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Cross-pollination in Olive Cultivars

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Foreword

The aim of this study was to determine the pollen donors for five major olive cultivars, using paternity analysis. Bloom time, pollen vitality, and weather conditions during the flowering period were also recorded. The study was done over two years at two sites and different compatibilities between cultivars were identified.

The olive industry will benefit from the results of this project through increased productivity. Many orchards have been established without due regard to the requirements of cross-pollination. These results will be valuable guidelines to anyone planning new plantings. In addition, they can be used to increase productivity in established groves through the addition of highly compatible pollen donors. The effect of introducing compatible pollen donors to a mono-varietal grove could improve yields from less than 1% up to 4%. New trees can be introduced through grafting onto old trees or inter-planting. This research also has environmental benefits as a significant increase in yields could be achieved without expansion of land use, irrigation or addition of fertilisers or chemicals for pest and disease control.

There were notable similarities between the results obtained at both sites. At least one of the major pollen donors identified for the mother trees at Gumeracha was also identified as a major donor for the trees at the NOVA site. This result indicates that these cultivars are effective pollen donors in more that one environment. The fact that they are major pollen donors at both sites also indicates that they are highly efficient donors for the respective mother trees.

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Peter O'Brien
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The aim of this study was to determine the pollen donors for five olive major cultivars, using paternity analysis. Bloom time, pollen vitality, and weather conditions during the flowering period were also recorded. The study was done over two years at two sites and different compatibilities between cultivars were identified.

Who is the report targeted at
The olive industry will benefit from the results of this project through potential increases in productivity. Many orchards have been established without due regard to the requirements of cross-pollination. The results from this project will provide valuable guidelines to anyone planning new plantings. In addition, they can be used to increase productivity in established groves through the addition of new pollen donors. The effect of introducing compatible pollen donors to a mono-varietal grove could improve yield (fruit set) from less than 1% up to 4%. New trees can be introduced through grafting onto old trees or inter-planting. This research also has environmental benefits as a significant increase in yields could be achieved without expansion of land use, irrigation or addition of fertilisers or chemicals for pest and disease control.

Background
Olives have been in cultivation for thousands of years, yet pollen compatibility relationships between cultivars and the mechanism of self-incompatibility have not been fully investigated. In common with many woody, forest trees, the olive is a wind-pollinated species. Several studies on the identification of compatible cultivars have led to conflicting results, possibly due to different environmental conditions under which the studies were conducted, or to confusion in cultivar identity (Mekuria *et al.* 1999). In addition, self-compatibility and the response to pollen donors can vary between seasons (Lavee *et al.* 2002).

The most commonly used methods for identification of compatible cultivars are based on artificial cross-pollination using pollination bags to regulate the pollen load, and subsequent observation of pollen tube growth (Wu *et al.* 2002) and/or fruit set (Lavee *et al.* 2002). However, conditions inside the pollination bag may affect fruit set (Rallo *et al.* 1990) and pollination bags may leak, allowing pollen from other genotypes to enter (de la Rosa *et al.* 2004). The results will also vary depending on whether the initial or final yield is considered because of the high rate of fruit drop in olives (Rallo *et al.* 1990).

The identification of the paternal parent using molecular techniques is a reliable method, because the genetic contribution of alleles is traced from the parents to the offspring. Microsatellite markers are useful for these studies as they have codominant segregation and a high level of polymorphism in olive (Sefc *et al.* 2000; Cipriani *et al.* 2002; de la Rosa *et al.* 2002). Several reports have used microsatellite markers and paternity analysis to study pollen movement in tree populations (Chaix *et al.* 2003, Isagi *et al.* 2004, Robledo-Arnuncio and Gil 2005). The effectiveness of microsatellite markers in identification of paternal parents of progeny obtained from an olive breeding program has been demonstrated by de la Rosa *et al.* (2004).

Results
There were notable similarities between the results obtained at both sites. At least one of the major likely pollen donors identified for the mother trees at Gumeracha was also identified as a major likely donor for the trees at the NOVA site. This result indicates that these cultivars may be effective pollen donors in more that one environment. The fact that they are likely pollen donors at both sites also indicates that they are potentially efficient donors for the respective mother trees. Selfing was not significant at the Gumeracha site but was predicted in Frantoio and at a higher rate in Koroneiki at the NOVA site, probably due to environmental influence.

Implications
To compensate for the variability in pollen viability and flowering times between seasons it is suggested that more than one pollen donor cultivar is present in a commercial grove, and preferably three or four. Under the conditions of this study the following combinations of mother trees and pollen donors were found likely to be compatible:

Frantoio: Kalamata, Mission, Coratina
Kalamata: Frantoio, Koroneiki, Barnea
Koroneiki: Mission, Hojiblanca
Mission: Koroneiki, Arbequina
Barnea: Kalamata, Mission

Introduction

Olives have been in cultivation for thousands of years, originating in the eastern Mediterranean region, and has since spread to many other parts of the world. The olive tree has played a role in the commerce and trade of Syria from as early as 3000 B.C. (Connell 1994), and the importance of the olive tree to the Mediterranean people has been documented by Voyiatzi *et al.* (1999) and Tsalikidis *et al.* (1999). Spain, Italy and Greece are the major producers and consumers of olive products in the world, although both production and consumption are increasing in areas outside the Mediterranean. The olive plant was introduced into Australia at the beginning of the nineteenth century (Booth and Davies 1996), and olives now comprise an important industry in Australia.

The viability of the olive industry depends on harvesting an economic yield of fruit, which can be used for oil production and table olives. Despite a profusion of flowers on olive trees, only a few set fruit in a growing season (Cuevas *et al.* 2001; Ghrisi *et al.* 1999; Marco *et al.* 1990; Martin 1990) and only about 1-2% of these fruits remain on the trees at maturity (Martin 1990). The reasons for this low fruit to flower ratio include the proportion of male to female flowers, climatic conditions during fruit set, and compatibility relationships among cultivars (Dal Pero Bertini 1960). Some cultivars are self-incompatible, which means that the flowers cannot be fertilised by pollen from the same cultivar, and some cultivars are cross-incompatible, where flowers cannot be fertilised by pollen from certain other cultivars. Therefore, it is important for growers to understand the cross-compatibility between cultivars when planning an olive orchard in order to maximise fruit set and yields. This is especially important when orchards are planted in isolated areas where the only sources of pollen available are within the orchard.

Since the pollination process may vary from year to year and can be affected by environmental factors, studying pollen flow amongst different cultivars at the molecular level is a new and exciting approach that is likely to be a more reliable and powerful method of determining compatibility than studies based on hand pollination, bagging, pollen tube growth, or fruit set. The identification of suitable pollen donors using paternity analysis studies analyses the paternal genetic contribution in the offspring and hence identify the most likely father.

Floral structure and pollen dispersal

Olive trees bear both hermaphrodite and staminate flowers (Ateyyeh *et al.* 2000) in the form of panicles (Griggs *et al.* 1975). The flowers are whitish and small and each bears a ‘short-toothed’ calyx and ‘short-tubed’ corolla (Hartmann and Opitz 1966). Hermaphrodite flowers generally have two stamens and a bi-locular ovary with a short style and stigma (Hartmann and Opitz 1966). In staminate flowers, the pistil is either rudimentary or absent. The flowers are wind pollinated, bear large quantities of pollen, and no nectar is produced as they do not possess nectaries (Martin 1994). Griggs *et al.* (1975) observed that olive flowers are morphologically adapted to either self- or cross-pollination. In some flowers, the anthers are close enough to the stigma so that when they dehisce, the pollen falls on the stigma and self-pollination could occur. At the same time there are flowers where the filaments are flattened so that the anthers spread away from the stigma, thus favouring cross-pollination. According to Cuevas *et al.* (2001), olives are naturally suited to cross-pollination by wind. The presence of flowers with male parts only indicates that these flowers are formed for the sole purpose of acting as pollen donors. The abundant amount of pollen, up to 200,000 pollen grains per flower, shows that they are adapted to be wind pollinated.

Factors affecting pollination

For successful cross-pollination to occur, it is necessary to have adequate amounts of compatible pollen available when the flowers are in bloom. This is possible if the compatible cultivars growing in the orchard have overlapping bloom times. Olive trees of the same cultivar growing under the same environmental conditions are known to bloom simultaneously (Dal Pero Bertini 1960). Griggs *et al.* (1975) and Ghrisi *et al.* (1999) observed that the bloom dates and duration vary among cultivars and between years. However, they observed that in most years the bloom time overlapped sufficiently for adequate pollination. Lavee *et al.* (2002) studied 36 olive cultivars over a period of 12 years and observed that the length of the flowering period depended on climatic conditions. Sanz-Cortes *et al.* (2002) studied three different cultivars and found that the start of blooming differed for the cultivars Temprana de Mont, Serrana de Esprad, and Penjoll. These studies highlight the importance of recording bloom period to ensure the availability of pollen when the stigma are receptive.

The proportion of complete flowers borne by a tree is another factor that has been studied and may affect the fruit set. Only the complete flowers can bear fruits while the staminate flowers can only act as pollen donors. Studies by Wu *et al.* (2002) showed wide variation in the percentage of complete flowers between cultivars, ranging from 23% in Kalamata to 87% in Picual. Cuevas and Rallo (1990) observed that trees with a low number of flowers had a higher proportion of complete flowers than those with more flowers. Rallo *et al.* (1981) and Lavee *et al.* (1996) found that although the proportion of complete flowers differed between cultivars there were no significant differences in the yield. This may be because of the exceedingly small number of flowers that actually produce fruit. However, they found that removal of half the inflorescences resulted in double the fruit set in most cultivars studied. This implies that competition between fruits and non reproductive organs for the resources of the plant may influence the final fruit set (Rallo *et al.* 1981). Griggs *et al.* (1975) observed that occasionally a tree may bear mostly staminate flowers and thus have a low yield due to unavailability of enough perfect flowers to mature into fruits. Reale *et al.* (2006) recently observed flowers and the rate of aborted flowers at the morphological and cyto-histological level and found that the number of panicles in the canopy is a major influence on yield rather than the number of flowers per tree.

Methods of measuring pollen vitality in olives have been studied by Pinney and Polito (1990). They used both the *in vitro* germination method and the fluorescein diacetate method to measure the viability of olive pollen and found that both methods were highly correlated. Similar studies by Wu (2002) showed that the coefficient of determination between the two methods is 0.86. Pinney and Polito (1990) also found pollen viability to vary among cultivars. Ascolano was found to have the highest pollen viability and Mission the lowest. Wu (2002) observed that pollen viability ranged from a low of 14% in Pendolino to a high of 79% in Frantoio. Occasionally, cultivars have been found to be male sterile (Moutier 2000; Villemur *et al.* 1984). It is important to consider the viability of pollen used in any pollination study since totally male sterile cultivars cannot be pollen donors. Also, there is no published information on whether the level of pollen viability has any effect on a cultivar being a pollen donor. Lavee *et al.* (2002) suggested that since the bloom time and pollen viability of cultivars vary from year to year, yields may benefit from having than more than one pollen donor in an orchard in order to ensure adequate pollination each year. Pollen vitality was observed to vary between years in Arbequina clones (Rovira and Tous 2000). Therefore, multi-year data must be obtained in order to observe the effect of changes in vitality on the ability of a cultivar to act as a pollen donor.

Several studies have reported that environmental conditions can affect the process of pollination. For example, Hartmann and Opitz (1966) observed that some cultivars had particular temperature requirements for optimum pollen tube growth. However, they found that fruit set was not affected by rain at the time of bloom. Bradley *et al.* (1961) found that the effect of temperature on growth of pollen tubes is dependent on the cultivar combination. Low temperatures have been found to reduce pollen tube growth, and as a result, the pollen tubes are unable to reach the embryo sac before it

degenerates (Martin 1994). On the other hand, high temperatures result in faster growth of pollen tubes (Griggs *et al.* 1975), although Fernandez-Escobar *et al.* (1983) and Cuevas *et al.* (1994) observed better pollen tube growth at 25°C than at 30-35°C. Androulakis and Loupassaki (1990) observed that cultivars differ in their compatibility when grown in different environments. They observed that high temperatures negatively affected pollination. Environmental factors have also been implicated in fruit set rates (Ghrisi *et al.* 1999), and the formation of perfect flowers (Lavee *et al.* 2002). Olives are wind pollinated and therefore the direction of wind during bloom time is an important consideration.

Problems with fruit set in olives

Several problems may affect the commercial production of fruit. The alternate bearing habit of the trees causes a heavy crop of small fruits in some years, and a light crop of large fruits in others. Radi *et al.* (1990) suggested that the phenomenon is an effect of competition among fruits for the limited nutritional resources of the tree. A high level of flower and fruit abscission is observed in olives (Cuevas *et al.* 2001; Ghrisi *et al.* 1999; Marco *et al.* 1990; Martin 1990). As many as 98% of the flowers have been observed to abscise and only 1 to 2% of the flowers develop into fruits (Martin 1990). However, before abscission, the large number of flowers causes a drain on the resources of the tree, and this may explain why the highest fruit set rates are followed by the highest fruit drop rates (Martin 1990). Ateyyeh *et al.* (2000) reported that there were two stages in perfect flower abscission. The perfect flowers that were not fertilised abscised three weeks after anthesis, while some of the fertilised flowers abscised during the next six weeks after anthesis. Olive yields may also be affected by the formation of shot berries, which are small parthenocarpic fruits of no commercial value (Rapoport and Rallo 1990). Some researchers have attributed the cause of shot berries to inadequate cross-pollination (Fernandez-Escobar and Gomez-Valledor 1985; Sibbett *et al.* 1992).

A major problem faced by both researchers and growers is the confusion regarding the compatibility relationships for successful fertilisation of olive cultivars. Studies have been conducted in several different countries to identify the most compatible cultivars (Androulakis and Loupassaki 1990; Rallo *et al.* 1990; Antognozzi and Standardi 1978; Sharma *et al.* 1976; Griggs *et al.* 1975). While most studies agree on the benefits of cross-pollination, there is still uncertainty about the most effective pollen donors for even the most commonly grown cultivars.

Self-incompatibility in olive cultivars

Cuevas and Polito (1997) observed that during self-pollination, most pollen tubes are unable to grow through the style and reach the ovules for fertilisation, while the pollen tubes arising from cross-pollination grew faster and reached the ovule. This indicates strongly that a self-incompatibility system operates in olives. In fact, several studies have shown that some olive cultivars are self-incompatible. Diaz *et al.* (2006) studied self-incompatibility by testing seeds obtained from Picual and Arbequina mother trees using paternity analysis with data obtained from four microsatellite markers. They found only three incidences of selfing out of the 90 seeds tested and concluded that these two cultivars were self-incompatible. Wu *et al.* (2002) tested self-incompatibility by hand pollinating and observing pollen tube growth and found the cultivars Frantoio, Kalamata, and Verdale to be self-incompatible. Moutier (2000) studied the compatibility relationships in sixteen olive cultivars and found that most of these cultivars needed cross-pollination for adequate fruit set. El-Kholy (2001) reported that the cultivars Pendolino, Leccino, and Coratina are self-incompatible, but he also found that the compatibility relationships of the cultivars change when grown under different environmental conditions. This may explain the contrasting results for some cultivars in the literature. The cultivar Moraiolo was found to be self-incompatible in studies conducted in Italy (Bini and Lensi 1981), while the same cultivar was found to be self-compatible in studies conducted in India (Singh and Kar 1980). Leccino was mostly found to be self-incompatible (Antognozzi and Standardi 1978; Ugrinovic and

Stampar 1996), although, Bartoloni and Guerriero (1995) found several selections of Leccino to be self-compatible.

Self-incompatibility could result in low fruit yields in orchards consisting of a single cultivar (Lavee and Datt 1978; Singh and Kar 1979). Sibbett *et al.* (1992) observed that cross-pollination could improve the quality of the crop by reducing the number of shot berries, and a topical application of Sevillano pollen on Manzanillo reduced the number of small, or parthenocarpic, fruits. Similar observations were made by Fernandez-Escobar and Gomez-Valledor (1985), and Cuevas and Polito (1997). Even cultivars that exhibit some level of self fertility have been observed to give higher yields following cross-pollination (Fontanazza *et al.* 1980; Lavee *et al.* 2002).

Cross-compatibility between olive cultivars

Pollen incompatibility has often been found to be the cause of poor performance of olive crops (Ghrisi *et al.* 1999; Sibbett *et al.* 1992). According to Lavee (1998), the pollinating cultivar should be selected on the basis of economic productivity, quality of pollen grains, compatibility with the receptor cultivar, and overlapping bloom time. Not all cultivars are cross-compatible, Griggs *et al.* (1975) and Cuevas *et al.* (2001) both found Mission and Manzanillo to be cross incompatible. Wu *et al.* (2002) found the cultivars Kalamata, Manzanillo, Pendolino and Picual to be cross-incompatible. Moutier *et al.* (2001) reported a high level of cross-incompatibility between French cultivars. They conducted 90 cross-pollinations and found that only 15 cultivar combinations produced good results. Lavee *et al.* (2002) also observed cross-incompatibility between cultivars using pollen germination in the presence of stigma extracts as well as fruit set data.

There is also confusion regarding the identities of some olive cultivars. Inconsistencies in the yield of cultivars led to DNA fingerprinting studies which have proved that there are problems pertaining to mistaken identities of cultivars (Guerin *et al.* 2002; Mekuria *et al.* 1999; Weisman *et al.* 1998). According to Bartolini *et al.* (1994) there are more than 1200 cultivars throughout the world with more than 3000 synonyms.

It has been proposed that self-incompatibility in olive is gametophytic (Cuevas and Polito 1997; Ateyyeh *et al.* 2000; Wu *et al.* 2002). In general, reciprocal compatibilities/incompatibilities are observed in systems under gametophytic control (Lewis 1994; Sedgley 1994). However, other reports have shown non-reciprocal relationships between olive cultivars (Moutier *et al.* 2001; Lavee *et al.* 2002). Lavee *et al.* (2002) suggested that multiple origins of the domesticated *Olea europaea* have resulted in a complex system controlling self-incompatibility. Further work is needed to understand the genetic system operating in olives. including the identification of the genes involved.

Methods of studying compatibility relationships in olives

Studies on compatibility relationships in olives have so far been based on artificial cross-pollination and subsequent observation of pollen tube growth (Bartoloni and Guerriero 1995; Cuevas *et al.* 2001) and/or fruit set (Singh and Kar 1980). However, when flowers are bagged the microenvironment differs from the natural conditions under which fruit set usually takes place. In addition, fruit set observations can be made at different times and may vary considerably as initial fruit set can be high, but after fruit abscission the final fruit set may not be so impressive. This was particularly evident in the study conducted by Rallo *et al.* (1990) where it was observed that although fruit set in self-pollinated trees was higher at full bloom, after 15 days, open pollinated trees had a higher fruit set at full bloom after 45 days. Studies in which pollen tube growth is used to determine cross-compatibility are based on the assumption that if the pollen tube reaches the embryo sac and successful fertilisation occurs, the fruit will reach maturity. However, it is a common phenomenon that, after the initial fruit set, a large proportion of the fruits abscise (Cuevas *et al.* 2001; Ghrisi *et al.* 1999). Other factors may be involved during post-fertilisation abscission, which may not necessarily be related to compatibility.

De la Rosa *et al.* (2004) used paternity analysis with data from four microsatellite markers to verify the parents of seedlings thought to be derived from selfing or controlled crossing. They found a high level of contaminating pollen had breached the pollination bags during hand pollinations. This finding throws many previous results using pollination bags into question, especially if the bags were not placed over the inflorescences well before anthesis. Compatibility relationships based on paternity analysis trace back the genes in mature fruits from the mother tree and its polliniser, so the true pollen donor can be identified.

Paternity analysis studies

Several paternity analysis studies have used isozymes as molecular markers to trace the movement of pollen between cultivars. For example, transmission of genes from parents to offspring in radish was studied using isozyme analysis where the genotype of the progeny was compared with that of the mother plant and then the maternal contribution was subtracted to study the paternal contribution of genes (Ellstrand *et al.* 1989). A similar approach was used to identify pollen donors for sweet cherry cultivars Stella and Summit (Brant *et al.* 1999). It was observed that the presence of a number of pollen donors in the orchard ensured the availability of adequate pollen at the time of full bloom, since effective pollination can occur only when the bloom times of the cultivars coincide. Isozymes were also used to study gene flow in almonds (Jackson and Clarke 1991), where the pollen gene contribution in the embryos of mature nuts was studied. However, the success of these studies depended on the availability of isozymes with sufficient polymorphisms to be able to distinguish between the different cultivars. When few pollen donors are involved, isozymes may prove to be a useful tool to study gene flow. However, since the polymorphism obtained with isozymes is less than that required to successfully distinguish between large numbers of cultivars, these markers are not suitable when many cultivars are present in the population.

Recent studies on paternity analysis have used microsatellite markers that amplify polymorphic repeat sequence regions of the genome. These markers have been successfully used for paternity analysis in sweet potato (Buteler *et al.* 2002) where two microsatellite loci were analysed in the experimental population. This study also showed that when one of the loci had a lower level of polymorphism in the population than is required to distinguish between cultivars, it was difficult to analyse the paternal contribution of genes. Hence, the level of polymorphism exhibited by the marker in the cultivars is an important factor in these studies. Microsatellite markers have been used confirm Mendelian segregation using paternity analysis in a population of olives derived from a cross between Leccino and Dolce Agogia (Rallo *et al.* 2000). Paternity analysis studies using microsatellite markers have been used for several tree species including oak (Gerber *et al.* 2000; Streiff *et al.* 1999), red pine (Lian *et al.* 2001), walnut (Polito *et al.* 2003) and *Eucalyptus* (Chaix *et al.* 2003).

The most likely method was proposed by Thompson and Meagher (1987) and can be used to identify potential fathers in populations with several genotypes based on the genetic data, since an individual cannot be a parent unless it has genes in common with the progeny. They use the concept of exclusion probability, which is the probability that an unrelated individual will be excluded from being a parent. The likelihood of paternity for each male parent is compared for a particular female parent and her progeny. The logarithm of likelihood ratios is thus the likelihood of an individual being the parent compared to all other individuals being the parent. Paternity is assigned to the male with the highest likelihood value. Thompson and Meagher (1987) used these approaches to identify the most likely parents in a population of *Chamaelirium luteum* seedlings based on 11 isozyme markers. The likelihood based method was also used by Gerber *et al.* (2000) on a population of oak trees. Gerber *et al.* (2000) included estimations of gene flow from outside the stand and gene flow from inside the stand to minimise errors, and their method has been developed into the computer program, FaMoz, which has been used successfully to estimate gene flow in a population of *Eucalyptus* using microsatellite markers (Chaix *et al.* 2003). This program was used for our study on olives.

Objectives

The aim of this research project is the identification of compatible and competitive pollen donors for five widely planted olive cultivars in Australia. Compatibility between cultivars will be investigated using DNA markers and paternity testing. The most likely pollen donors of olive fruits will be determined by analysing the DNA of embryos collected from trees within the orchard.

Methodology

Plant material

Trees

Olive trees growing at Milano Olives, were used for the experiments for the Gumeracha site, South Australia. Gumeracha is located in the Adelaide Hills at an altitude of 355 m above sea level, latitude 34° 49’ 33” S and longitude 138° 52’ 10” E. The property was laid out in sections supplied by separate irrigation valves. Some of these sections were planted with a single cultivar, while others were planted with a mixture of cultivars (Figure 1). The trees in the orchard ranged from one to four-and-a-half years old at the time of study. The orchard was irrigated with water of 1550 Total Dissolved Solids (ppm). Tree sprinklers were used at 35 litres per hour and the orchard was irrigated every two weeks for 10 to 12 hours from December to end of March.

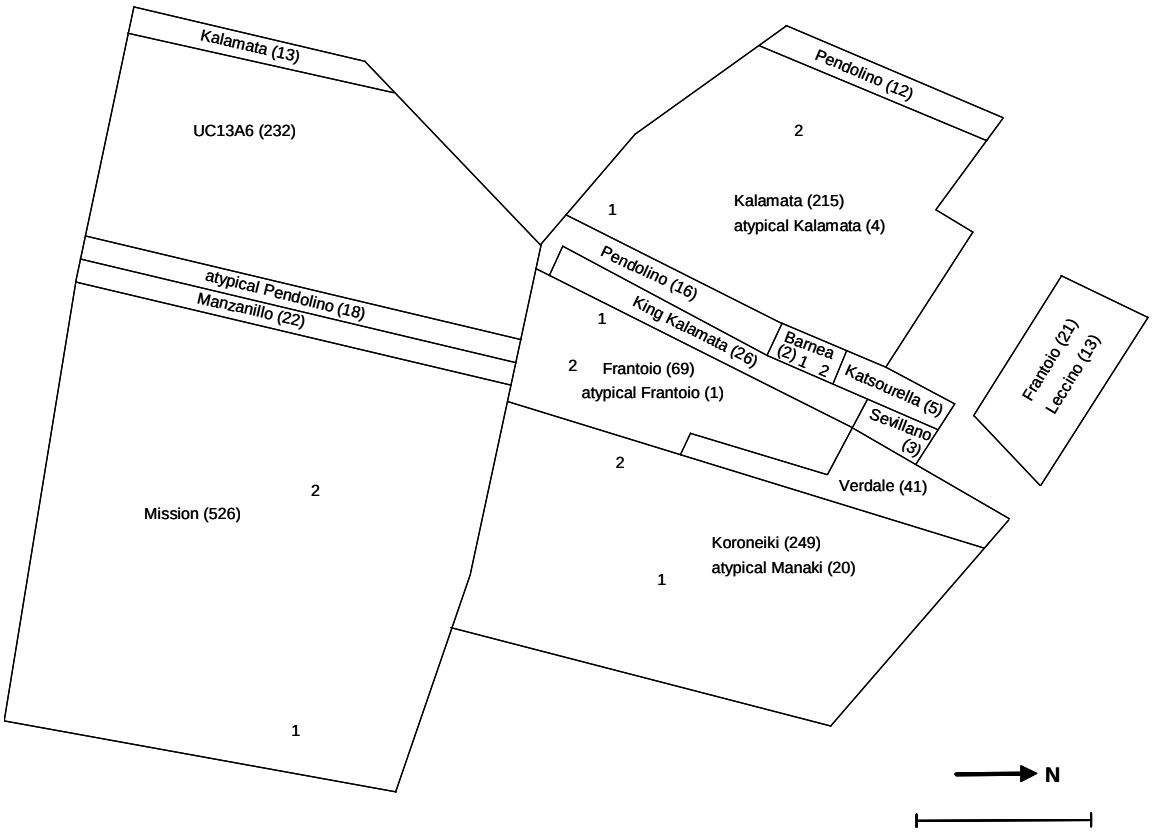


Figure 1. Field plan of the orchard showing the position of cultivars and the selected mother trees (indicated by 1 and 2). The number of trees for each cultivar are indicated in parentheses. Scale bar = 50m.

Fruits collected from mother trees of five cultivars (Barnea, Frantoio, Koroneiki, Kalamata, and Mission) were used for paternity analysis. Two mother trees of the cultivars were selected, one from a region of the orchard where it was surrounded by trees of the same cultivar, and one from a location where it had other cultivars nearby. The reason behind such selection was that the possibility of self-fertility and the effect of nearby pollen donors could be assessed. Additional trees were used for collecting fruits when the selected mother trees did not have enough fruits for analysis. All the genotypes present in the orchard were considered to be potential pollen donors.

Trees at the Roseworthy site (-34 052'S, 138069'E), were planted for the National Olive Variety Assessment project, established to scientifically evaluate most of the known olive varieties in Australia, at the University of Adelaide Roseworthy campus (-34 052'S, 138069'E), 50 km north of Adelaide, South Australia. Roseworthy has a Mediterranean-type climate with an average annual rainfall of 440mm with 330mm (75%) occurring between the months of April to October. Details of irrigation and soil conditions are outlined in the RIRDC Publication No 03/054.

The National Collection was planted as a resolvable incomplete block design, consisting of 3 replicates by 2 trees per replicate of 100 accessions sourced from nurseries and old government collections across Australia. Tree spacing was 6 metres within rows by 7 metres between rows. A barrier row of olive trees was planted around the 3 replicate blocks. Three mother trees were used, one from each replicate, so that different neighbours or potential pollen donor varieties surrounded each one. Figure 2 shows the position of the mother trees relative to the other genotypes planted in the trial. The mother trees used at this site were Frantoio, Kalamata, Koroneiki and Mission.

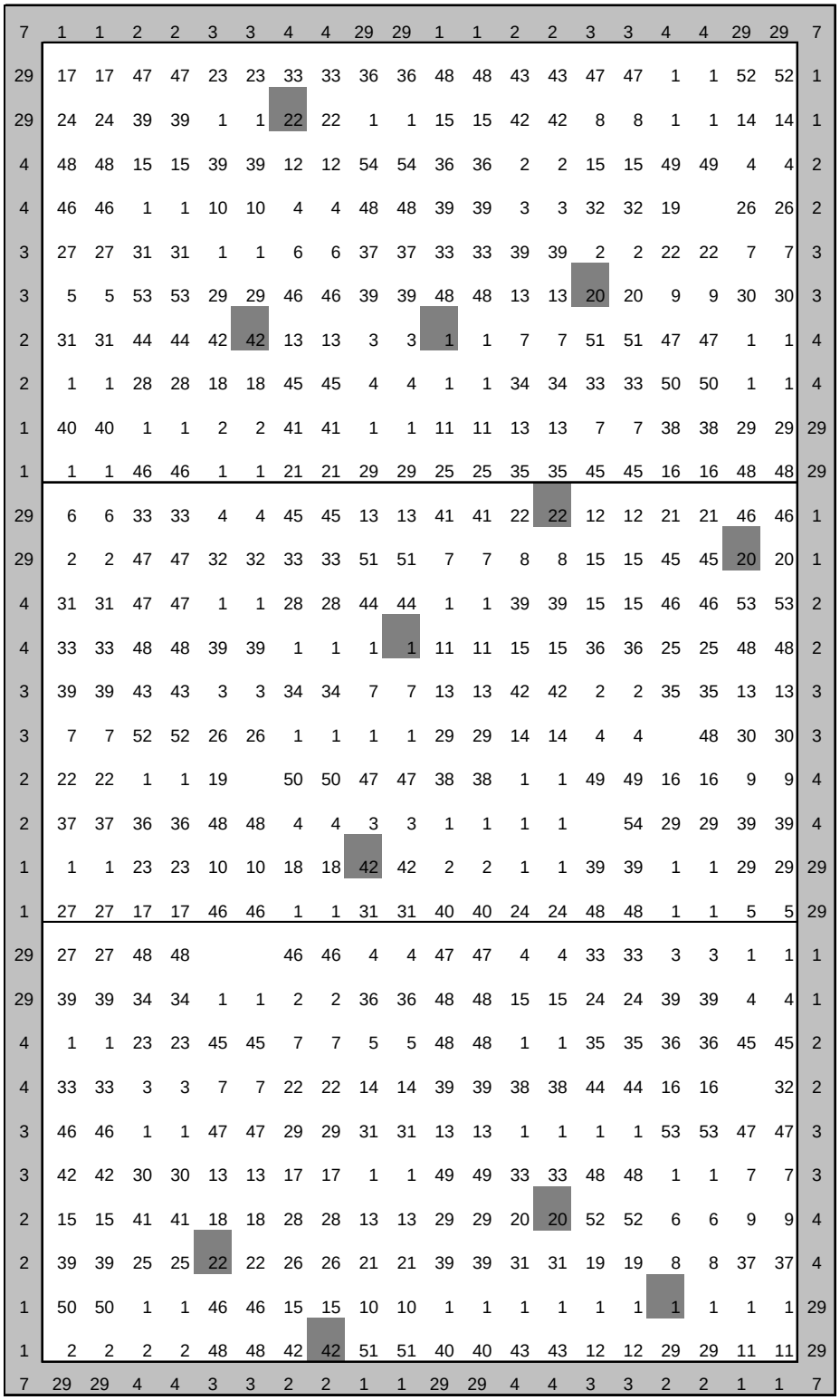


Figure 2. Field plan of the NOVA site showing the three replicate blocks R1, R2 and R3. Border row is shaded in light grey. The genotype number of each tree is shown and the mother trees from each block are indicated by a dark grey square. Mother trees are Frantoio (1), Kalamata (20), Koroneiki (22) and Mission (42).

Flowers

Flowers were collected when the plants were in full bloom. The flowers were collected in plastic bags and kept on ice during transport from the field to the laboratory. They were then stored at 4°C for a maximum of 2 days until observations on the percentage of complete flowers were made.

Pollen

Anthers from freshly collected flowers were transferred with forceps to 2 mL Eppendorf tubes, and left overnight at room temperature in a plastic container containing silica gel. The shed pollen was used for vitality tests.

Fruits

Fifteen fruits were collected from four sides of each of the selected mother trees and stored in separate bags. Ten embryos from the fruits in each bag were used for DNA analysis, the extra fruits being collected to allow for the possibility of parthenocarpic fruits lacking embryos. The fruits were collected in labelled paper bags and then packed into plastic bags. They were kept on ice while transporting them from the field to the laboratory and then stored at 4°C until they were used for DNA extraction.

Methods

Bloom time

The trees were observed at regular intervals of 3-4 days from the commencement of flowering. Start of bloom was regarded as the time when approximately 10% of the flowers were in bloom, full bloom when approximately 80% of the flowers were in bloom, and end of bloom when about 80% of the flowers were spent and the petals had fallen off. The dates for start of bloom, full bloom, and end of bloom were recorded.

Determination of percentage of complete flowers

Five inflorescences were collected, one from each side of the tree facing north, south, east, and west, and one from the centre of the tree, and used to study the percentage of complete flowers. Olives have two types of flowers: complete flowers with well-developed stamens and pistils, and staminate flowers where pistils are rudimentary or absent. All flowers from each of the five inflorescences were observed and the number of complete and staminate flowers was recorded.

Pollen vitality tests

Anthers from flowers of five inflorescences of each plant were used for pollen vitality tests. A small amount of pollen was transferred with a pair of forceps onto a slide and a drop of the vital stain fluorescein diacetate (2 mg/mL in acetone) was added (Pinney and Polito 1990). A cover glass was placed on the pollen and the suspension was observed under a Zeiss photomicroscope (Axiophot) at 520nm using an exciter filter of 395 - 440 nm. Pollen grains with active enzymes were observed to fluoresce whereas non-vital ones did not. The numbers of stained and non-stained pollen grains were counted in five fields of observation.

Weather data

The weather data were obtained from the Australian Bureau of Meteorology. The nearest weather station to the Gumeracha olive grove was the Mt. Crawford station. Mt. Crawford is located in the Adelaide Hills at an altitude of 525 m above sea level, latitude of 34° 43’ 31” S, longitude 138° 55’ 40” E and is 11.5 km northeast of Gumeracha. It was assumed that the weather at Gumeracha was similar to that at Mt. Crawford. The data for the NOVA site was collected from the Roseworthy

Agricultural College station at an altitude of 65m above sea level, latitude of 34° 30’ 38” S, longitude 138° 40’ 35” E which is 2 km south of the NOVA site.

DNA extraction from leaves

The method developed by Mekuria *et al.* (1999) was used to extract DNA from the leaves of the selected mother trees. About 2 g of leaves were ground in liquid nitrogen to a fine powder using a chilled mortar and pestle. This leaf tissue was added to 7.5 mL of cold extraction buffer (3% w/v cetyltrimethyl ammonium bromide (CTAB) (SIGMA), 2 M NaCl, 0.02 M EDTA, pH 8.0, and 1 M Trizma base) (Steenkamp *et al.* 1994) and 15 mg of PVP-40T (SIGMA) and 15 µL of 2-mercaptoethanol (Merck, Damstadt, Germany) were added to the extraction buffer just before use. The mixture was thoroughly shaken and placed on ice. The samples were incubated at 60°C for 30 min with inversion every 10 min and then cooled on ice. After addition of 7.5 mL of chloroform-isoamyl alcohol (24:1), the tubes were spun on a rotary wheel for 10 min at approximately 60 rpm followed by centrifugation in a swing-out head centrifuge at 4,000 rpm. The upper aqueous layer was transferred to a clean tube and two-thirds volume of cold isopropanol added. The tubes were covered with Parafilm® and inverted gently. The DNA was spooled onto a glass rod and left in 20 mL of wash buffer (76% (v/v) ethanol and 10 mM ammonium acetate) until it was white. Then the DNA was dissolved in 1mL of TE buffer (10 mM Tris HCl, 0.1 mM EDTA, pH 8.0). RNA was removed by the addition of 2 µL of 100 µg/mL DNase-free RNaseA (AMRESCO®, Solon, Ohio, U.S.A.,) and incubation for 30 min at 37°C. The solution was transferred to a sterile tube and 2 mL of TE buffer and 1 mL of 7.5 M ammonium acetate was added and mixed. The tubes were kept on ice for 20 min to allow the proteins to precipitate, and then centrifuged at 10,000 rpm for 20 min at 4°C. The supernatant was transferred to another tube and 2 volumes of cold ethanol were added. The tubes were again kept on ice for 20 min for the DNA to precipitate, and then centrifuged at 8,000 rpm for 10 min at 4°C. The supernatant was removed and the excess ethanol was allowed to evaporate before the DNA was dissolved in 1 mL of TE buffer and stored at –20°C till required.

The DNA quality was checked by observing the absorbance in a UV-160A, UV-visible spectrophotometer (Shimadzu Corporation, Tokyo, Japan). The ratios of the absorbances at 260/280 and 260/230 were calculated. DNA having ratios of 1.8 or above was considered to be of good quality (Ausubel *et al.* 1987) and were used for further analysis. The DNA concentration was calculated from the absorbance at 260 nm, considering that 1 O.D. corresponds to 50 µg/ml of double-stranded DNA.

DNA extraction from fruits

Since DNA was to be extracted from a large number of embryos, a quicker method of DNA extraction than the one used for leaves was required. A DNA extraction method modified from the protocol obtained from the web page of Tree Genetic Engineering Research Cooperative (www.fsl.orst.edu/tgerc/dnaext.htm) was used to extract DNA from embryos. The flesh was sliced from the olive fruits and the stone cracked open with a vice. The embryo was separated from the endosperm using a pair of forceps and immersed in 500 µL of grinding buffer (100 mM Tris, pH 8.0, 20 mM EDTA, pH 8.0, with 4 mg/mL diethyl dithiocarbamic acid sodium salt and 100 µg/mL DNase free RNase (AMRESCO®, Solon, Ohio, U.S.A.) added just before use). The embryo was ground in the buffer using a micropestle (Eppendorf) attached to a drill and kept on ice until all the samples were ready. Then the samples were incubated for 10 min at 65°C followed by the addition of 500 µL of lysis buffer (100 mM Tris, pH 8.0, 20 mM EDTA, pH 8.0, with 1 M NaCl, 2% SDS, and 1% sodium metabisulphite added just before use) and incubated for another 30 min at 65°C. The tubes were inverted every 10 min during this time to mix the contents. The tubes were cooled on ice and then 1 mL of phenol:chloroform:isoamyl alcohol (25:24:1) was added. The tubes were spun on a rotary wheel for 10 min at approximately 60 rpm, centrifuged for 10 min at 14,000 rpm and the supernatant removed to a fresh 1.5 mL tube. 500 µL of isopropanol was added to each tube and kept on ice for 15 min. The tubes were centrifuged for 5 min at 14,000 rpm and the supernatant removed.

The pellets were washed with 1 mL wash buffer (76% ethanol and 10 mM ammonium acetate) on a rotary wheel for 10 min at approximately 60 rpm**.** The tubes were spun at 13,500 rpm and the supernatant was then decanted and the DNA pellet dried under reduced pressure in a Labconco Centrivac Concentrator at 45°C for 10-15 min. The pellet was suspended in 50 µL of TE buffer (10 mM Tris HCl, 0.1 mM EDTA, pH 8.0). The DNA quality and concentration were determined as described in Section 3.2.5.

PCR

DNA amplification was performed in a volume of 25 µL containing 60 ng genomic DNA, 1.5 mM $MgCl_2$, 1 x PCR Buffer (200 mM Tris-HCl (pH 8.4), 500 mM KCl), 0.2 µM of each dNTP, 0.4 mM of forward and reverse primers, and 1.25 units of *Taq* DNA Polymerase (Invitrogen). A negative control was also added which contained the reagents of the PCR mix but did not contain the DNA.

A Programmable Thermal Controller (M.J. Research Inc. USA) was used for amplification of the DNA. The DNA amplification procedure consisted of the following steps: initial denaturation at 95°C for 5 min, 34 cycles of 45 s at 95°C, 45 s at the appropriate temperature for the primer, 45 s at 72°C, and finally an extension step at 72°C for 45 min.

Primers and their annealing temperatures were selected from published articles: SSR3, SSR4, and SSR14 (Sefc *et al.* 2000) UDO6, UDO8, UDO24, UDO31 (Cipriani *et al.* 2002) EMO 2 (de la Rosa *et al.* 2002) were used to type the parents and progeny from the Gumeracha grove. The primer UDO31 was replaced by SSR9 (Sefc *et al.* 2000) for the NOVA population as the rate of PCR failure with UDO31 was high. The forward primers were labelled with fluorescent phosphoramidites (FAM, NED, or HEX), which are detected at wavelengths of 518 nm, 580 nm and 556 nm to enable scoring after passing through the ABI Prism 3700 DNA Analyser. An internal size standard (ROX), which is detected at the wavelength of 605 nm was included in each run.

ABI Prism 3700 DNA Analyser

For the Gumeracha data the PCR products were analysed using a 96-capillary ABI Prism 3700 DNA Analyser (Applied Biosystems). FAM and HEX labelled primers were obtained from GeneWorks Pty. Ltd., and the NED labelled primers were obtained from Applied Biosystems. The data were analysed using the program Genescan version 3.5.1 (Applied Biosystems) and the alleles for each locus were scored by comparing with the bands obtained with ROX which gave peaks at known intervals of molecular weight. All PCR amplifications and capillary electrophoresis separation were done in duplicate. A third replicate was done only if consistent results were not obtained with the first two experiments.

The NOVA data were analysed on an ABI 3770 DNA Capilliary sequencer using Genotyper software (Applied Biosystems). The PCR and data analysis were done by the Australian Genome Research Facility (AGRF) at the Waite Campus, SA. PCR results that were ambiguous were repeated.

Data analysis

Identification of parental genotypes

The presence or absence of alleles was recorded in a Microsoft Excel spreadsheet. The Numerical Taxonomy Systems Software Package, NTSYS-pc version 2.02k (Rohlf, 1993) was used to compare the fingerprints of the potential parents by preparing the data in the form of dendrograms. A similarity matrix was prepared using SIMQUAL (Similarity of Qualitative Data) and a dendrogram was constructed using the UPGMA (Unweighted Pair Group Mathematical Average) and SAHN (Sequential, Agglomerative, Hierarchial, and Nested Clustering Methods) algorithm (Rohlf, 1993).

Paternity Analysis

Paternity analysis (Gerber *et al.* 2003) was generated using the software program FAMOZ (www.pierroton.inra.fr/genetics/labo/Software/Famoz/index.html). The statistical analysis used in this program is based on the most likely method described by Meagher and Thompson (1986). A simulation was done using 10000 offspring to obtain the threshold for paternity in order to minimize errors due to gene flow from inside and outside the stand. Distributions of LOD scores from the simulated population were plotted against the offspring generated from the genotyped parents. The crossover point between the two graphs was taken as the threshold as described by Chaix *et al.* (2003). The genotype with the highest log of the odds ratio (LOD) score was considered as the most likely father. The LOD score represents the likelihood of a particular genotype being the father compared to all other genotypes. Famoz settings error was set at 1% which is the error likely to be generated with microsatellite markers (Oreilly *et al.* 1998).

Estimating the probability of null alleles present in the data set.

When only one allele was present it could not be determined whether a homozygote or heterozygote with a null allele was measured, so the second allele was scored as missing data to avoid incorrect exclusion of potential fathers. This rule was used for embryo data from the Gumeracha property.

For data from the NOVA site we estimated the probability of null alleles according to (Khadari *et al.* 2003) and three of the primers (SSR3, SSR9 and SSR14) were found to be unlikely to have null alleles at the 1% level. In these cases where only one allele was detected we assumed homozygosity, and using this method more pollen donors were identified above the threshold level.

Identification of genotypes in the orchards

Genetic similarities among all pairs of individuals were estimated using the simple matching coefficient. Cluster analysis was performed on the estimated similarities using the unweighted pair group method with arithmetic average (UPGMA) and the SAHN algorithm, and the resulting clusters were expressed as a dendrogram using NTSYS-pc (Exeter Software v.1.8) (Rohlf, 1993).

Results

Identification of different genotypes in the olive orchards using microsatellite polymorphisms

1. Gumeracha

DNA that was extracted from the leaves of the parent trees was compared to DNA from standard cultivars from the DNA database using the DNA fingerprinting technique described in the methods section. Table 1 shows the list of standards used for the cultivars in the grove. The data obtained from the DNA analyses of the selected trees showed that the fingerprints of most of the mother trees tested matched their corresponding standard DNA samples. However, a few trees that did not match the standards were found. The cultivar referred to as Manaiki by the grower did not match with the standard Manaiki in the database and was referred to as atypical Manaiki (a-Mk). The Pendolino plants in the orchard were sourced from two different nurseries. The Pendolino obtained from one nursery matched the standard Pendolino, but that sourced from the other did not and was referred to as atypical Pendolino (a-Pen). One Frantoio plant that was morphologically different to the other Frantoio trees showed a different fingerprint compared to the standard Frantoio DNA and was referred to as atypical Frantoio (a-Fra). Some trees that were identified by the grower had a different appearance from the other Kalamata trees. DNA fingerprinting confirmed that these trees were a different genotype and they were named atypical Kalamata (a-Kal).

A dendrogram showing the relationship of the tested trees with the standards is shown in Figure 3. It can be seen on the dendrogram that all the cultivars matched the standards except for the atypical genotypes mentioned above. Seventeen different genotypes were identified in the grove using the DNA marker set.

Table 1. Olive cultivars used for paternity analysis study at Milano Olives, Gumeracha, South Australia, and the origins of the standards used for the comparison of DNA fingerprints.

Cultivar	Source of the standard
Barnea	The Volcani Centre, Bet-Dagan, Israel
Frantoio	Consiglio Nationale delle Ricerche, Instituto di Ricerca Sulla Olivicultura, Perugia, Italy.
Kalamata	Consiglio Nationale delle Ricerche, Instituto di Ricerca Sulla Olivicultura, Perugia, Italy.
Katsourella	NOVA Collection, Roseworthy Campus, University of Adelaide, South Australia, Australia.
Koroneiki	Subtropical Plants and Olive Trees, Institute of Chania Agrokipio, Chania, Greece.
King Kalamata	NOVA Collection, Roseworthy Campus, University of Adelaide, South Australia, Australia.
Leccino	The Volcani Centre, Bet-Dagan, Israel
Manzanillo	The Olive World Collection, Centro de Investigacion y Desarollo Agrario, Cordoba, Spain.
Manaiki	NOVA Collection, Roseworthy Campus, University of Adelaide, South Australia, Australia.
Mission	Foundation Plant Material Service, University of California, Davis, California, United States.
Pendolino	Consiglio Nationale delle Ricerche, Instituto di Ricerca Sulla Olivicultura, Perugia, Italy.
Sevillano	The Olive World Collection, Centro de Investigacion y Desarollo Agrario, Cordoba, Spain.
UC13A6	NOVA Collection, Roseworthy Campus, University of Adelaide, South Australia, Australia.
Verdale	Foundation Plant Material Service, University of California, Davis, California, United States.

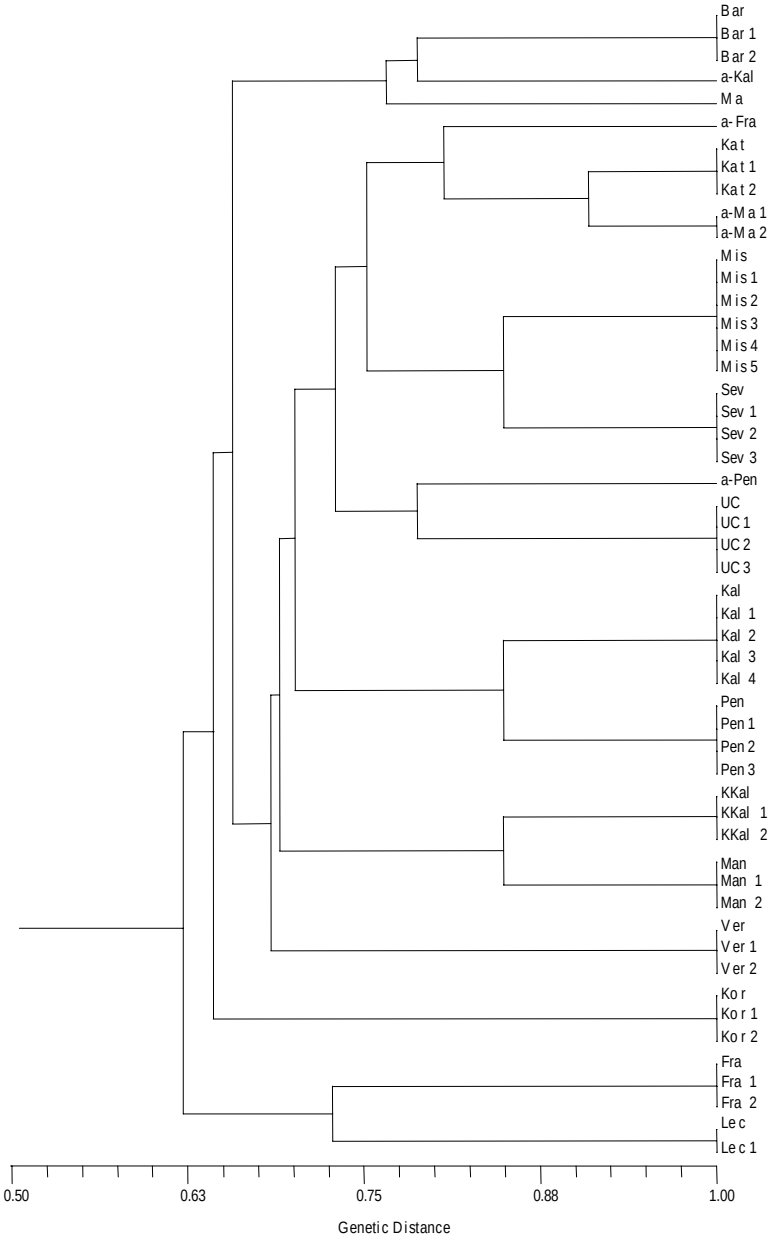


Figure 3. Dendrogram showing comparison of the cultivars present at Milano Olives, Gumeracha, SA with the standard cultivars (Table 1).
Bar: standard Barnea; Bar1 and Bar2: Barnea trees; Fra: standard Frantoio; Fra1, Fra2: Frantoio trees; a-Fra: atypical Frantoio; Kal: standard Kalamata; Kal1, Kal2, Kal3, Kal4: Kalamata trees; a-Kal: atypical Kalamata; Kat: standard Katsourella; Kat1 and Kat2: Katsourella trees; Kor: standard Koroneiki; Kor1, Kor2: Koroneiki trees; KKal: standard King Kalamata; KKal1, KKal2: King Kalamata trees; Lec: standard Leccino; Lec1: Leccino tree; Man: standard Manzanillo; Man1, Man2: Manzanillo trees; Ma: standard Manaiki; a-Ma1 and a-Ma2: atypical Manaiki trees; Mis: standard Mission; Mis1, Mis2, Mis3, Mis4, Mis5: Mission trees; Pen: standard Pendolino; Pen1, Pen2, Pen3: Pendolino trees; a-Pen: atypical Pendolino; Sev: standard Sevillano; Sev1, Sev2, Sev3: Sevillano trees; UC: standard UC13A6; UC1, UC2, UC3: UC13A6 trees; Ver: standard Verdale; Ver1, Ver2: Verdale trees.

2. NOVA

Results from DNA fingerprinting the trees in the NOVA collection are shown as a dendrogram in Figure 4. The results are very similar to those previously obtained with RAPD markers (Guerin *et al.* 2002) and several differently named cultivars are shown to have the same genotype. Only one difference was detected with the microsatellite markers; the Mission accession #10 was slightly separated from the Frantoio group. A total of 54 individual genotypes were distinguished with the set of primers used. The genotype numbers of the cultivars planted at NOVA are shown in Table 2. Each genotype was considered as a potential pollen donor in the paternity analysis.

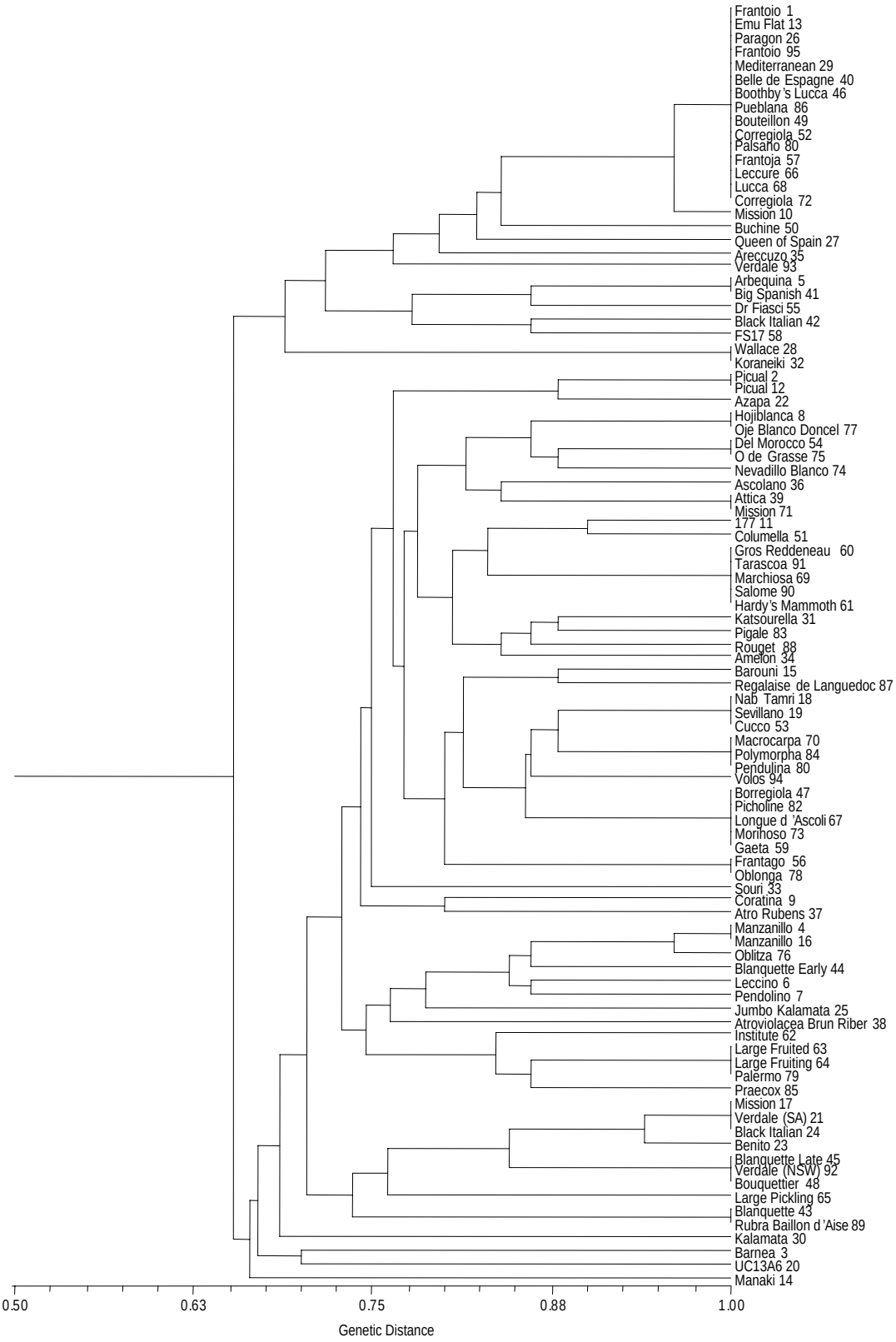


Figure 4. Dendrogram derived from the data obtained with 8 microsatellite markers for each of the cultivars planted at the NOVA site, NOVA accession numbers are shown in parenthesis. Groups of identical genotypes have a genetic similarity of 1.

Table 2. Genotypes in the NOVA collection. The table shows the given genotype numbers and the cultivar names we have used for that genotype number in this report. The NOVA numbers of the cultivars that have the same genotype and the names of the synonymous cultivars in the collection are also shown.

Genotype #	Cultivar Name	NOVA #'s	Synonyms from NOVA
1	Frantoio	1, 13, 26, 29, 40, 46, 49, 52, 57, 66, 68, 72, 80, 86, 95	Corregiola, Paragon, Emu Flat, Mediterranean, Belle de Espagne, Boothby's Lucca, Pueblana, Bouteillon, Palsano, Frantoja, Leccure, Lucca.
2	Picual	2, 12, 96	
3	Barnea	3, 97	
4	Manzanillo	4, 16, 98	
5	Leccino	6	
6	Pendolino	7	
7	Hojiblanca	8, 100	
8	Coratina	9	
9	Mission (WA)	10	
10	177	11	
11	Manaiki	14	
12	Barouni	15	
13	Sevillano	18, 19, 53.	Nab Tamri, Cucco.
14	UC13A6	20	
15	Verdale (SA)	17, 21, 24	Mission, Black Italian.
16	Azapa	22	
17	Benito	23	
18	Jumbo Kalamata	25	
19	Queen of Spain	27	
20	Kalamata	30	
21	Katsourela	31	
22	Koraneiki	28, 32	Wallace
23	Souri	33	
24	Amelon	34	
25	Areccuzo	35	
26	Ascolano	36	
27	Atro Rubens	37	
28	Atroviolacea Brun R	38	
29	Arbequina	5, 41, 99	Big Spanish
30	Black Italian (BEO)	42	
31	Blanquette	43, 89	Rubra Baillon d'Aise
32	Blanquette Early	44	
33	Bouquettier	45, 48, 92	Verdale (NSW), Blanquette Late
34	Buchine	50	
35	Columella	51	
36	Del Morocco	54, 75	O' de Grasse
37	Dr Fiasci	55	
38	FS17	58	
39	Hardy's Mammoth	60, 61, 69, 90, 91	Gros Reddeneau, Marchiosa, Salome, Tarascoa.
40	Institute	62	
41	Large Pickling	65	
42	Mission	39, 71	Attica
43	Nevadillo Blanco	74	
44	Oblitza	76	
45	Oblonga	56, 78	Frantago
46	Palermo	63, 64, 79	Large Fruited, Large Fruiting
47	Pendulina	81	
48	Picholine	47, 59, 67, 73, 82	Borregiola, Gaeta, Morihoso, Longue d'Ascoli.
49	Pigale	83	
50	Praecox	85	
51	Regalise de Langued	87	
52	Rouget	88	
53	Verdale (BEO)	93	
54	Volos	94	

A note about Frantoio and Verdale genotypes.

Many differently named cultivars had the same genetic fingerprint as Frantoio with the markers used in this study as well as the RAPD markers used in a previous study analysing the genotypes planted in the NOVA site. The cultivar planted at the Gumeracha grove which was named Corregiola had the same fingerprint as the Frantoio standard from Italy. We have referred to it as Frantoio for the purposes of this report.

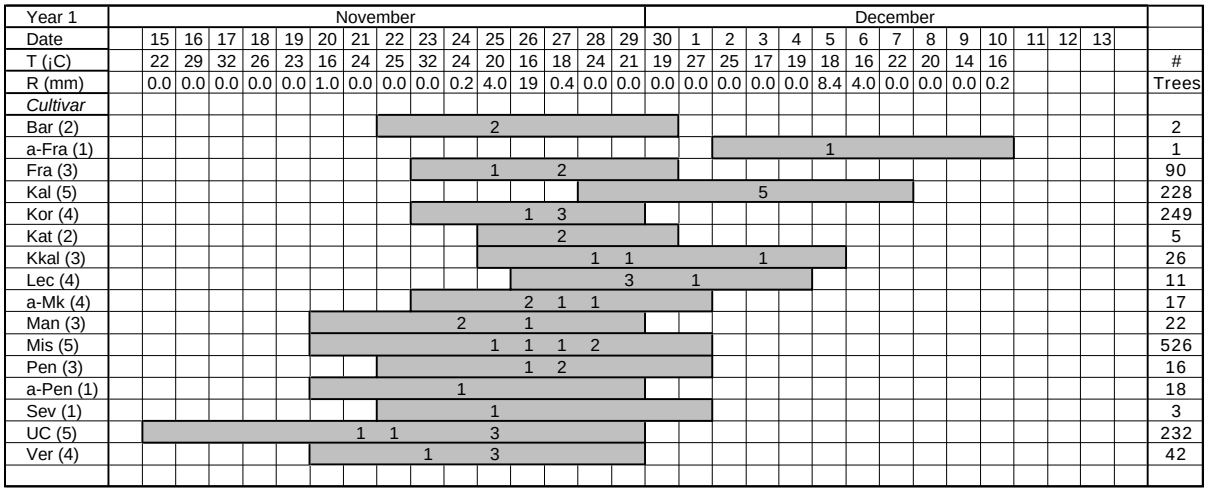
In contrast three cultivars named Verdale were planted at the NOVA site and all had different genotypes, known as homonyms. Verdale with the NOVA number 21 is commonly known as SA Verdale and had the same genotype as the Verdale at Gumeracha and the Verdale sample obtained from The University of California. We have referred to it as Verdale for the purposes of this report.

Floral characteristics

Events at bloom time contribute significantly towards fruit yield in olive. Studies by Cuevas and Polito (1997), Fernandez-Escobar and Gomez-Valledor (1985) and Wu *et al.* (2002) showed that some cultivars may be self-incompatible and others cross-incompatible, and concluded that olive orchards should contain several cultivars in order to maximise the possibility that all cultivars will receive compatible pollen. This raises the question of identifying cultivars that are not only cross-compatible, but also have overlapping bloom times, to ensure adequate pollen transfer at the time the stigmas are receptive. The ultimate fruit yield in any given season depends to a large extent on the processes that occur during the flowering period. We measured bloom time and pollen vitality for the cultivars grown at the two sites as well as sex ratio for the mother trees and some other commonly grown cultivars, over the two years of study.

Bloom time and pollen viability

As cross-pollination is most likely to occur when compatible cultivars flower simultaneously, the bloom periods for each genotype were recorded in the two years of the study (Figures 5-7). The dates for the beginning of flowering, full bloom and the end of flowering are shown in Figure 5 for the Gumeracha property and Figures 6 and 7 for the NOVA site. The maximum temperature and rainfall are also shown on the graphs.



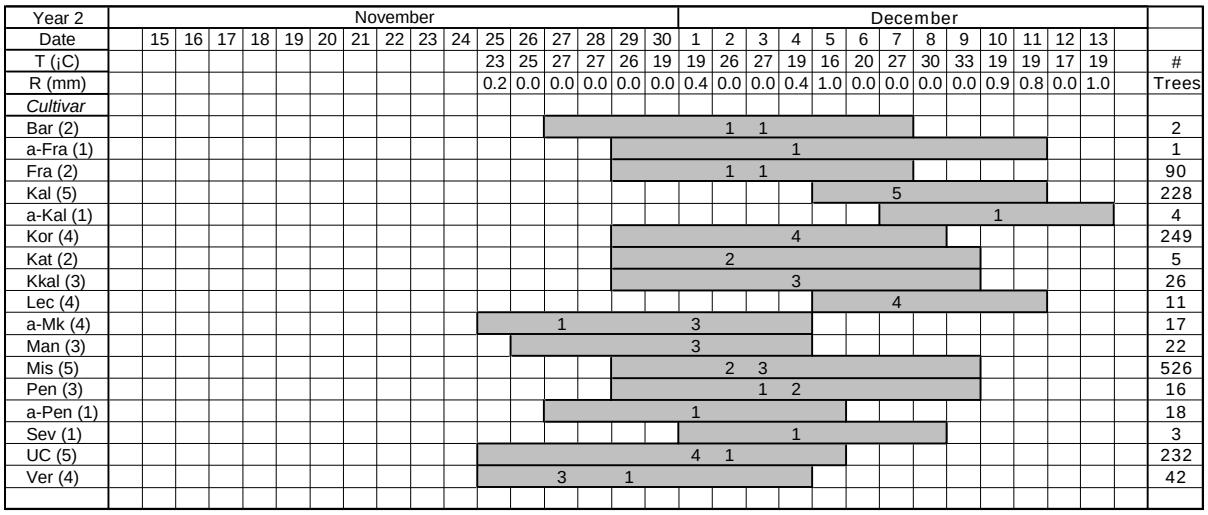


Figure 5. Bloom Time in Olive Cultivars at Gumeracha, during the two years of study. The bars indicate the time from start (10% of flowers open) until the end (80% spent) of flowering. T: Temperature (°C) and R: Rainfall (mm) for each day are shown. Cultivar names are abbreviated as follows: Bar, Barnea; a-Fra, atypical Frantoio; Fra, Frantoio; a-Kal, atypical Kalamata; Kal, Kalamata; Kor, Koroneiki; Kat, Katsourella; KKal, King Kalamata; Lec, Leccino; a-Mk, atypical Manaiki; Man, Manzanillo; Mis, Mission; Pen, Pendolino; a-Pen, atypical Pendolino; Sev, Sevillano; UC, UC13A6; Ver, Verdale trees. The number of trees tested from each cultivar are shown in parentheses, and of those, the number of trees reaching full bloom on each day are shown on the graph. Total number of trees for each cultivar in the grove are shown in the final column.

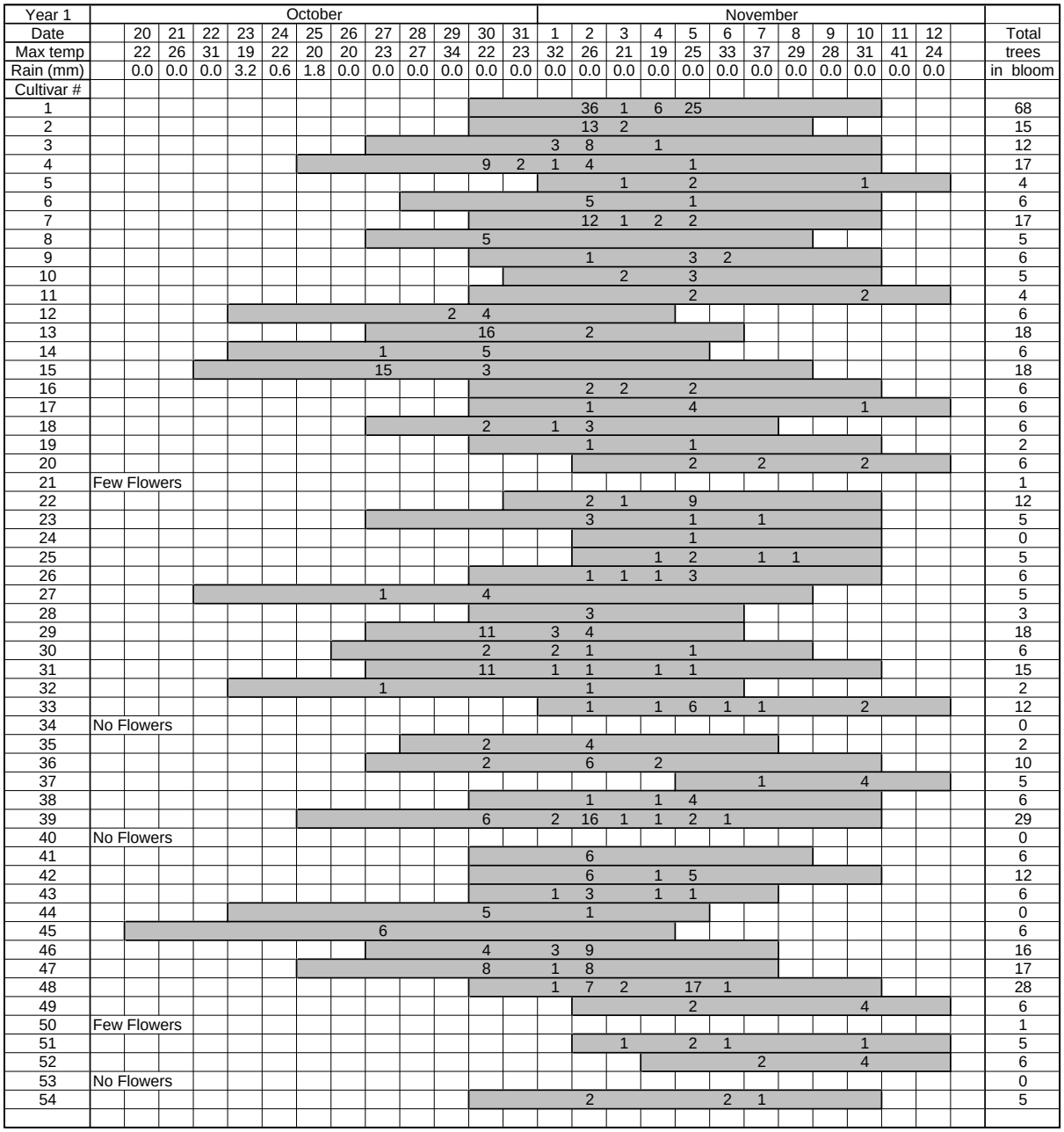


Figure 6. Bloom Time in Olive Cultivars at the NOVA site in year 1. The bars indicate the time from start (10% of flowers open) until the end (80% spent) of flowering. T: Temperature (°C) and R: Rainfall (mm) for each day are shown. The genotype number for each cultivar is shown in the first column. All of the trees at the site were tested and the number of trees reaching full bloom on each day are shown on the graph. Total number of trees for each cultivar that bloomed in the grove are shown in the final column.

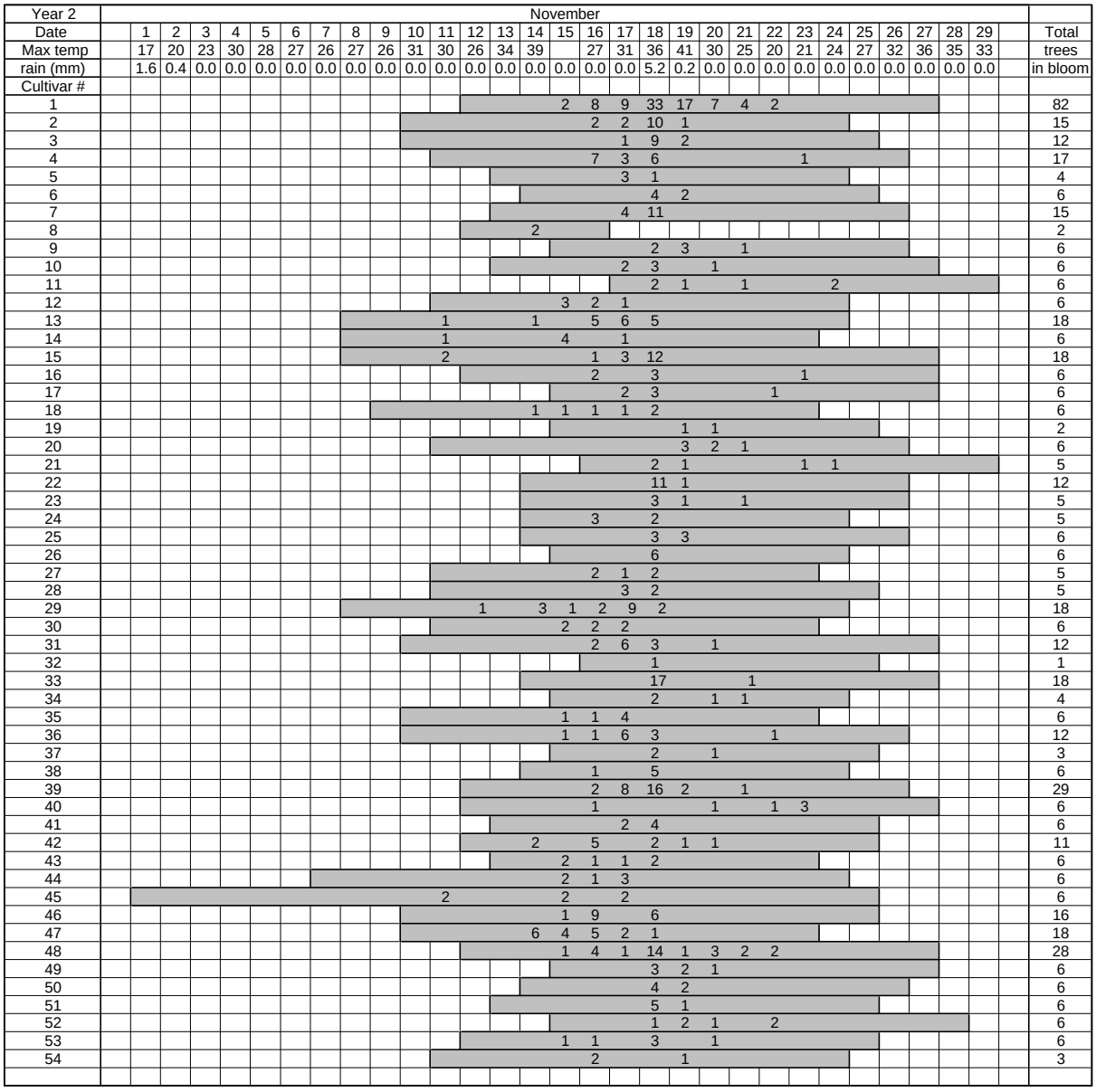


Figure 7. Bloom Time in Olive Cultivars at the NOVA site in year 2. The bars indicate the time from start (10% of flowers open) until the end (80% spent) of flowering. T: Temperature (°C) and R: Rainfall (mm) for each day are shown. The genotype number for each cultivar is shown in the first column. All of the trees at the site were tested and the number of trees reaching full bloom on each day are shown on the graph. Total number of trees for each cultivar that bloomed in the grove are shown in the final column.

The cooler climate in the Adelaide Hills had the effect of a delayed bloom time by approximately 24 days in year 1 and 17 days in year 2 at Gumeracha, compared to the NOVA site in the northern plains at Roseworthy. During the first year several trees did not bloom in the NOVA collection as they were still overcoming juvenility. All cultivars flowered in the second year. At Gumeracha, Frantoio, Verdale and atypical Frantoio had fewer flowers than the other cultivars as they had only been planted for one year compared to the other trees that were between 2 and 4.5 years old.

Bloom times of the potential pollen donors overlapped to some extent with the maternal trees during both years of the study. The only exception was in year 1 when atypical Frantoio did not begin to flower until the flowers of Barnea, Frantoio, Koroneiki, and Mission were already predominantly spent. The atypical Kalamata trees were not identified until the second year and, therefore bloom times were not recorded for these trees in the first year of study.

Pollen vitality was measured to confirm that the trees produced viable pollen for fertilization. At Gumeracha, percent pollen vitality ranged from 24% in King Kalamata to 72% in Koroneiki in the first year and from 20% in UC13A6 to 66% in Leccino in the second, as shown in Figure 8. Very little pollen was collected from Verdale and atypical Manaiki in both years, indicating that, at least for the trees sampled, these two cultivars were predominantly male sterile. Pollen vitality varied between years and was generally higher in the first year of study.

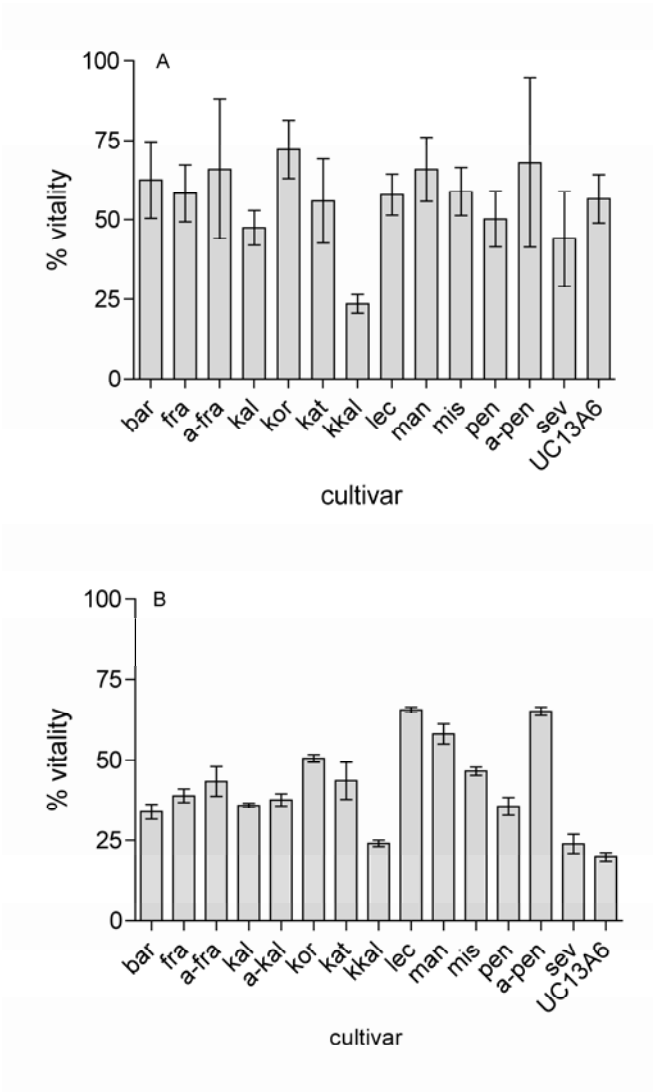


Figure 8. The % vitality of pollen from the cultivars in the grove at Gumeracha tested in A) year1 and B) year 2.

At the NOVA site pollen vitality ranged from 11% for UC13A6 to 71% for Arecuzo in the first year and from 8% for Regalaise de Langedoc to 78% for Mission in the second year, shown in Figure 9.

Verdale was also found to be predominantly male sterile at the NOVA site in both years. No general trend was observed at this site regarding increased pollen vitality in either year.

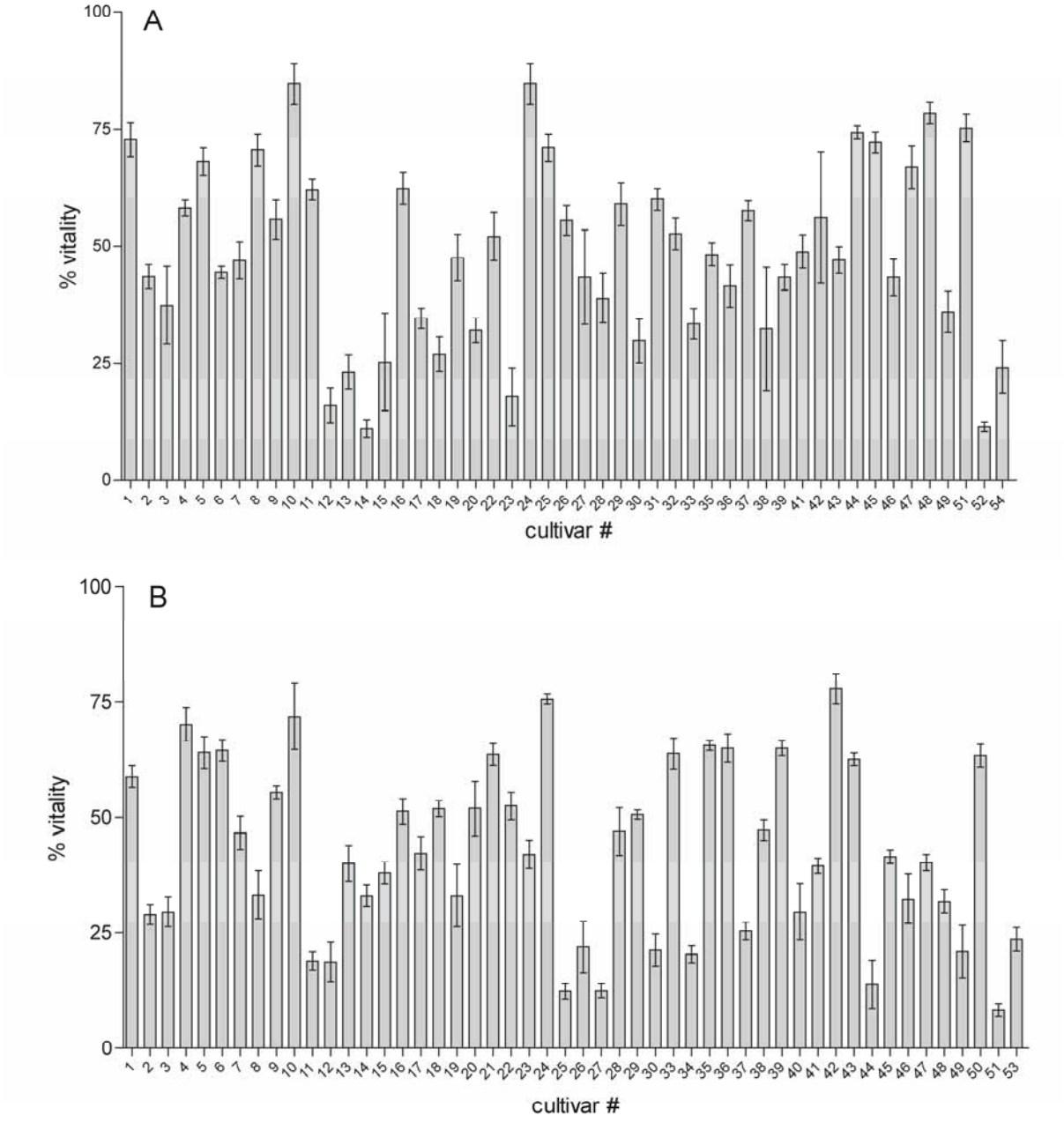


Figure 9. The % vitality of pollen from the genotypes at the NOVA site tested in A) year1 and B) year 2.

Floral structure

The inflorescence of the olive flower is a panicle with flowers distributed along the rachilla, or main axis, in an opposite pattern. Olive flowers can be complete, having both pistil and anthers or staminate, where they lack a functional pistil and cannot set fruit. The number of complete flowers per inflorescence is expressed as % complete flowers and data from both study sites are shown in Figure 10. The mean % complete flowers per panicle at the Gumeracha site ranged from 42 to 98 and differed significantly between years for 9 out of the 16 cultivars tested. At the NOVA site the means ranged from 14 to 100% and there were marked decreases in the number of complete flowers in the cultivars Pendolino, Manzanillo and Mission in the second year. The low levels seen at this site would still be capable of producing a good yield for olives, approximately 2 - 4% fruit set. However if the number of flowers capable of setting fruit were below about 10% it may result in low yields.

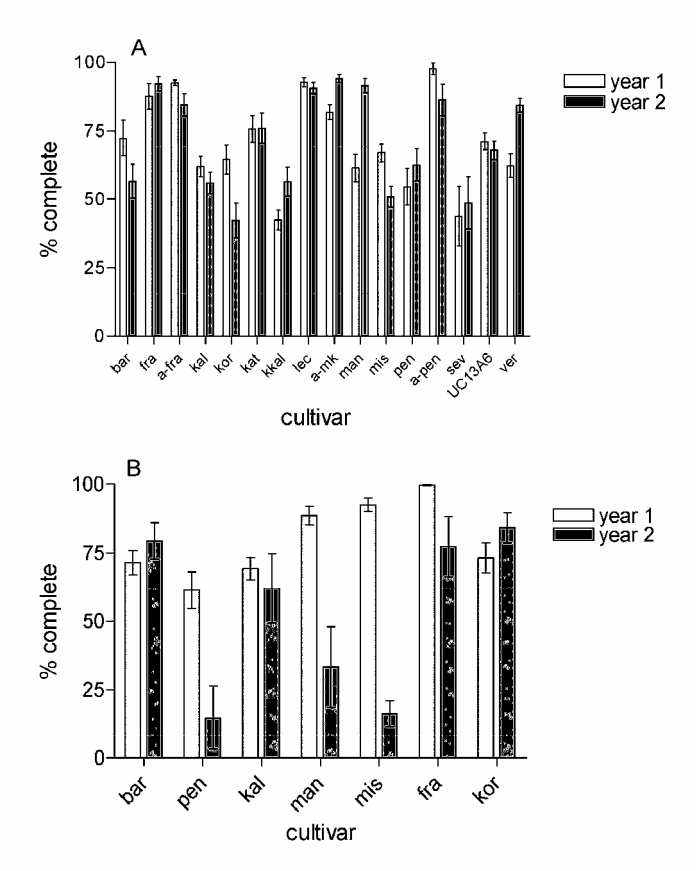


Figure 10. The % complete flowers from five panicles tested over two years from selected cultivars at A) Gumeracha and B) NOVA.

Weather data

Olives are anemophilous, requiring dry conditions and adequate air movement for pollen distribution. The weather during the bloom periods was recorded from the Bureau of Meteorology. The weather station used for the Gumeracha property was at Mt Crawford, which is located at a distance of 11.5 km north-east of the study site. The bloom periods were mostly dry, with rainfall occurring on only a few days. The maximum temperatures during the bloom period of both the years ranged from 15°C to 33°C with an average of 21°C. However, it was between 20°C to 30°C on most days, which is suitable for pollen tube growth (Cuevas *et al.*1994). Maximum temperatures and mm rainfall are shown in Figure 5. Winds were predominantly easterly and westerly in the mornings and mainly easterly in the afternoons, with winds of lower intensities blowing in other directions through out the day.

The NOVA site data were recorded from the weather station at Roseworthy Agricultural College. Only a small amount of rain was recorded during bloom time at this site over both years. Maximum temperatures were warmer on the northern plains and ranged from 17°C to 41°C with an average of 28°C. On several days the maximum reached higher than 30°C in both years which may have effected pollen tube growth (Figures 6 & 7). Wind movement was predominantly north-easterly in the mornings, shifting to south-westerly in the afternoons.

In general, conditions were suitable for effective pollen dispersal in both of the orchards during the flowering periods.

Paternity analysis

Gumeracha

The pollen donors that were assigned to the embryos sampled from the maternal trees are shown in Table 3. The LOD scores for the most-likely fathers ranged from 0.10 to 10.88, with an average of 3.63, and were above the estimated threshold for paternity, which ranged from 0.01 to 0.06, with an average of 0.02. The numbers of embryos assigned to pollen donors ranged from 169 for Koroneiki, represented by 249 trees, to one embryo for atypical Frantoio represented by only one tree in the grove. Paternity could not be assigned to approximately 22% of embryos; these are listed as having an unknown pollen donor. Unknown donors were most commonly found for Mission embryos, with only 57% having been assigned a pollen donor from the cultivars in the grove.

Table 3. Numbers of embryos assigned to each of the likely pollen donors over two years.
Cultivar names were abbreviated as follows: Bar, Barnea; Fra, Frantoio; Kor, Koroneiki; Kal, Kalamata; Mis, Mission; Kat, Katsourella; KK, King Kalamata; Lec, Leccino; Pen, Pendolino; Man, Manzanillo; Sev, Sevillano; UC, UC13A6; Ver, Verdale; a-F, atypical Frantoio; a-K, atypical Kalamata; aMk, atypical Manaiki; a-P, atypical Pendolino; Ukn, unknown.

Pollen donor:	**Bar**	**Fra**	**Kor**	**Kal**	**Mis**	**Kat**	**KKal**	**Lec**	**Pen**	**a-Mk**	**Man**	**Sev**	**UC13A6**	**Ver**	**a-F**	**a-Kal**	**a-Pen**	**Unknown**
Mother Tree																		
Barnea	0	4	0	18	30	3	8	3	29	0	0	15	9	13	0	3	2	23
Frantoio	1	0	0	29	45	1	0	2	6	27	2	6	0	3	0	0	2	36
Koroneiki	3	0	0	12	68	4	1	3	8	8	1	2	18	2	0	2	2	26
Kalamata	0	26	87	0	3	0	2	2	1	8	0	2	3	1	1	0	2	22
Mission	1	6	82	0	2	1	0	0	0	0	0	0	0	0	0	0	0	68
Total	5	36	169	59	148	9	11	10	44	43	3	25	30	19	1	5	8	175

The major difference between the two years of the study was due to the activity of UC13A6, which was estimated to pollinise one embryo in year 1 compared to 29 in the second year. UC13A6 had the third largest number of trees after Mission and Koroneiki, yet its overall contribution to the embryos was relatively low (4%) compared to Mission (19%) and Koroneiki (21%).

Kalamata was a major pollen donor for Barnea in both the years. However, Pendolino and Sevillano were also among the top pollen donors in the first year, while Mission, Verdale, UC13A6, and King Kalamata were more significant pollinisers in the second year. The main pollen donors identified for Frantoio were Mission, Kalamata, and atypical Manaiki. Kalamata was predominantly pollinised by Koroneiki and Frantoio. Mission and Koroneiki had a clearly reciprocal relationship, as each was the major pollen donor for the other. The only incidence of self-pollination detected occurred in two embryos from Mission.

The spatial relationship between the mother tree and the most likely pollen donors, and the frequencies of pollen donation are represented in Figure 11. In some cases it appears that incompatibilities exist between the maternal trees and potential pollen donors. For example, pollen from Koroneiki fertilised many flowers from Kalamata and Mission, but did not contribute to the embryos tested for Barnea or Frantoio. Similarly, Kalamata was assigned as the likely pollen donor for many embryos from Koroneiki, Frantoio, and Barnea, but not for those from Mission. UC13A6 was a significant pollen donor for Koronieki, but made a weak contribution to Kalamata and Barnea, and was not represented among the donors for the embryos from either Frantoio or Mission. The results of this study have been published previously (Mookerjee *et al.* 2005).

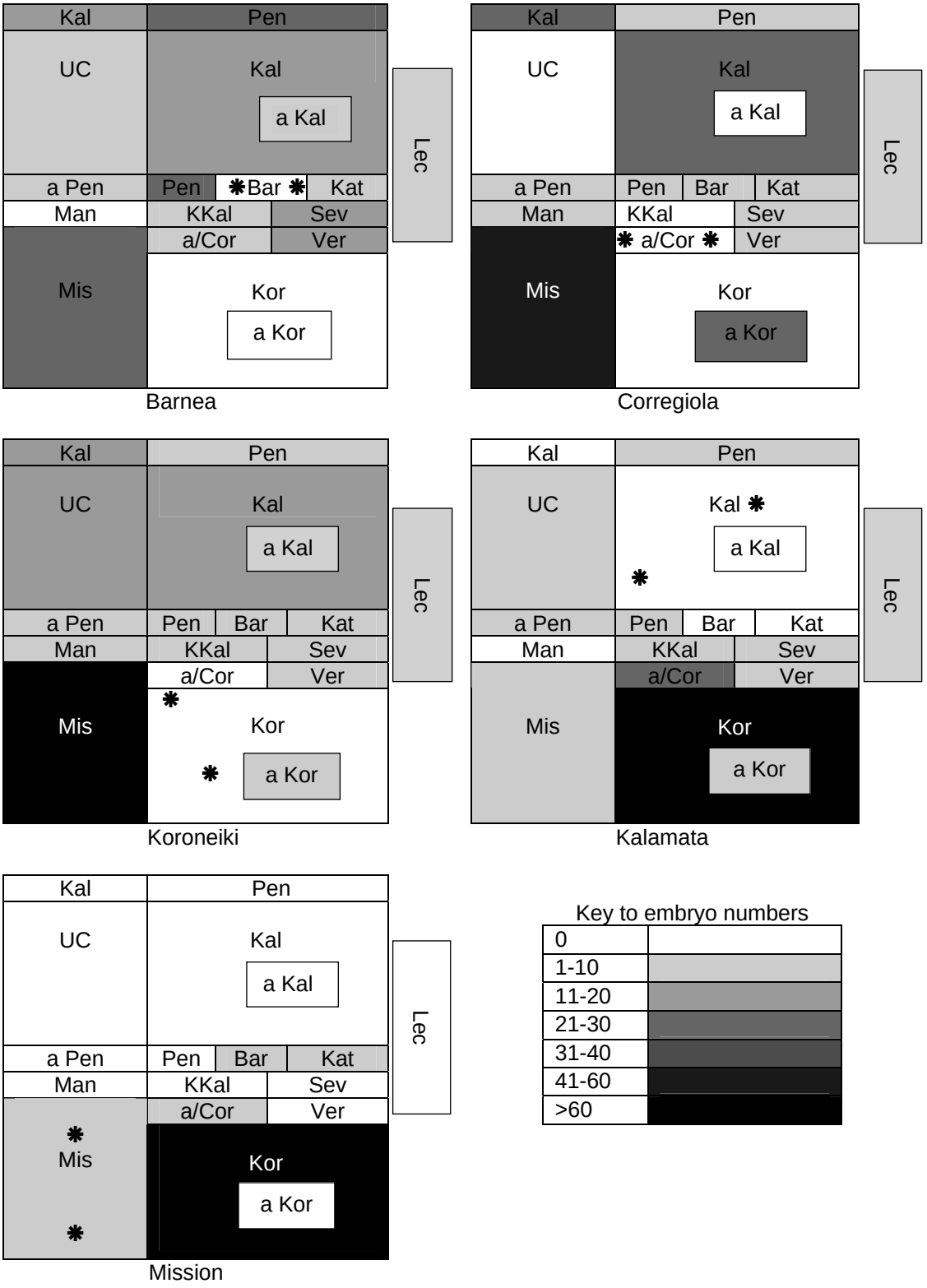


Figure 11. Diagrammatic representation of the olive grove showing the spatial relationship between the mother trees studied (❋) and the most likely pollen donors. Shaded areas indicate the number of embryos fathered by that cultivar ranging from none (white) to >60 (black) as indicated by the key. Cultivar names were abbreviated as follows: a/Fra, atypical Frantoio a Kal, atypical Kalamata; a Man, atypical Manaiki; a Pen, atypical Pendolino; Bar, Barnea; Kal, Kalamata; Kat, Katsourella; KKal, King Kalamata; Kor, Koroneiki; Man, Manzanillo; Mis, Mission; Pen, Pendolino; Sev, Sevillano; UC, UC13A6; Ver, Verdale.

NOVA

Likely pollen donors that were assigned to the embryos sampled from the maternal trees are shown in Table 4. The LOD scores ranged from 1.10 to 6.16, however the threshold was determined at 2.2 and only scores above this were recorded as indicating the true parent (Gerber *et al.* 2000). A total of 586 likely pollen donors were identified out of 920 embryos analysed. The most embryos assigned to pollen donors were from Koroneiki (45), Hardy's Mammoth (25), Manaiki (23), Coratina (21), Mission WA (21) and Kalamata (20). The cultivars with the least number of embryos identified were Bouquettier (0), 177 (1), Verdale (1), and Oblitza (1).

Table 4. Most likely pollen donors identified for the mother trees analysed at the NOVA site. The cultivar name, replicate # and year are shown for the mother trees in the left-hand column. The genotype number of the likely pollen donors are shown in the top row. The number of embryos estimated to be pollinised by each genotype are indicated in the table. The total number of embryos attributed to pollen from each genotype are shown in bold for each mother cultivar and the grand total over the whole study. For some of the embryos the likely pollen donor could not be distinguished between two cultivars and these have been placed under the genotypes 1/9, 2/16 and 4/44.

genotype #	1/9	1	2/16	2	3	4/44	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	total
Frantoio 1 yr 1			4	1		3			1	1	1				2		1						6		1		1	1							1		1				2						2	1	2									32
Frantoio 1 yr 2		2	2	1							1	1		1	2				1	1	1		1				1											1				1			2							1			1						21
Frantoio 2 yr 1		2	2	1		1				1	2	1					1		2	1		1	1				1				1						1				5			1			1	1	1				1				29
Frantoio 2 yr 2		2	1			1			1		2			3		1						1	2			1		1													2			2					2			1						23
Frantoio 3 yr 1	1	3	1	2		1					3	1		2					1	1			3				1								1					1		2			1						1					1		27
Frantoio 3 yr 2	1	1	5	4					1			3		4	1							1			1																1	1														1		26
Total	**2**	**10**	**15**	**9**	**0**	**6**	**0**	**0**	**3**	**2**	**9**	**6**	**0**	**10**	**5**	**1**	**2**	**0**	**4**	**3**	**1**	**3**	**13**	**0**	**2**	**0**	**3**	**4**	**1**	**0**	**0**	**1**	**0**	**0**	**1**	**0**	**2**	**2**	**0**	**1**	**1**	**13**	**0**	**0**	**6**	**0**	**0**	**3**	**2**	**5**	**1**	**0**	**2**	**0**	**2**	**2**	**0**	**158**
Koroneiki 1 yr 1			1			1			2	1	1									3	2	1		1	5		1	1									2					2			1					1	1			1				28
Koroneiki 1 yr 2						2				1		1		1					1	1			1		1				1				1								3		1	1				1	1						1		20
Koroneiki 2 yr 1			1	1						5	1			1					2	1		1	3		1	1	1	2													1			2				2	1				1				28
Koroneiki 2 yr 2		1	3	2		1				2				1						1		1			3			2		1								2			1			2	1					2				1		27
Koroneiki 3 yr 1			5								3				1				2		1				2		1	2	1															2					1						1			22
Koroneiki 3 yr 2			3	2		1					2	1										1			4			2													1			1				1	1					1			21
Total	**0**	**1**	**13**	**5**	**0**	**5**	**0**	**0**	**2**	**9**	**7**	**2**	**0**	**3**	**1**	**0**	**0**	**0**	**5**	**6**	**3**	**4**	**4**	**1**	**16**	**1**	**3**	**9**	**1**	**2**	**0**	**0**	**0**	**1**	**0**	**0**	**2**	**0**	**2**	**0**	**1**	**8**	**0**	**1**	**9**	**1**	**0**	**0**	**4**	**5**	**3**	**0**	**1**	**2**	**1**	**2**	**0**	**146**
Kalamata 1 yr 1								1	1	2	1	2		1					1	1					7															1		2		2	1						3		1		1		29
Kalamata 1 yr 2	3				2							1		2		1					2	1	1	2	2								1				1				1			1						1		1				24
Kalamata 2 yr 1					2			1	1			2													5		1	1					1			1		1							1							1		1		3	23
Kalamata 2 yr 2	4		1		2						1	1		2		1					2	1	1	3	2															1			1		1					1		1				27
Kalamata 3 yr 2	3				2						1	4								2				2			1			1					1					1		1				2		1	1						23
Total	**10**	**0**	**1**	**0**	**8**	**0**	**0**	**2**	**2**	**2**	**3**	**10**	**0**	**5**	**0**	**2**	**0**	**0**	**1**	**3**	**4**	**2**	**2**	**7**	**16**	**0**	**2**	**1**	**0**	**1**	**0**	**0**	**1**	**1**	**3**	**0**	**1**	**3**	**1**	**1**	**2**	**1**	**0**	**4**	**0**	**5**	**1**	**2**	**0**	**1**	**3**	**4**	**2**	**2**	**0**	**1**	**3**	**126**
Mission 1 yr 1					1			1	1			1					3				3						3					3		1	1						1			2			1		1		1					24
Mission 1 yr 2					1	1			1	1				3									1				2				1	4	2	4	1						1			2							1			26
Mission 2 yr 1															1		1							1	4	1				1			2			1	1							2						2			2	21
Mission 2 yr 2			1							2	1	2									1						1	1			2	3		3							1	1		2									1	22
Mission 3 yr 1			1	1						1				2			2	1	1			3			4	1	2	1	3				1	2					1		1				1							1	2	1	34
Mission 3 yr 2					5					1					2							1			3		3											2				2		6			1						2	1				29
Total	**0**	**0**	**2**	**1**	**7**	**1**	**0**	**1**	**2**	**4**	**2**	**3**	**0**	**5**	**3**	**0**	**6**	**1**	**1**	**0**	**4**	**4**	**1**	**1**	**11**	**2**	**11**	**2**	**3**	**1**	**3**	**10**	**3**	**12**	**2**	**0**	**1**	**1**	**3**	**1**	**1**	**3**	**1**	**8**	**4**	**0**	**0**	**9**	**0**	**1**	**0**	**5**	**1**	**1**	**1**	**2**	**4**	**156**
Grand Total	**12**	**11**	**31**	**15**	**15**	**12**	**0**	**3**	**9**	**17**	**21**	**21**	**0**	**23**	**9**	**3**	**8**	**1**	**11**	**12**	**12**	**13**	**20**	**9**	**45**	**3**	**19**	**16**	**5**	**4**	**3**	**11**	**4**	**14**	**6**	**0**	**6**	**6**	**6**	**4**	**4**	**25**	**1**	**13**	**19**	**6**	**1**	**14**	**6**	**12**	**7**	**9**	**6**	**5**	**4**	**7**	**7**	**586**

The total number of Frantoio embryos where likely pollen donors were identified was 158. The predominant likely donors were Kalamata, Hardy's Mammoth, Coratina and Manaiki. There were also some selfed embryos identified in Frantoio. Selfing also occurred in Koroneiki with 16 embryos identified as being selfed progeny. Likely pollen donors were identified for 146 embryos analysed from Koroneiki. Other prominent donors estimated for Koroneiki were Mission, Hardy's Mammoth, Hoji Blanca and Arecuzo. Kalamata had likely pollinisers identified for 126 of the embryos analysed, they were mainly attributed to Koroneiki, Mission WA and Barnea. Frantoio was also a potential pollen donor as pollinisers for 10 embryos were either from Mission WA or Frantoio. Mission had likely pollen donors identified for 156 embryos. The major donors were Blanquette, Koroneiki, Amelon, Arbequina and Oblonga.

The cultivars Frantoio and Mission WA appear to be closely related as their genotypes were not distinguished using RAPD markers and only differed by one microsatellite peak in this anlaysis. They appear close together on the dendrogram, Figure 4, and were difficult to distinguish from each other as pollen donors. In several cases they received the same LOD score so the true parent could not be identified. Similarly the cultivars Picual and Azapa, as well as Manzanillo and Oblitza received the same LOD score for some of the embryos. In these cases scores were added to a separate column where either cultivar could have donated pollen to the embryo: Frantoi/Mission WA (1/9), Picual/Azapa (2/16), and Manzanillo/Oblitza (4/44). In this way these potential parents were not eliminated from the data set.

Flowering survey from different growers properties

Several growers were asked to mark down the flowering times for three olive trees from each of the main cultivars grown on their properties. The property locations and cultivars are listed in Table 5 below.

Table 5. Property locations and cultivars used in the flowering survey

Property Location	**Cultivars**
Keith SA	Frantoio, Barnea, Leccino, Picual, Manzanillo
Willunga SA	Frantoio, Corregiola, Manzanillo, Kalamata, Mission, Koroneiki
Nangkita SA	Frantoio, Barnea, Leccino, Kalamata, Koroneiki, Pendolino, UC13A6
Muswellbrook. NSW	Corregiola, Hardy's Mammouth, Manzanillo
Gunnedah NSW	Barnea, Manzanillo, Hardy's Mammouth, Kalamata, Jumbo Kalamata, Mission

The results from the growers are shown in Figure 12, and clear differences in flowering times were observed between the different environments. However, within each property bloom times for the cultivars overlapped such that it would have been possible for any cultivar to be a pollen donor for the others providing that they were cross compatible. However, full bloom, when the maximum amount of pollen is released, did not always coincide for all of the cultivars.

Frantoio or Corregiola was present in the SA properties and the NSW properties. Flowers from these trees first opened in Muswellbrook approximately one month before the trees at Keith. Trees at Willunga and Nangkita reached full bloom simultaneously about a week later than the Keith property.

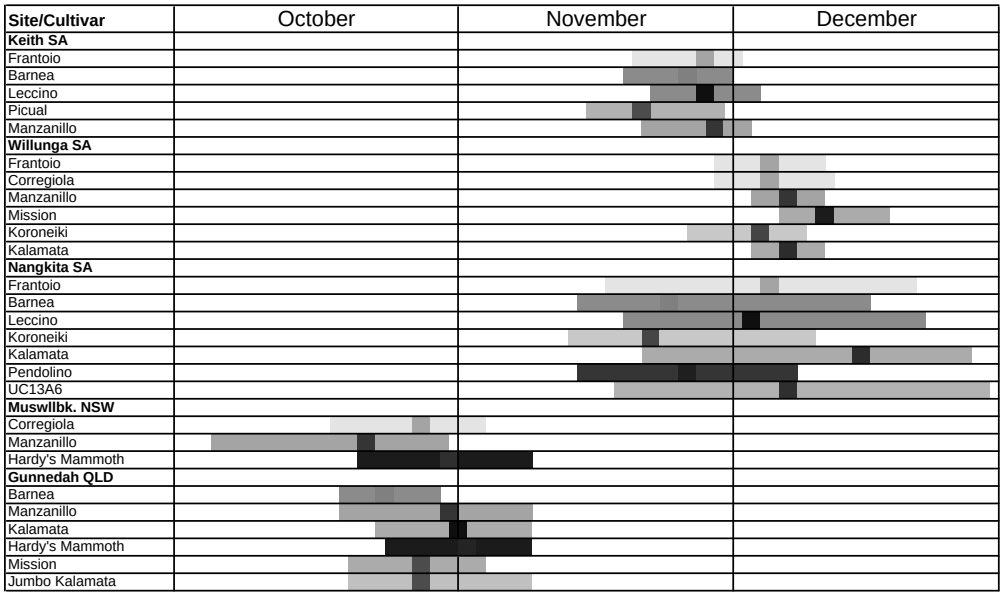


Figure 12. Results from the flowering survey. Data supplied by participating growers for 3 trees of each cultivar. Bands show the bloom time for each cultivar from the start of flowering (10% of flowers open), full bloom (darker coloured squares) to the end of flowering (80% of flowers spent).

Discussion

The results presented in this report demonstrate that microsatellite markers and paternity analysis are effective tools to survey the frequency of pollen donors for selected maternal trees in a mixed orchard. This report documents the first studies using these techniques to monitor pollination patterns in an olive grove. By using paternity analysis to study the distribution of pollen donors within the grove the actual events of fertilization under the conditions of free, open pollination have been observed. The results from this study show that at the time of stigma receptivity in the maternal tree, viable pollen from the donor was present in the air, could travel to the recipient flower and successfully compete with other pollen grains on the stigma to reach the egg cell. The successful pollen donors identified in this report are therefore not only compatible with the maternal tree but have been demonstrated to function effectively under field conditions.

Identification of different genotypes

The identification of the different cultivars in the groves was done primarily to determine the genotypes present that could act as pollen donors. The marker set was adequate for distinguishing all the genotypes in the stands. In the case of the Gumeracha property the DNA fingerprinting found that some a-typical trees had been planted as different cultivars. The identification of atypical genotypes was useful information for the grower, and also ensured that these genotypes could be included as potential pollen donors. Such a situation may often be encountered in an olive orchard since there is a lot of confusion about the genetic identity of cultivars.

The cultivars planted in the NOVA collection had previously been DNA typed using 10 RAPD markers (Guerin *et al.* 2002), and were typed again with microsatellite markers. The two sets of markers gave remarkably similar results, which shows that the RAPD analyses were robust. However, it was necessary to type the collection again with the microsatellites for the paternity study so the parents and embryos could be analysed with the same marker set. The codominant segregation of microsatellites allows for more efficient parentage analyses compared with dominant markers (Gerber *et al.* 2000) such as RAPDs.

Floral characteristics

Bloom time

Recording the bloom times was important to determine that all the potential pollen donors were shedding pollen at the same time as the maternal trees were in flower. However, the results also showed that some of the other cultivars were not synchronized in their bloom times. In the first year at the NOVA site, Dr Fiasci flowered late and did not overlap with the earlier flowering cultivars Barouni, and Oblonga. In year 2 Coratina had a very short flowering time, which finished at the time that Manaiki was just beginning to flower. These results show that not all cultivars will bloom at the same time and certain cultivars may not be suitable as pollinisers for some trees purely on the basis of bloom time synchronicities.

Pollen viability

The lack of pollen grains in the anthers taken from Verdale indicated that the trees tested were male sterile in both years from both sites. However, paternity analysis showed that Verdale contributed pollen to 19 embryos at Gumeracha but to only one embryo from the NOVA samples. Pollen vitality observations were taken on only a sample of flowers collected at full bloom stage. It is possible that some flower clusters that were not sampled had viable pollen, and also that viable pollen developed

later in the season after the trees had been sampled. The high incidence of male sterility in this Verdale genotype means indicates that it would not be recommended as a pollen donor cultivar in a commercial olive grove.

The cultivars UC13A6 also had low pollen vitality in both sites in one of the years and King Kalamata had low levels measured in both years at Gumeracha. These cultivars were not major pollen donors at either of the sites, which may be due to a lack of pollen vitality. However, pollen vitality levels are not the sole determinant for pollen donor success. It is not known at what level pollen vitality becomes a limiting factor for competitive pollinisation.

Paternity analyses

In this study the same method was applied to two different field layouts. In the case of the Gumeracha site the analysis worked well with the smaller number of cultivars in the grove. The NOVA site had a large number of cultivars present so that a wider range of pollen donors could be identified for the mother trees. However, the increase in potential pollen donors presented a greater challenge for the marker system to identify the paternal parent. For 64% of the embryos studied, pollen parents having LOD scores above the threshold were identified after the analyses. For the remaining 36 % of the embryos analysed the LOD scores were below the threshold range and pollen donors could not be identified. The application of more markers to these embryos would help to resolve the pollen parents.

Likely cross-compatibilities found at Gumeracha

Top likely pollen donors identified at the Gumeracha site for the following mother trees were:

- Frantoio: Kalamata, Mission
- Kalamata: Frantoio, Koroneiki.
- Koroneiki: Mission, UC13A6, Kalamata.
- Mission: Koroneiki.
- Barnea: Kalamata, Mission, Pendolino.

An interesting observation at this site was the difference in receptivity to pollen donors for each of the maternal cultivars within the orchard. The dominant pollinisers were not necessarily the closest neighbours, indicating that compatibility, rather than proximity, has a major role in pollen success. This has also been reported in other studies using isozyme markers to identify pollen donors in orchards of cherry and avocado (Brant *et al.* 1999; Sulaiman *et al.* 2004). Barnea was receptive to several pollen donor cultivars, whereas the majority of Mission embryos were pollinised by Koroneiki. Twelve of the seventeen genotypes identified in the orchard did not contribute pollen to the Mission embryos, indicating that Mission may be cross-incompatible with most of the cultivars in the grove.

The two major pollen donors found in the orchard were Koroneiki and Mission. These two cultivars had the largest number of trees in the grove, and would therefore have had a favourable bias on the overall pollen load. Koroneiki appeared to be a strong pollen donor for both Mission and Kalamata. However, the fact that none of the embryos from either Frantoio or Barnea were assigned to Koronieki, even though the mother trees were near the Koroneiki block, indicates possible incompatibility between these cultivars. In contrast, Mission, pollinated at least some embryos from all the cultivars tested. The results confirm the findings of previous reports, that self-incompatibility and cross-incompatibility commonly occur in olive (Griggs *et al.* 1975; Moutier *et al.* 2001; Wu *et al.* 2002; Lavee *et al.* 2002).

Pollen donors for a significant proportion of the embryos tested could not be identified. It is proposed that the mother trees were pollinated either by pollen from unidentified cultivars in the orchard or by

wind-borne pollen from trees outside the orchard. Analysis of the paternal alleles identified in these embryos revealed a similar contribution in both the years and indicated that more than one unknown genotype was involved.

Likely cross-compatibilities found at the NOVA site

Top likely pollen donors identified at the NOVA site for the following mother trees were:

- Frantoio: Kalamata, Coratina, Hardys Mammoth, Manaiki, Picual/Azapa
- Kalamata: Koroneiki, Frantoio/WA Mission, Barnea
- Koroneiki: Mission, Hojiblanca, Hardy's Mammoth, Arecuzo
- Mission: Koroneiki, Arbequina, Amelon, Blanquette, Oblonga

There were notable similarities between the results obtained at both sites. At least one of the major likely pollen donors identified for the mother trees at Gumeracha was also identified as a major likely donor for the trees at the NOVA site. This result indicates that these cultivars may be effective pollen donors in more that one environment. The fact that they are likely pollen donors at both sites also indicates that they are potentially efficient donors for the respective mother trees. In the NOVA site cultivars such as Koroneiki, Kalamata and Mission are not as highly represented in the grove as they are at Gumeracha, which shows that they are likely to be the preferred pollen donors for the mother trees.

Similarly some potential cross-incompatibilities were evident at both sites. Koroneiki only pollinised 2 embryos from Frantoio, whereas it was a significant donor to other mother trees. Conversely, Frantoio only pollinised one embryo from Koroneiki at NOVA and none at Gumeracha indicating reciprocal cross-incompatibility between these two cultivars. Kalamata and Mission also exhibited reciprocal cross-compatibility at both sites.

Selfing

Another point of interest arising from the results is that the olive trees tested at Gumeracha rarely self-fertilised. Only two cases of self-pollination were observed out of the 800 embryos tested, and both of these were from Mission. It has previously been reported that self-pollination in Mission resulted in very low fruit set (Griggs *et al.* 1975). The low incidence of self-fertilization was not unexpected as olives are predominantly allogamous, exhibiting high levels of heterozygosity and DNA polymorphism among individuals (Rallo *et al.* 2000).

In contrast we found an increase in self-fertilised embryos in both Koroneiki and Frantoio at the NOVA site. Mission and Kalamata had a low incidence of selfing with 3% and 2% of embryos identified as selfed, respectively. Frantoio had 7% and Koroneiki, 15% of embryos as being products of selfing. The environment may have affected the self-incompatibility response. Koroneiki has been reported previously to be self-compatible (Lavee *et al.* 2002), and Androulakis and Loupassaki (1990) found Koroneiki to be partially self-fertile. It has been shown that self-incompatibility breaks down due to an increase in temperatures in sweet cherry (Choi and Andersen 2005), lily (Suzuki *et al.* 2001) and perennial ryegrass (Wilkins and Thorogood 1992) and this may also be the case in olives.

Conclusions

The study identified compatible pollen donors for five commercially important olive cultivars growing under natural conditions, thus avoiding the adverse effects of artificial cross-pollination. Bloom time and pollen viability varied between cultivars and years and may have affected the ability of a cultivar to contribute pollen to the pollen load. The results obtained are a valuable source of information for planning new olive orchards for optimum yield. Continued research in this area is needed to offer growers more guidance with orchard design. The identification of the incompatibility mechanism acting in olives would facilitate compatibility studies through the use of molecular markers for incompatibility genes.

Implications

The olive industry will benefit from the results of this project through increased productivity. Many orchards have been established without due regard to the requirements of cross-pollination. These results will be valuable guidelines to anyone planning new plantings. In addition, they can be used to increase productivity in established groves through the addition of highly compatible pollen donors. The effect of introducing compatible pollen donors to a mono-varietal grove could improve yield (fruit set) from less than 1% up to 4%. New trees can be introduced through grafting onto old trees or inter-planting. This research also has environmental benefits as a significant increase in yields could be achieved without expansion of land use, irrigation or addition of fertilisers or chemicals for pest and disease control.

The impact of different pollen donors on oil quality was not specifically addressed in this research project. This impact of pollen donors on characteristics of the fruit is called metaxenia. Metaxenia is only found in a few species and is not common to all fruit and nut crops. Griggs et al (1975) showed that there is no physical effect of the pollen donor on fruit size or shape which indicates that metaxenia does not occur in olives. A comprehensive study would require paternity testing of fruit samples combined with individual fatty acid testing of the oils in the fruit flesh to determine if there is any effect.

Recommendations

Planting recommendations

To compensate for the variability in pollen viability and flowering times between seasons it is suggested that more than one pollen donor cultivar is present in a commercial grove, preferably three or four.

The following combinations of mother trees and pollen donors were found to be compatible under the conditions of this study:

Mother tree	Recommended Pollen Donor
Frantoio	Kalamata, Mission, Coratina
Kalamata	Frantoio, Koroneiki, Barnea
Koroneiki	Mission, Hojiblanca
Mission	Koroneiki, Arbequina
Barnea	Kalamata, Mission

Plantings found to be incompatible under the conditions of this study

Single cultivar blocks are not recommended due to the low incidence of selfing in most olive cultivars.

The following combinations of mother trees and pollen donors were found to be incompatible under the conditions of this study.
Frantoio and Barnea
Kalamata and Mission
Mission and Kalamata
Kalamata and Manzanillo
Koroneiki and Frantoio

Verdale (SA) is not recommended as a pollen donor due to frequent male sterility, observed in this study.

Future research

It is strongly recommended that continued research be conducted in the area of pollen compatibility in olives. Further work is needed to understand the genetic system operating in olives, including the identification of the genes involved. Information on compatibilities between more cultivars will assist in orchard planting designs to maximize fruit yields.

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