequina’ and ‘Koroneiki’, while many local cultivars were found to be good pollinators. The information presented here will be useful to growers for planning their orchards with suitable pollinators and for our breeding program aiming at obtaining new low vigour olive genotypes. In addition, our results suggested that the recent model of attribution of *S*-alleles and the prediction of suitable pollinizers for a given variety should be more cautious and always based on controlled crosses and paternity testing of seed from those crosses.

Keywords: Breeding; microsatellites; *Olea europaea*; paternity test; self-fruitfulness; *S*-allele prediction model.
Abbreviations: S.P._self-pollination; O.P._open-pollination.

Introduction

The cultivated olive, *Olea europaea* (L.), is an allogamous species that is wind-pollinated and economically important for fruit and oil production, and most olive cultivars are thought to be self-incompatible (Mookerjee et al., 2005). Studies of self and inter-compatibility between olive cultivars have produced ambiguous results, with the compatibility status often influenced by environmental conditions (Mekuria et al., 1999; Lavee et al., 2002). For example the cultivar Manzanillo was described as self-compatible by Androulakis and Loupassaki (1990), partially self-incompatible by Wu et al. (2002) and self-incompatible in other studies (e.g. Lavee and Datt, 1978; Cuevas et al., 2009). Cuevas (2005) reported a certain degree of self-compatibility for ‘Arbequina’ and ‘Picual’, whilst Diaz et al. (2006) reported both cultivars as self-incompatible, even in the most favourable environmental condition for selfing and in monocultural orchards. The cultivar Koroneiki has been reported self-compatible by Lavee (1986) and Gharibzadeh et al. (2007) and self-incompatible by Mookerjee et al. (2005) and Seifi et al. (2011) in South Australia.

It is thought that self-incompatibility in olive is temperature dependent (Griggs et al., 1975; Lavee et al., 2002; Koubouris et al., 2009; Rejón et al., 2013) and that high temperatures promote self-incompatibility (e.g. Androulakis and Loupassaki, 1990; Ayerza and Coates, 2004). The presence of suitable pollinators in olive orchards is recommended to ensure adequate pollination and fruit set (Lavee and Datt, 1978; Moutier, 2002); and in addition it has been reported that cross-pollination increases fruit set and results in more regular yields even for partially self-fertile cultivars (e.g. Bradley et al., 1961; Breton and Bervillé, 2012). The literature presents contrasting reports on the possible self-incompatibility system acting in olive. Some works stated that a gametophytic (GSI) self-incompatibility system might exist in olive, controlled by *S*-RNase proteins (Serrano and Olmedilla, 2012). Other works provided cyto-histological observations and molecular data indicating instead the existence of a sporophytic self-incompatibility (SSI) system (Collani et al., 2012). It has been inferred that SSI may involve six *S*-alleles, with possible dominance relationships among them (Breton and Bervillé, 2012; Breton et al., 2014).

The determination of self and inter-(in)compatible olive cultivars has been based on cross-pollination tests and observation of fruit set (Lavee et al., 2002; Moutier, 2002). In the last ten years, molecular techniques and, in particular microsatellite markers, have been proved to be reliable for paternity test in olive (De la Rosa et al., 2004; Mookerjee et al., 2005; Diaz et al., 2006).

Recently, super-intensive planting systems of olive have been established in Southern Italy and in particular in Sicily

(Caruso et al., 2014a), with the introduction of foreign low vigorous and early-bearing cultivars: the Spanish ‘Arbequina’ (Del Río et al., 2005) and the Greek ‘Koroneiki’ (Androulakis and Loupassaki, 1990). Self-fertility and inter-compatibility relationships among ‘Arbequina’ and ‘Koroneiki’ are unknown in Southern Italian areas. The present work was undertaken to ascertain whether these cultivars are self-(in)compatible and cross(in)-compatible in intensive olive orchards in Sicily, in an attempt to raise new low vigour genotypes derived from the self-fertilisation or inter-cross of low vigour cultivars, under the breeding programme of the Department Agricultural and Forest Sciences (SAF) - University of Palermo, which was initiated in 2010.

Results and Discussion

In this work, we examined the self and cross-(in)compatibility and self-fruitfulness of the olive cultivars Arbequina (IRTA17) and Koroneiki, recently introduced in Sicily in high density plantation, by S.P. and O.P. pollination tests, observation of fruit set, and paternity test of the embryos with microsatellite markers. We discovered, among the local cultivars, appropriate pollinators for both varieties.

Fruit set in self-pollination and open pollination tests

Fruit set (Number of fruit set / total number of flowers × 100) in both cultivars was extremely different in self-pollination and open pollination tests. In the cultivar Arbequina, only 20 seeds were recovered from the self-pollination test, of which 15 had plump embryos while five presented aborted embryos (fruit set = 0.33%). Concerning the cultivar Koroneiki, 250 seeds were collected from the self-pollination test (fruit set = 4%), 35 of which were used for the molecular analysis: 21 of them showed plump embryos, while 14 were aborted. A total of 499 seeds of ‘Arbequina’ O.P. and 992 seeds of Koroneiki O.P. were collected, 50 each of which were used for the molecular analysis. Fruit set in open pollination trials, was around 16.5% for ‘Koroneiki’ and 8.31% for ‘Arbequina’.

SSR analysis and paternity testing

SSR markers were polymorphic and successfully amplified in all the samples. The average number of alleles per locus was 7.12, mean *He* was 0.69, mean *Ho* was 0.77, PIC was 0.65, and the combined exclusion probability for identity (NE-I) was low $1.8*10^{-7}$, ranging from 0.046 with the SSR locus UDO43 to 0.211 with the SSR locus DCA09, implying a very low probability to assign an incorrect pollen donor (Table 1). This set of SSRs provided sound molecular support in paternity test, discriminating seeds derived from self-pollination or outcrossing and allowed to discover suitable pollinators. The paternity test of the seeds was based on the calculation of LOD scores (Kalinowski et al., 2007). The critical LOD scores were 4.26 with 95% level of confidence and -0.09 with 80% level of confidence. True fathers were assigned to the embryos when the LOD scores were higher than the critical scores. Observed assignments were 63% with an 80% level of confidence.

Paternity test of ‘Arbequina’ S.P. and O.P. seeds

The paternity test (Table 2) of seeds derived from the self-pollination (S.P.) of ‘Arbequina’ showed that none of these seeds originated from self-fertilization, resulting instead from crosses with foreign airborne pollen, indicating that the air in the bag was already contaminated with foreign pollen or most likely it was on the branches of the trees. Inflorescences at balloon stage were isolated with double bags about a week before full bloom; bagging was not performed earlier since in our experience the presence of the bag may have a depressive effect on anthesis, stigma receptivity, pollen germination and fruit set, due to change in the temperature and humidity inside the bag. Putative male parents of 50% of ‘Arbequina’ seeds (S.P.) identified with 80% confidence, comprised the Sicilian cultivars Brandofino, Erbano, Giarraffa, Minuta, Nocellara del Belice and Nocellara Messinese and the Spanish cultivar Arbosana. For the remaining 50% of the seeds, we found one or two trio loci mismatching by using Cervus therefore we chose to refuse the paternity of these seeds, although again the most likely fathers were the cultivars Arbosana, Erbano, Nocellara del Belice, Nocellara Messinese, and Lumiaro. We also performed the paternity test of seeds originated from O.P. of ‘Arbequina’ (50 seeds) (Table 2). None of these seeds were derived from self-fertilization or originated from ‘Koroneiki’ or ‘Arbosana’. Possible male parents of 60% of these seeds, with 80% confidence, were found and reported in the Table 2. For the remaining 40% of the seeds the paternity was not accepted since one or two trio loci mismatching were detected. Therefore, the Spanish cultivar Arbequina (IRTA-17) was determined to be self-incompatible in the Mediterranean climate of Sicily, in agreement with the findings of Diaz et al. (2006).

Paternity test of ‘Koroneiki’ S.P. and O.P. seeds

The paternity test of 66% of seeds coming from self-fertilization of ‘Koroneiki’, presented in Table 2, showed, with a strict level of confidence (95%), that 70% of them were derived from self-fertilization, while the remaining 30% of seeds resulted, with a relaxed level of confidence (80%), from crosses with foreign airborne pollen of the cultivars Aitana, Erbano, Indemoniata and Arbosana. Additionally, one seed originated from a cross with ‘Arbequina’. For the remaining 34% of the seeds the paternity was not accepted because one or two trio loci mismatching were found. Regarding the paternity test of seeds originated from O.P. of ‘Koroneiki’, 11% of them were derived from self-fertilization, while the most likely fathers of the remaining seeds, with relaxed level of confidence, were five local cultivars and the Spanish cultivar Arbosana; whilst two seed were derived from ‘Arbequina’ (Table 2). For 14% of the seeds we found two trio loci mismatching using Cervus, again the most likely pollen donors were ‘Aitana’, ‘Biancolilla’, ‘Nerba’, ‘Piricuddara’ and in addition ‘Ogliarola Messinese’. However since we decided to accept the paternity of seeds with no locus mismatching at all, we cannot exclude the possibility that they may derived from crosses with unknown pollen donors. Thus, ‘Koroneiki’ was found to be highly self-compatible in self-bagged branches, in agreement with the report of Lavee (1986) and Gharibzadeh et al. (2007), but in contrast with the study of Mookerjee et al. (2005) and Seifi et al. (2011). However, a low incidence of self-fertilization was found in open pollinated trees, indicating that ‘Koroneiki’ is still predominantly allogamous. The level of cross-compatibility among ‘Arbequina’ and ‘Koroneiki’ was very low, although they were planted in close rows and bloom times of all putative pollen donors, including ‘Arbequina’ and ‘Koroneiki’, overlapped during the year of the experiment. This finding is in contrast with the work of El-Hady et al. (2007), who reported that these two cultivars are reciprocally

Table 1. Parameters of genetic variability for eight microsatellite markers. N_A, number of alleles; H_O, observed heterozygosity; H_E, expected heterozygosity, calculated in the 27 parental cultivars and the embryos; PIC, polymorphic information content; NE-I, non-exclusion probability (identity), computed only in the 27 parental cultivars.

Locus name	Reference	N_A	*Ho*	H_E	PIC	NE-I
ssrOeUA-DCA03	Sefc et al. (2000)	6	0.69	0.7	0.73	0.093
ssrOeUA-DCA05	Sefc et al. (2000)	6	0.84	0.6	0.56	0.203
ssrOeUA-DCA09	Sefc et al. (2000)	11	0.81	0.79	0.56	0.211
ssrOeUA-DCA15	Sefc et al. (2000)	6	0.68	0.64	0.60	0.170
UDO-43	Cipriani et al. (2002)	11	0.92	0.79	0.82	0.046
Oelgp06	Sabino Gil et al. (2006)	6	0.59	0.64	0.62	0.158
EMO-90	de La Rosa et al. (2002)	5	0.74	0.58	0.592	0.183
Gapu71b	Carriero et al. (2002)	6	0.89	0.77	0.73	0.091
Mean		**7.1**	**0.77**	**0.69**	**0.65**	
Combined						**0.8 * 10^{-7}**

N_A, number of alleles; H_O, observed heterozygosity; H_E, expected heterozygosity; PIC, polymorphic information content; NE-I, non-exclusion probability (identity).

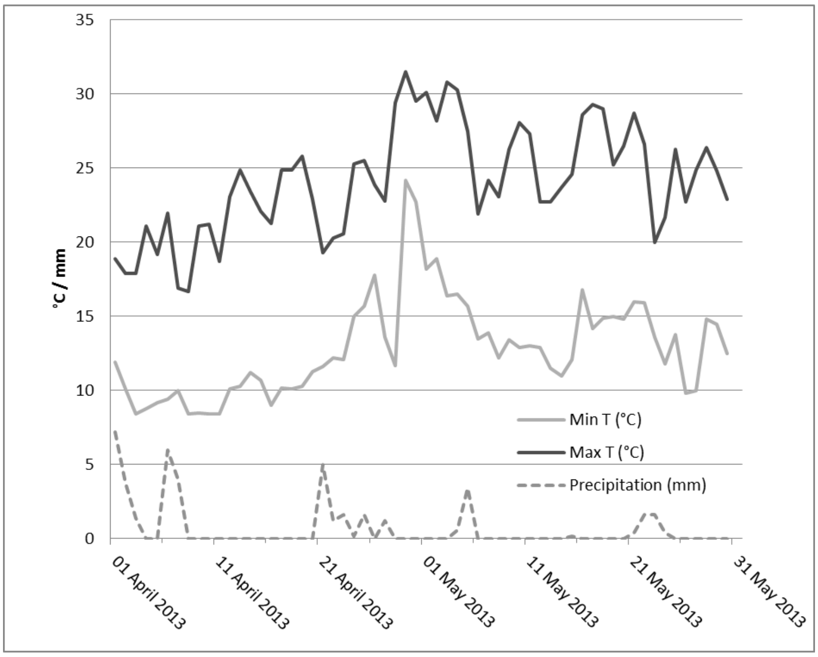

Fig 1. Climatic condition of flowering months (April-May) in the field of the experiment. Minimum temperature (Min T, °C), maximum temperature (Max T, °C), and precipitation (mm).

inter-compatible and that 'Arbequina' is the best pollinator for 'Koroneiki', on the basis of controlled pollination and fruit set, but without performing paternity test of the seeds. Thus, we can suggest to the local growers that is possible to establish high-density monocultural orchard of 'Koroneiki', although it would be better to consider implanting the low vigour and inter-compatible cultivar Arbosana, as cross-pollination may improve yield. Concerning the self-incompatible 'Arbequina' we recommend that some self-compatible pollinators are established in commercial orchards, such as the Sicilian cultivars Aitana, Brandofino, Nerba, Nocellara del Belice and Nocellara Messinese.

Consideration on current model of attribution of S*-alleles in olive and prediction of pollinators*

From the perspective of the SSI system proposed by Breton and Bervillé (2012), Breton et al. (2014) and Farinelli et al. (2015) the following *S*-alleles have been attributed to 'Arbequina' (R1R3), 'Koroneiki' (R4R6) and also to other Sicilian cultivars 'Giarraffa' (R2R3), 'Nocellara Etnea' (R2R4), 'Nocellara Messinese' (R4R5) and 'Moresca' (R4R5), that are also present in the orchard were the trial was conducted. In the present work, we did not find any embryo resulting from the cross 'Arbequina' × 'Koroneiki' and only two embryos of 'Koroneiki' could have 'Arbequina' as a father, indicating that 'Arbequina' and 'Koroneiki' are not reciprocally good pollinators, and that they may be partially inter-incompatible, although they were predicted to be cross-compatible, on the basis of statistical modelling, by Farinelli et al. (2015). According to our findings, Diaz et al. (2007) reported that 'Arbequina' and 'Koroneiki' were reciprocally inter-incompatible on the base of paternity test of the seeds by using SSR markers. Thus, the *S*-allele series or the dominant relationship among *S*-alleles should be further investigated, supposing that a SSI system acts in olive. We

Table 2. Percentage of self-fertilization of 'Arbequina' and 'Koroneiki' in self-pollination test and open pollination test, the most probable fathers (*at least 80% of confidence; error rate = 0; mismatch for the trio loci = 0).

		Self-pollination test (bagged branches)			Open-pollination test		
Orchard	Cv	Putative fathers* (N. embryos)		% of self-fertilization	Putative fathers* (N. embryos)†		% of self-fertilization
mixed	'Arbequina'	'Arbosana' 'Biancolilla' 'Brandofino' 'Erbano' 'Giarraffa' 'Minuta' 'Nocellara del Belice' 'Nocellara Messinese'	(2) (1) (1) (2) (1) (2) (2) (2) (2)	0%	'Aitana' 'Biancolilla' 'Brandofino' 'Crastu' 'Erbano' 'Indemoniata' 'Lumiaro' 'Nerba' 'Nocellara del Belice' 'Nocellara Etnea' 'Nocellara Messinese' 'Ogliarola Messinese' 'Pizzo di Corvo' 'Piricuddara'	(2) (1) (2) (1) (1) (1) (1) (4) (4) (1) (3) (1) (1) (1)	0%
mixed	'Koroneiki'	'Aitana' 'Arbosana' 'Erbano' 'Indemoniata'	(2) (1) (1) (2)	70%	'Aitana' 'Arbosana' 'Arbequina' 'Biancolilla' 'Giarraffa' 'Indemoniata' 'Minuta' 'Nerba' 'Piricuddara'	(3) (2) (2) (2) (1) (2) (1) (2) (2)	11%

() N. of embryos originated from each putative father

do not know if our Sicilian cultivars are genetically identical to those described in the literature, especially because many Sicilian cultivars are derived from cultivar-populations, thus sharing sibling relationships (Caruso et al., 2014b) however assuming that they may correspond, 'Arbequina' (R1R3) seemed inter-compatible with 'Nocellara Etnea' (R4R5) and 'Nocellara Messinese' (R4R5), according the prediction of Farinelli et al. (2015). On the other hand, one seed S.P. of 'Arbequina' (R1R3) was derived from a cross with 'Giarraffa', reported to be R2R3, which should be inter-incompatible. However we know that 'Giarraffa' is derived from a cultivar-population, composed of genotypes sharing sibling relationships, thus our 'Giarraffa' may have different *S*-alleles to the genotype used in the study of Farinelli et al (2015). In our opinion, the prediction and the attribution of *S*-alleles, formulated on computations of the index of self-incompatibility and the index for cross inter-compatibility without performing the paternity test of seeds, should be interpreted more cautiously, and predictions should ideally be based on controlled crosses always followed by paternity test of the obtained seeds, performed with reliable molecular markers, since the proposed model does not work precisely in this small scale experiment.

Materials and Methods

Plant materials

Pollination tests

Experiments were carried out in 2013 in a high density plantation orchard located at Menfi, Sicily, using eight year old 'Arbequina' (IRTA17) and 'Koroneiki' trees, placed in rows at a distance of 2.5 x 3.5 meters, trained to a *palmette* shape, under the same horticultural practices. In the same field 24 Sicilian cultivars and the foreign cultivar Arbosana, a low vigorous and early-bearing variety (Del Río et al., 2005) were planted in rows (Supplementary Fig 1S). All these cultivars are well characterised with microsatellite markers; for 20 of them, SSR profiles were reported in Marra et al. (2013) and Caruso et al. (2014b). Temperatures in the period of pollination (from the 1 of April to the 31 of May) were typical for the Sicilian Spring, and precipitation was modest (Fig 1). For each cultivar, five trees were chosen to perform self-pollination (S.P.) and open-pollination (O.P.) tests. For self-pollination tests, shoots were bagged when flowers were at the balloon stage, and open flowers were removed by hand. Fifteen double-bags of parchment paper were placed (three for each tree), containing an average of 400 flowers each; no pollen was added at all. Bags were removed two weeks later. Concerning the open-pollination test, flowers from three branches for each tree were left to pollinate under natural conditions. Fruits from self-pollination and open pollination were collected in November.

DNA extraction from embryos

The seed-coat was removed from seeds since it is of maternal origin and embryo tissues were excised from the endocarp. Genomic DNA was extracted from the embryos following the protocol developed by Doyle and Doyle (1987), with modifications. DNA was quantified and checked with a Nanodrop spectrophotometer (ND1000 Thermo fisher

Scientific); usually 50-70 ng of genomic DNA were recovered.

Data analysis and paternity test with SSR markers

Genomic DNA was amplified with eight fluorescently labeled microsatellite (SSR) markers, seven of which were combined in three multiplexed primer sets as follow DCA: 03, 05 (Sefc et al., 2000); EMO90 (De la Rosa et al., 2002), GAPu71b (Carriero et al., 2002) and UDO43 (Cipriani et al., 2002); Oelgp06 (Sabino Gil et al., 2006) and DCA15 (Sefc et al., 2000), while DCA09 (Sefc et al., 2000) was used in a single PCR reaction (Supplementary Table S1). PCRs were carried out in a reaction volume of 8 µl, containing 10 ng of genomic DNA, 1x Multiplex PCR master mix (Qiagen) and 0.2 µl of each primer pairs, using the following cycling steps for touch down PCR: 95°C for 15 min; ten cycles: 94°C for 30 s, 60°C for 1 min 30 s, with 1°C of temperature reduction for each cycle, 72 °C for 1 min; 25 cycles: 94°C for 30 s, 50°C for 1 min 30 s; and finally 65°C for 30 min. Amplicons were separated on an automatic sequencer ABI3130 (Applied Biosystems) and alleles were sized using GeneMapper 4.1 software (Applied Biosystems).

The following parameters of genetic variability of the SSR loci were calculated with Cervus (Kalinowski et al., 2007) for the embryos, the two known maternal plants and the pollen donors (27, including 'Arbequina' and 'Koroneiki'): number of alleles per locus, observed heterozygosity (H_o) and expected heterozygosity (H_e); while the PIC (polymorphic information content) and the average exclusion probability for identity of two unrelated individuals (NE-I), were calculated only in the 27 putative paternal genotypes (Table 1).

Seeds having only maternal SSR alleles were considered as derived from self-pollination. Paternity test of the seeds was performed with Cervus (Kalinowski et al., 2007), using the paternity analysis (mother known/father unknown) with the maximum likelihood approach (LOD) (Marshall et al., 1998; Kalinowski et al., 2007), including all the SSR profiles available for Sicilian cultivars (Marra et al., 2013; Caruso et al., 2014b) or present in the database that we are developing for olive genotyping (data not shown). Paternity simulations were first performed to estimate the critical values of LOD associated with strict (95%) and relaxed (80%) levels of confidence in assignment (Kalinowski et al., 2007). The error rate was taken equal to zero, the mismatch for the trio (i.e. the genotypic inconsistency among the offspring and one or both parents, based on Mendelian patterns of inheritance) of even one allele led to exclusion of paternity; putative fathers were considered as the cultivars with the highest LOD score, presenting zero mismatch and interval of confidence at least 80% (Table 2).

Conclusions

The Spanish cultivar Arbequina (IRTA-17) was determined to be self-incompatible in the Mediterranean climate since none of the seeds from S.P. and O.P. tests originated from self-fertilization. The Greek cultivar Koroneiki was found highly self-compatible in the S.P. trial but it was still found to be predominantly allogamous in the O.P. trial. Knowledge on self(in)compatibility is important for the growers from Southern Italian regions to allow them to better manage newly introduced cultivars in their orchards and for choosing suitable pollinators among the local olive cultivars. The self-compatibility test and the paternity results are extremely useful for our breeding program aiming at obtaining new genotypes adapted to intensive mechanized orchards and low vigour rootstocks by using 'Arbequina', 'Koroneiki' and 'Arbosana' as parents. Promising low vigour genotypes with an upright habit have already been obtained by the Department SAF of the University of Palermo, and are currently under evaluation.

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