Identification of Pollen Donors for Olive Cultivars

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Abstract

The olive industry has emerged as an important industry in Australia with increasing demand for both olive oil and table olives. To meet the domestic demand for olive products, it is necessary to increase production. Studies have shown that only 1-2% of olive flowers mature into fruits (Martin, 1990). Insufficient pollination due to self and cross incompatibility is a major factor affecting fruit set.

The various methods used for studies on compatibility relationships have often shown conflicting results, with the same cultivar being found to be self-compatible in some studies, and self-incompatible in others (Sibbett *et al.*, 1992; Caruso *et al.*, 1993). Also, most of these studies have been conducted in the northern hemisphere where the environmental conditions and combination of cultivars growing nearby are expected to be different from Australia. It is therefore necessary to carry out studies on compatibility relationships under natural conditions in the Australian environment.

The use of molecular markers has been found to be an effective and reliable method for paternity analysis studies. Using polymorphic and codominant markers, fingerprints of embryos may be compared to markers present in the mother plant, and therefore, the paternal contribution of alleles may be identified. By comparing these alleles with the genotype of all the potential pollen donors the pollinating genotype can be identified.

The aim of this project was to identify the most compatible pollen donors for five olive cultivars (Barnea, Corregiola, Koroneiki, Kalamata, and Mission) and to observe the effect of morphological characters (bloom time, percentage pollen vitality, and percentage of complete flowers) and weather conditions (temperature, rainfall, and wind direction) on pollination. The study was conducted in a mixed olive orchard in Gumeracha, South Australia over the 2002-2003 and 2003-2004 growing seasons.

Prior to the study, the genotypes of the trees were compared with the standards in the database (Guerin *et al.*, 2002) and it was found that most of the trees matched with the standard cultivars. However, the trees considered to be Manaki by the grower did not match with the standard Manaki and were therefore referred to as atypical Manaki. Also, some Pendolino, Corregiola, and Kalamata trees did not match with the standard and were also referred to as atypical.

The maximum and minimum temperatures, rainfall, and wind direction were recorded for the bloom period of both the years. The range of maximum temperature minimum temperatures during the bloom period was similar in both years. There was more rainfall in the bloom period during the first year than during the second year. Wind direction data during the bloom period showed that the wind direction was similar in both years. The winds were mainly easterly or westerly in the mornings and mainly westerly in the afternoon. However, there were winds of lower intensities blowing in the other directions as well, thus ensuring adequate wind movement for pollen dissemination.

Dates of the start of bloom, full bloom and end of bloom for each cultivar were recorded for both years. It was observed that most of the cultivars overlapped in their bloom time, although some such as Kalamata flowered late in both years. Bloom time dates for replicate trees of a cultivar were similar, but there were differences in the dates between cultivars.

The percentage of complete flowers was recorded for all cultivars in both years and it was observed that King Kalamata had the lowest value (42.5%) in the first year and Koroneiki had the lowest value (29%) in the second year. Leccino, atypical Manaki, and Corregiola had high percentages of complete flowers in both years.

Percentage pollen vitality observations ranged from 23.5% in King Kalamata to 72.3% in Koroneiki in the first year. In the following year, UC13A6 had the lowest percentage pollen vitality (19.7%) and Leccino had the highest value (65.5%). The flowers sampled from Verdale and atypical Manaki did not contain pollen in both the years.

Paternity analysis showed that: Barnea embryos were mainly fertilised by Pendolino and Mission; Corregiola embryos were mainly fertilised by Mission, Kalamata, and atypical Manaki; Koroneiki embryos were mainly fertilised by Mission; Kalamata embryos were mainly fertilised by Koroneiki; and Mission embryos were mainly fertilised by Koroneiki. There were also unidentified pollen donors pollinating a significant proportion of embryos. No apparent effect of direction of canopy and distance of pollen donors was observed and it was concluded that wind movement was not a limitation for movement of pollen in the orchard. Temperature and rainfall

did not have any apparent effect on the overall bloom period. Pollen vitality, time of flowering, number of trees in the orchard, and tree age may have affected the effectiveness of some cultivars as pollen donors.

The results highlighted the importance of cross-pollination for fruit set. Only two instances of self-pollination were observed suggesting that cross-pollination is more effective than selfing. The results also suggest that there is genetically controlled compatibility relationship operating among the cultivars and this determined which pollen type lead to successful fruit formation. However little is known about the mechanism of incompatibility operating in olives. There were differences in the effectiveness of some pollen donors over the two years which suggests that having more than one compatible pollen donor in the orchard is important.

The results obtained in this study may be used as a basis for studying the mechanism of incompatibility in olives. The compatible pollen donors identified can be used to make recommendations to olive growers regarding the combinations of olive cultivars that will maximise yield and hence boost the production of olives in Australia. The method can also be extended to other cultivars to identify compatible pollen donors and also to compare the effect of different environmental conditions on pollination.

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Chapter One

General Introduction

Olives have been in cultivation for thousands of years. Since their origin in the eastern Mediterranean region, use of olives has now spread to 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 in the lives of Mediterranean people has been well documented by Voyiatzi *et al.* (1999) and Tsalikidis *et al.* (1999). Countries in the Mediterranean region are the major producers and consumers of olive products in the world, although both production and consumption are spreading to some non-Mediterranean countries (Dal Pero Bertini, 1960). For example, the olive plant was introduced into Australia at the beginning of the nineteenth century (Booth and Davies, 1996), and olives now form an important industry in Australia.

Although the soil and climate of South Australia are suitable for olive growing, olives failed to gain economic importance after their introduction in the 1830s (Dal Pero Bertini, 1960). However, the industry gained impetus after the 1939-45 war when large numbers of immigrants of Mediterranean origin entered Australia (Dal Pero Bertini, 1960), and since then there has been increasing interest to invigorate the industry in order to meet the demand for olive products.

The viability of the olive industry depends on economic yield of the fruit, which can be used for both oil production and consumption as table olives. Even though olive

trees flower profusely, only a few of these flowers set fruit in a growing season (Marco *et al*., 1990; Martin, 1990; Ghrisi *et al*., 1999; Cuevas *et al*., 2001) 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 may be self-incompatible, which means that the flowers cannot be successfully fertilised by pollen from the same cultivar, and some cultivars are cross-incompatible, whereby the flowers cannot be fertilised by pollen from certain other cultivars. Therefore it is important for growers to know which cultivars are cross-compatible when planning an olive orchard in order to maximise fruit set and hence achieve good yields. This is especially important when orchards are planted in isolated areas where the only sources of pollen are within the orchard.

In the past, the main problem has been to identify cross-compatible cultivars. Compatibility studies on olive cultivars have often given variable results (Antognozzi and Standardi, 1978; Singh and Kar, 1980; Bini and Lensi, 1981; Wu *et al*., 2002), possibly due to the different environmental conditions under which the studies were conducted, or due to mistakes in the identities of the olive cultivars studied (Mekuria *et al*., 1999). For example, Wu *et al* (2002) showed that under Australian conditions the cultivars Frantoio, Kalamata, Pendolino, and Picual were self-incompatible, and Manzanillo was partially self fertile. They also showed that Manzanillo, Kalamata, Pendolino and Picual were cross-compatible with Frantoio but not with each other. Other studies have reported Frantoio, Manzanillo, and Pendolino to be self fertile (Sharma *et al*., 1976; Sibbett *et al*., 1992; Caruso *et al*.,

1993; Ugrinovic and Stampar, 1996). Wu *et al.* (2000) also observed that crosses in one direction may be compatible but the same crosses in the reverse direction may not. Moutier *et al.* (2001) observed that while some cultivars show reciprocal compatibility, others do not.

Since the pollination process may vary from year to year and can be affected by environmental factors, a molecular approach towards studying pollen flow amongst different cultivars may prove to be a more reliable and powerful method of determining compatibility than studies based on hand pollination, bagging, pollen tube growth, or fruit yield. The identification of suitable pollen donors may be possible through paternity analysis studies, which can analyse the paternal contribution of alleles in the offspring and hence identify the most likely father.

Paternity analysis studies involve the analysis of genes transmitted by the male parent trees to their progeny. Such studies can be successfully conducted if polymorphic and codominant markers are available. Microsatellite markers fulfil these two criteria and therefore are ideal for determining paternity.

Thesis Aims

This project aims to identify the compatible cultivars in an olive orchard containing several different cultivars.

Specifically, the project aims to:

- Identify pollen donors through paternity analysis in five cultivars of olive growing in an olive orchard containing 14 cultivars.
- Identify possible effects on compatibility of morphological characters such as bloom period, pollen vitality, and percentage of complete flowers.
- Identify possible effects on compatibility of environmental factors such as temperature, rainfall, and wind direction.

Chapter Two

Literature Review

2.1 Origin of olives:

Olives originated in the Mediterranean region where the cool wet winters and warm dry summers provide an ideal climate for these trees (Booth and Davies, 1996). According to Dal Pero Bertini (1960), the olive tree was first cultivated west of the Iranian highlands and along the eastern coast of the Mediterranean, in Syria and Palestine, and then spread to the Greek Islands and the rest of the Mediterranean region. The olive tree played an important role in the social and economic lives of the Mediterranean people, mainly because the tree is an important source of table olives and olive oil (Dal Pero Bertini, 1960). The Mediterranean region is the world's major producer of olive products. About 78% of the fruit and 85% of olive oil production in the world is contributed by Spain, Italy, Greece, and Tunisia (Booth and Davies, 1996).

2.2 Classification and morphology of olive tree:

Olives are members of the family Oleaceae, and comprises two main subspecies: wild olives or oleaster, and cultivated olives, which have the Latin binomial, *Olea europaea* L.

The olive tree has a shrub-like appearance, and normally grows up to a height of 5 to 7 m (Martin, 1994). It is a shallow-rooted evergreen tree with a long life span

extending up to 1000 years (Hartmann and Opitz, 1966). The branches of the tree are compactly arranged and bear opposite lanceolate leaves (Dal Pero Bertini, 1960). The plants 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 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 anemophilous, bear large quantities of pollen, and no nectar is produced as they possess no nectaries (Martin, 1994).

As described by Hartmann and Opitz (1966); Martin (1994); and Ateyyeh *et al.* (2000) the fruit is a drupe with a thin epicarp (skin), fleshy mesocarp, and hard endocarp (stone). The seed is located inside the endocarp and consists of a thin seed coat, inside which is the starch-filled endosperm, and tapering flat cotyledons with a radicle and plumule.

The olive plant is suited to the Mediterranean climate of hot, dry summers and cool winters with adequate rainfall. The regions where olives are grown are located within 30° to 40° north and south latitudes (Dal Pero Bertini, 1960). The tree can grow in temperatures as low as −8°C, requires a minimum rainfall of 500 mm, and prefers moderately acid or alkaline soils (Dal Pero Bertini, 1960).

2.3 The olive industry in Australia:

The climate and soil conditions in South Australia are suited to olive growing and the

area has the potential to develop a strong, vibrant industry (Guerin *et al.*, 2000). The suitability of the Australian conditions to olive growing is further asserted by the fact that, in some parts of southern Australia, olive trees are regarded as weeds because of natural propagation (Sedgley and Wirthensohn, 2000; Spennemann and Allen, 2000).

Olives were introduced into Australia in 1812 (Booth and Davies, 1996), but in spite of conducive environmental conditions, the industry was disadvantaged by factors such as the high cost of labour, and cheap imports of olive products from the Mediterranean countries (Booth and Davies, 1996). Since the end of the 1939-45 war, the number of people migrating from the Mediterranean countries to Australia has increased, and also the population is becoming more aware of the health benefits of olives and olive oil (Bertrand, 2002). This has led to a greater demand for olive products, which has increased from 7,000 tonnes in 1988 to 19,000 tonnes in 1995 (Guerin *et al.*, 2000). However, the olive industry in Australia has so far been unable to meet the internal demands for olive products, and as a result the import of olive oil has increased from 12,345 tonnes in 1990 to 28,447 tonnes in 2003, while that of table olives has increased from 5,345 kg in 1990 to 14,483 kg in 2003 (data sourced from Australian Olive Association website). In recent years, the olive industry in Australia has aimed to meet internal demands for olive products as well as to occupy a position in the world market (Guerin *et al.*, 2000). However, the success of the olive industry depends on a close interaction between the industry and researchers to maximise economic yields. It is also important that this research be carried out in the Australian environment so that the specific requirements of the olive cultivars can be identified under local conditions.

2.4 Problems with fruit set in olives:

Olive growers have to face several problems which may affect the commercial production of fruit. One major problem is the alternate bearing habit of the trees. In some years there may be a heavy crop of small fruits, while in others, the crop may consist of a light crop of large fruits. Neither of these crops is commercially valuable. Mort (1952) explained this situation to be the result of having more staminate flowers in the trees in some years. Since it is only the hermaphrodite flowers which can bear fruit, it is important to have enough flowers of this type in the trees to achieve good commercial yields. Radi *et al.* (1990) have explained the phenomenon to be 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 olive (Marco *et al.*, 1990; Martin, 1990; Ghrisi *et al.*, 1999; Cuevas *et al.*, 2001). 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) observed that there were two stages in perfect flower abscission. The perfect flowers which were not fertilised abscised three weeks after anthesis, while some of the perfect flowers which were fertilised, 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).

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 (Weisman *et al.*, 1998; Mekuria *et al.*, 1999). According to Bartolini *et al.* (1994) there are more than 1200 cultivars throughout the world with more than 3000 synonyms.

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 around the world to identify the most compatible cultivars (Griggs *et al.*, 1975; Sharma *et al.*, 1976; Antognozzi and Standardi, 1978; Androulakis and Loupassaki, 1990; Rallo *et al.*, 1990). However, there is still uncertainty as to the correct combination of cultivars to be planted in an orchard. While most studies point towards the benefit of cross-pollination, growers need to know which cultivars are most compatible. Some of these studies have shown contradictory results, leading to increased confusion. For example, a study conducted by Sibbett *et al.* (1992) showed that Manzanillo is self-compatible, while another study conducted by Caruso *et al.* (1993) showed the cultivar Manzanilla to be self-sterile. It is not known whether the cultivars used in these two studies are genotypically the same.

2.5 Studies on compatibility relationships in olives:

2.5.1 Adaptations to cross-pollination: Griggs *et al.* (1975) observed that olive flowers are morphologically adapted to 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 occurs. 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. Again the abundant amount of pollen, up to 200,000 pollen grains per flower, shows that they are adapted to be wind pollinated. 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.

2.5.2 Self-sterility in cultivars: Several cultivars have been found to be self-sterile. Wu *et al.* (2000) and Wu *et al.* (2002) found the cultivars Frantoio, Kalamata, and Verdale to be self-sterile. Moutier (2000) studied compatibility relationships in sixteen olive cultivars and found that most of these cultivars need cross-pollination for adequate fruit set. Pollen incompatibility has often been found to be the cause of poor performance of olive crops (Sibbett *et al.*, 1992; Ghrisi *et al.*, 1999). El-Kholy (2001) observed that the cultivars Pendolino, Leccino, and Coratina are self-incompatible, but he also pointed out that the compatibility relationships of the cultivars change when grown under different environmental conditions. It is

therefore important to have more than one compatible cultivar in an orchard to allow for cross-pollination.

2.5.3 Effect on yield: Lack of self-compatibility may lead to the failure of pollination, and hence low fruit yield, in an orchard with 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, as even though topical application of Sevillano pollen on Manzanillo did not increase the yield, it reduced the number of small fruits. Similar observations were made by Fernandez-Escobar and Gomez-Valledor (1985) and Cuevas and Polito (1997). Even cultivars that exhibit self fertility have been observed to give higher yields following cross-pollination (Fontanazza *et al.*, 1980; Lavee *et al.*, 2002).

2.5.4 Studies on cross compatibility: The choice of cultivars to be interplanted in an orchard is an important issue. Not all cultivars are cross-compatible. Griggs *et al.* (1975) and Cuevas *et al.* (2001) found Mission and Manzanillo to be cross incompatible. 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.

In a mixed variety orchard, it is important that the different cultivars be cross-compatible so that there can be successful pollination. Studies conducted by Wu *et al.* (2002) showed that the compatibility relationships are not always reciprocal. Verdale and Kalamata were found to be mainly cross-compatible, although cross

incompatibility was observed between one Verdale and one Kalamata tree (Wu *et al.*, 2000). Only occasionally were some combinations found to be reciprocal (Moutier *et al.*, 2001).

In many cases, studies on compatibility relationships among olive cultivars led to opposing conclusions even when the same cultivars were used. 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. One possible reason for these contradictions in compatibility relationships could be differences in the environmental conditions under which the studies were conducted. El-Kholy (2001) reported that the environmental factors at a particular place may affect pollination processes in different cultivars. There is also confusion regarding the identity of the different cultivars. The cultivars may have the same name but may have different genotypes, and mistakes in nomenclature are common (Mekuria *et al.*, 1999). Therefore, it is possible that the cultivars used in these studies may not be the same genotypically.

Such confusion may mislead the grower during the selection of cultivars for an orchard. It is therefore essential to study the pollination requirements of olive cultivars under Australian environmental conditions so that the information can serve as a guide while planning orchards. Open pollination has been found to result in

higher fruit set than controlled cross-pollination (Rallo *et al.*, 1990). It is possible that the cross-pollinations carried out under artificial conditions affect fruit set. Often, paper bags are used to cover the flowers when they are in bloom to prevent the entry of foreign pollen. The conditions inside the bag may hamper fruit set and this may be the reason why the yield is better when the flowers are left uncovered during open pollination (Bartoloni and Guerriero, 1995). It is therefore necessary to study the compatibility relationships among cultivars under natural conditions, and it is important to know which cultivars will be most compatible when pollen from a number of cultivars is available.

2.6 Factors that may affect 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 bloom period started on 5th April for Temprana de Mont, on 12th April for Serrana de Esprad, and on 20th April for Penjoll. All 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. Studies by Wu *et al.* (2002) showed wide variation in the percentage of complete flowers between cultivars, ranging from 22.9% in Kalamata to 86.6% 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. Only the complete flowers can bear fruits while the staminate flowers can only act as pollen donors. 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 yields. 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). Again, 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. The presence of an abnormal embryo sac has been found to be responsible for preventing fruit set in the ornamental cultivar Swan Hill (Rallo *et al.*, 1981). This cultivar was also found to have very low pollen production (Rallo *et al.*, 1981) due to inhibition of dehiscence of anthers (O'Rourke and Buchmann, 1986).

Methods of measuring pollen vitality, which is assumed to reflect viability, 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 13.8% in Pendolino to a high of 78.7% in Frantoio. Occasionally cultivars have been found to be male sterile (Villemur *et al.*, 1984; Moutier, 2000). 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, it may be better to have 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 it is necessary to monitor pollen vitality over several years in order to observe the effect of changes in vitality on the ability of a cultivar to act as a pollen donor.

Studies on the morphological characteristics of olive trees have reported that these differ both between cultivars and between years. Orlandi *et al.* (2003) studied the cultivars Ascolano, Tenera, and Giarraffa and concluded that the cultivars differ significantly in tree canopy volume and number of inflorescences. Based on the average tree size, Orlandi *et al.* (2003) concluded that Ascolano had a higher

productive potential, and since the three cultivars showed synchrony in their flowering period, would cross-pollinate successfully.

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) observed 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 and relative humidity decreased 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. For good pollen dispersal from pollinating cultivars within a commercial orchard, it is necessary to have adequate wind flow in the required direction.

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, these conditions do not reflect the natural conditions under which fruit set will take place. Also, results based on yield will depend on the criteria used by the researcher. Some studies consider a cross to be compatible if there is any fruit set, while others consider it to be compatible only if the fruit set is high. Besides, fruit set observations can be made at different times. Initial fruit set may reflect a large number of fruits, while 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 +15 days, open pollinated trees had a higher fruit set at full bloom +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 (Ghrisi *et al.*, 1999; Cuevas *et al.*, 2001). So other factors may be involved during post-fertilisation abscission which may not necessarily be related to compatibility. Therefore it is important to study compatibility relationships based on paternity analysis, which will trace back the genes in mature fruits to the pollen donor. With the use of molecular markers it is possible to record the composition of markers in a particular embryo and to identify the pollen donor using paternity analysis.

2.7 Studies on mechanism of self-incompatibility in plants:

Studies on mate choice in plants (reviewed by Marshall and Folsom, 1991) have shown that there is a tendency to have multiple pollen donors for the seeds of any particular plant. As a result, when there are a number of different types of compatible pollen available to a flower, the fertilisation process may not be random. The characters that influence choice of pollen donor have been classified into prepollination factors (morphology and phenology), prezygotic factors (interaction on stigma surface and growth of pollen tube through the style to reach the ovule), and postzygotic factors which lead to embryo abortion. Both the vigor of the embryo and the effect of maternal tissues have been found to be responsible for abortion of embryos. Plants not only distinguish among compatible mates in general, but against relatives that are more distantly related than others. There is also evidence that presence of a choice of compatible pollen increases the fitness of the offspring.

Self-incompatibility in plants can be classified into three types: gametophytic self-incompatibility, sporophytic incompatibility, and late-acting self-incompatibility (Lewis, 1994; Sedgley, 1994). Gametophytic self-incompatibility is controlled by the haploid genotype of the pollen grain due to the action of specific genes which are expressed in the pollen and the pollen tubes are unable to reach the ovule because of retarded growth and discharge of contents in the style. Sporophytic incompatibility is determined by the diploid genotype of the pollen parent and the mother plant due to the expression of specific genes before the meiotic division, and the incompatible pollen is unable to germinate on the stigma. In late-acting self-incompatibility, pollen tubes reach the ovule, however, fertilisation may not take place or post-

zygotic abortion may occur.

Gametophytic self-incompatibility is more common in occurrence than sporophytic incompatibility. Examples of families characterised by gametophytic self incompatibility are Solanaceae, Rosaceae, and Papaveraceae (Newbegin *et al.*, 1993), while sporophytic incompatibility has been found to occur in Brassicaceae (Nasrallah, 2002) and *Ipomea* (Kowyama *et al.*, 2000).

Since gametophytic incompatibility depends on the haploid genotype of the pollen, they are not influenced by the dominance-recessive relationship of the alleles (Sedgley, 1994). However, sporophytic incompatibility depends on the diploid genotype and is therefore affected by the dominance-recessive relationships of the alleles. In general, gametophytic incompatibility is associated with the presence of wet stigma, while sporophytic incompatibility is associated with presence of dry stigma (Elleman and Dickinson, 1994).

The mechanism of self-incompatibility has been investigated in several plants and these studies have been reviewed by Nettancourt (1997); McCubbin and Kao (2000); and Franklin-Tong and Franklin (2003). If the alleles present in the pollen and the pistil are the same, then the pollen is rejected as incompatible. This rejection occurs at the surface of the stigma in sporophytic incompatibility, and in the style in gametophytic incompatibility. The exact mechanism by which self-pollen is degraded and non-self pollen is accepted varies with the species.

Wu *et al.* (2002) studied the penetration and growth of pollen tubes through the style in five cultivars of olives and found that the number of pollen tubes declined from the stigma down to the style. As discussed in their paper, gametophytic self-incompatibility may possibly be the mechanism operating in olives. Bradley *et al.* (1961) proposed that if the pistil and pollen have certain identical genes, then the pollen tube stops growing and is unable to reach the embryo. However, they also observed that even when the pollen reaches the embryo sac, fertilization may not occur if the pollen tube is too slow and the sac begins to degenerate. Little is known about the genetic control of this system in olive. Identification of compatible olive cultivars may be a preliminary step towards the study of the mechanism of self-incompatibility in olive cultivars.

2.8 Use of molecular markers for genotype identification:

Morphological characters are affected by the environment and also require extensive and time consuming observation of the mature plant (Belaj *et al.*, 1999). This may be a limitation in olives which have a long juvenile phase (Dal Pero Bertini, 1960). Molecular markers are used to identify specific regions of the genome and, because they are not affected by either environmental factors or phenological stages (Vergari et al., 1998; Belaj *et al.*, 2003), are a reliable means for establishing cultivar identity (Angiollo *et al.*, 1999; Cipriani *et al.*, 2002). In contrast, morphological observations are possible only at certain times of the year, such as when the tree is in bloom or when the fruits are formed (Sanz-Cortes *et al.*, 2001).

Most olive cultivars cannot be distinguished on the basis of phenotypic characters

because they have similar appearance (Sweeny, 2003). Genetic variability in olives has been studied by Randomly Amplified Polymorphic DNA (RAPD) (Bogani *et al.*, 1994; Weisman *et al.*, 1998; Belaj *et al.*, 1999; Mekuria *et al.*, 1999; Guerin *et al.*, 2002; Mekuria *et al.*, 2002), Amplified Fragment Length Polymorphism (AFLP) (Angiollo *et al.*, 1998; de la Rosa *et al.*, 2000), and Inter Simple Sequence Repeats (ISSRs) (Hess *et al.*, 2000). However, the above-mentioned techniques produce dominant markers that are less suitable for a paternity analysis study than codominant markers. Isozymes are a class of codominant markers that may be used for paternity analysis study. Kamoun *et al.* (2002) used isozymes for characterising olive cultivars and found that these markers had a low level of polymophism with an average of only 2.6 alleles per locus. Since isozymes depend on gene expression (Fabbri *et al.*, 1995; Hormaza, 2002) and have a low level of polymorphism (Hormaza, 2002; Kamoun *et al.*, 2002), they are not markers of choice in paternity analysis studies in olive.

Microsatellites or Simple Sequence Repeats (SSR) are another class of codominant markers that may be used for paternity analysis studies. SSRs are polymorphic repeat sequences of between one and six base pairs present in the genomes of eukaryotes and prokaryotes (Toth *et al.*, 2000). As reviewed by Toth *et al.* (2000), SSRs may result from DNA polymerase slippage or unequal recombination. Plants have a higher frequency of AG/CT repeats as compared to AC/GT (Morgante *et al.*, 2002). Variations in microsatellite sequences can be detected using primers designed from the conserved flanking regions of the repeat sequences (Saghai Maroof *et al.*, 1994). Microsatellites have been found to have a higher level of polymorphism as

compared to RAPDs and isozymes (Fahima *et al.*, 2002). One disadvantage of using microsatellites is the lengthy process of identifying these sequences and designing primers for them.

Hormaza (2002) demonstrated the usefulness of microsatellites in the characterisation of apricots and the cross transportability of these markers between species of *Prunus*, while Alvarez *et al.* (2001) demonstrated the same in *Lycopersicon*. Transferability of microsatellite markers developed in olive into other species of the genus *Olea* has been demonstrated by Rallo *et al.* (2003).

In a study by Jones *et al.* (1997), the reproducibility of RAPD, AFLP, and SSR markers were tested across various laboratories. Jones *et al.* (1997) found that RAPDs had the least reproducibility, while SSRs showed only minor differences among the laboratories. Russel *et al.* (1997) found that SSRs had a higher level of polymorphism compared to AFLPs, RFLPs, and RAPDs.

Microsatellite markers have been developed and used in olives in several studies (Sefc *et al.*, 2000; Bandelj *et al.*, 2002; Carriero *et al.*, 2002; Cipriani *et al.*, 2002; de la Rosa *et al.*, 2002). Rallo *et al.* (2000) developed primers for 13 microsatellite loci and confirmed their codominant nature by testing for Mendelian segregation in a population obtained from a cross between two known parents. The number of alleles per locus in these markers ranged from 9 to 17. Since these markers are codominant and highly polymorphic, they are ideal markers for paternity analysis studies. Codominant microsatellites have proved better than dominant AFLPs in parentage

analysis studies (Gerber *et al.*, 2000).

2.9 Paternity analysis and gene flow studies:

Gene flow has been defined as 'the process by which parental genes are reassociated in the next generation, affecting and/or being affected by the distribution patterns of individuals and population structure' (Kameyama *et al.*, 2002).

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 microsatellites as molecular markers. 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 such 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 in oak (Streiff *et al.*, 1999; Gerber *et al.*, 2000), red pine (Lian *et al.*, 2001), *Neobalanocarpus heimii*, (Konuma *et al.*, 2000) and *Eucalyptus* (Chaix *et al.*, 2003).

Most of the paternity analysis studies are based on three main approaches: simple exclusion, fractional paternity assignment, and the most likely parent method.

The simple exclusion technique compares the genotype of the progeny with that of the female parent. The maternal contribution is subtracted and the remaining genotype is compared with all the potential fathers. Those individuals that could not have contributed the genotype are excluded and the remaining individuals are assigned as the possible parents. This approach has been used by Roeder *et al.*

(1989) and Velde *et al.* (2001).

The most likely method was proposed by Thompson and Meagher (1987). This method involves identifying potential fathers in a natural population of plants involving several genotypes based on the genetic data, since an individual cannot be the parent unless it has some alleles in common with the progeny. Thompson and Meagher (1987) introduced 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. This method has been used by Marshall *et al.* (1998) who applied the technique to a population of red deer (*Cervus elaphus*) and also developed a Windows-based computer program, called Cervus, which can be used for parentage analysis. 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). Other studies that have used the likelihood-based approach are those of Kaufman *et al.* (1998); Lian *et al.* (2001); Kameyama *et al.*

(2002); and Schueler *et al.* (2003). Buteler *et al.* (2002) compared the paternity exclusion and most likely parent methods. While neither of the methods assigns a father for all the progeny (Devlin *et al.*, 1988), Buteler *et al.* (2002) found that the most likely parent method was able to assign fathers to more of the progeny.

Fractional paternity assignment is similar to the likelihood method, but paternity is assigned to all the non-excluded males based on the proportion of each male's likelihood of paternity. This proportion is estimated on the basis of the statistical likelihood of each of the non-excluded males to be the father of the offspring compared to all other males (Devlin *et al.*, 1988). This technique assigns paternity to all the offsprings.

2.10 Conclusion:

The success of the olive industry in Australia depends on the consistent production of high yields of good quality fruit. Since olive trees have a long juvenile phase, proper planning of orchards at the time of planting the trees is important. Carefully planned orchards with suitable pollinator cultivars planted amongst the mother trees will increase fruit set due to increased pollination and also result in superior quality fruits as a result of cross-pollination (Sibbett *et al.*, 1992). Planting several cultivars in an orchard can have additional advantages, such as ensuring good commercial yields in spite of the alternate bearing habit of olives, having different cultivars for olive oil with different flavours, and extending the harvest season (Archer, 1996). This will help meet Australia's domestic requirements of olive products and also help Australia become an exporter of olive products. The identification of the most

suitable pollen donors for the cultivars grown commercially in Australia will be an important step towards achieving these goals. This project will be a start in this direction, by using microsatellite markers for paternity analysis studies in a commercial orchard in South Australia to determine the most likely pollen donors for the cultivars under investigation. This study will also contribute to the theoretical understanding of olive reproduction.

Chapter Three

Material and Methods

The materials and methodologies used in this project are described in this chapter.

3.1 Material:

3.1.1 Trees: Olive trees growing at Milano Olives, Gumeracha, South Australia were used for the experiments. 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. The trees in the orchard ranged from one to four-and-a-half years old at the time of study. A field plan of the property is shown in Fig. 3.1. The cultivars on the property were Barnea, Corregiola, Katsourella, Kalamata, King Kalamata, Koroneiki, Manzanillo, atypical Manaki (cultivar thought to be Manaki by the grower but found to be genotypically distinct from the standard, refer Chapter Four), Mission, Leccino, Pendolino (from Samrea Nurseries, South Australia), atypical Pendolino (Pendolino sourced from Nuriootpa Nursery, South Australia and found to be genotypically distinct from the standard, refer Chapter Four), Sevillano, UC13A6, and Verdale. Some other unidentified trees were also present in the orchard and these have been referred to as atypical Corregiola and atypical Kalamata (considered to be Corregiola and Kalamata respectively by the grower but found to be genotypically distinct from the standards, refer Chapter Four).

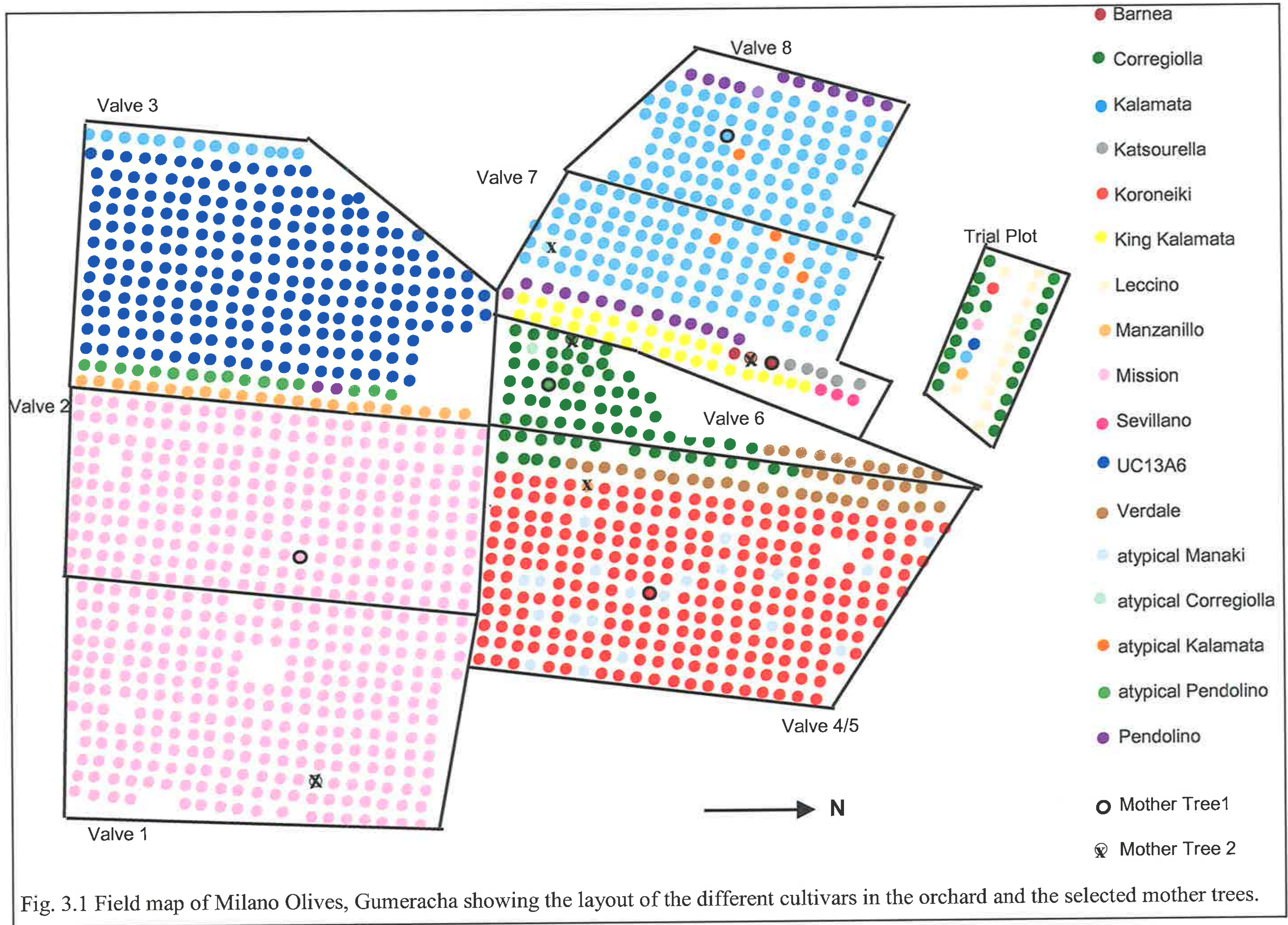


Fig. 3.1 Field map of Milano Olives, Gumeracha showing the layout of the different cultivars in the orchard and the selected mother trees.

The trees of Barnea, Corregiola, Katsourella, Leccino, atypical Pendolino, Mission, Sevillano, UC13A6, and Verdale were propagated from cuttings. Kalamata, King Kalamata, Koroneiki, atypical Manaki, Pendolino, and atypical Kalamata were grafted on to wild rootstocks. There were a few Kalamata trees which were grafted onto Manzanilla rootstocks.

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.

Fruits collected from mother trees of five cultivars (Barnea, Corregiola, 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.

3.1.2 Leaves: Leaves were collected from two trees of each cultivar for DNA analysis. Leaves were also collected from trees that were thought to have a different appearance from the others of the same cultivar in order to compare their genotypes.

The leaves were collected in plastic bags and kept on ice while transporting them from the field to the laboratory. They were then stored at 4°C, and extracted for DNA within a week of collection. Field (1997) reported that olive leaves stored in this way yielded DNA of high quality up to 12 weeks after collection.

3.1.3 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.

3.1.4 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.

3.1.5 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.

3.2 Methodology:

3.2.1 Bloom time: The trees at Gumeracha 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.

3.2.2 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. The percentage of complete flowers was calculated by the formula:

Percentage of Complete Flowers = {Number of complete flowers / (Number of staminate flowers + Number of complete flowers)} x 100.

3.2.3. 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 and the percentage pollen vitality was calculated using the formula:

Pollen vitality (%) = {Number of stained pollen grains/(Number of stained pollen grains + Number of non-stained pollen grains)} x 100

It was assumed that the vital stain reflected the viability of the pollen (Pinney and Polito, 1990). During the first year, pollen from five inflorescences of each tree was pooled and used for the vitality tests. During the second year, pollen from inflorescences collected from different sides of the tree was tested separately to facilitate statistical analysis.

3.2.4 Weather data: The weather data were obtained from the Australian Bureau of Meteorology, 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.

3.2.5 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., prepared according to Sambrook *et al.*, (1989)) 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 (Sambrook *et al.*, 1989).

3.2.6 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 alchol (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.

3.2.7 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. The primers used, along with their annealing temperatures, are listed in Table 3.1.

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 appropriate annealing temperature (Table 3.1), 45 s at 72°C, and finally an extension step at 72°C for 45 min.

Table 3.1. Primers used for genotyping and paternity analysis.

Primer	Sequence	5' Label	Annealing Temperature	Number of alleles scored	Range of alleles scored (bp)
ssrOeUA-DCA3 AJ279854 Sefc *et al.*, 2000	F: CC CAA GCG GAG GTG TAT ATT GTT AC R: TGC TTT TGT CGT GTT TGA GAT GTT G	HEX	57°C	6	230-252
EMO2AJ416320 de la Rosa *et al.*, 2002	F: CTC GCA CTT TAA ATT CAT ATG GGT AGG T R: GCG TGC TTG GGT GCT TGT TTG	NED	57°C	4	202-215
UDO99-006 Cipriani *et al.*, 2002	F: TC AGT TTG TTG CCT TTA GTG GA R: TTG TAA TAT GCC ATG TAA CTC GAT	FAM	57°C	6	146-170
UDO99-008 Cipriani *et al.*, 2002	F: AA AAA CAC AAC CCG TGC AAT R:AAA TTC CTC CAA GCC GAT CT	HEX	57°C	7	155-170
UDO99-031 Cipriani *et al.*, 2002	F: TA TCC TCT ATG TGG CGA TG R: TGC TTT TGT CGT GTT TGA GAT GTT G	FAM	57°C	7	108-151
ssrOeUA-DCA4 AJ279855 Sefc *et al.*, 2000	F: CTT AAC TTT GTG CTT CTC CAT ATC C R: AGT GAC AAA AGC AAA AGA CTA AAG C	NED	55°C	8	131-187
ssrOeUA-DCA14 AJ279863 Sefc *et al.*, 2000	F: AA TTT TTT AAT GCA CTA TAA TTT AC R: TTG AGG TCT CTA TAT CTC CCA GGG G	FAM	50°C	7	148-188
UDO99-024 Cipriani *et al.*, 2002	F: GG ATT TAT TAA AAG CAA AAC ATA CAA A R: CAA TAA CAA ATG AGC ATG ATA AGA CA	HEX	57°C	6	166-194

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 (Section 3.2.8.3). An internal size standard (ROX) which is detected at the wavelength of 605 nm helps in the scoring of the bands. The details of the dyes are proprietary information of Applied Biosystems, 850 Lincoln Centre Drive, Foster City, CA 94404, USA.

3.2.8 Gel electrophoresis: Gel electrophoresis, using both agarose and polyacrylamide, was used to observe the PCR products.

3.2.8.1 Agarose gel electrophoresis: Agarose gels, 1.75% (w/v) in 1 x TBE buffer (90 mM Trizma base, 90 mM boric acid, 2 mM EDTA, pH 8.0) were used to observe the PCR products. Nine µL of each PCR reaction were loaded per well along with 1 µL of the 10 x loading dye (1 mM cresol red in 20% sucrose solution). A 100 bp DNA ladder (DMW-100M, GeneWorks Pty. Ltd., 150 ng/mL) (225ng) was mixed with loading buffer (5% v/v glycerol, 7.5 mM EDTA, 0.02% (w/v) SDS, 0.01% (w/v) bromophenol blue, 0.01% (w/v) xylene cyanol) and loaded into one of the wells to estimate the size of the bands observed. The gels were run at 190 V for about 1.5 hr until the bromophenol blue had moved three quarters of the length of the gel.

The gels were stained in 0.5 µL/mL ethidium bromide solution for 10 min, destained in RO water for 10 min, and observed under UV light. A digital image of the gel was taken using the Program Tekcap, version 1.0 (copyright Tekram Corporation,

1997-1998) and then saved using the program PaintShop Pro, version 5 (copyright JascSoftware Inc, 1991-1998) in paint shop pro image format. Polaroid 667 films were also used to take photographs of the gels.

3.2.8.2 Polyacrylamide gel electrophoresis: Sequagel 8 (National Diagnostics) was used for the 8% polyacrylamide gels. Forty mL of the sequencing gel solution was mixed with 10 mL of buffer reagent and 400 µL of 10% ammonium persulphate. The gels (22 cm x 20 cm) were run in 1x TBE. The PCR products were mixed with an equal volume of 6 x formamide loading dye (95% deionised formamide, 10 mM EDTA, pH 8.0, 0.02% xylene cyanol and 0.02% bromophenol blue), heated at 95°C for 5 min to denature the DNA, and then cooled on ice. Ten µL of the samples were loaded into the wells. One µL of *p*UC19/*Hpa*II ladder (GeneWorks Pty. Ltd.), 1 µL of 10 x PCR buffer, 1.6 µL of 6 x formamide loading dye, and 6.4 µL of water was denatured in a similar way and loaded onto the gel. The gel was preheated for 20 min at 25 mA and then the samples were loaded and run at 25 mA. The gel was observed by ethidium bromide staining as described in Section 3.2.8.1.

3.2.9 ABI Prism 3700 DNA Analyser: PCR reaction products were first observed on 1.75% agarose gels (Section 3.2.8.1) to check the amount of DNA products amplified. Depending on the intensity of the bands on the agarose gel, from 1 to 4 µL of the PCR products were added to 100 µL of autoclaved milli Q water in a 96 well PCR plate. The PCR products from three PCR reactions, each using FAM, NED, and HEX labelled primers, were combined and loaded into each well. Three µL of the diluted PCR products were transferred to each well of another 96 well plate

and mixed with 5 μL of ROX-Formamide (6 μL ROX Genescan® 400MD, Applied Biosystems + 500 μL of Hi Di Formamide, Applied Biosystems) which is the internal size standard marker. The products were analysed using a 96-capillary ABI Prism 3700 DNA Analyser (Applied Biosystems, 850 Lincoln Centre Drive, Foster City, CA 94404, USA). FAM and HEX labelled primers were obtained from GeneWorks Pty. Ltd., and the NED labelled primers were obtained from Applied Biosystems.

The Genescan data were analysed using the program Genescan version 3.5.1 (copyright: Applied Biosystems 1989-2000) and the alleles for each locus were scored by comparing with the bands obtained with ROX which gave red peaks at known intervals of molecular weight. Products obtained using the FAM-labelled primer were observed as blue peaks, those with the HEX-labelled primer as green peaks, and those with NED-labelled primer as black peaks. Because of the difference in size and colour of the peaks obtained with the different primers, PCR products with three sets of fluorescent markers were added when used for Genescan.

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.

3.2.10 Data analysis:

3.2.10.1 Analysis of bloom time, percentage of complete flowers and pollen vitality data: The data were recorded in a Microsoft Excel spreadsheet and the standard deviations and confidence intervals were calculated using Smith's Statistical

Package, version 2.5, 2001, Gary Smith, Pomona College, Claremont, California 91711. Microsoft Excel was used for graphical representation of the data. The percentage of complete flowers and pollen vitality data were analysed using MINITAB (Version 13.20, Minitab Inc.).

3.2.10.2 Analysis of Genescan data: The presence or absence of alleles was recorded in a Microsoft Excel spreadsheet. The Numerical Taxonomy Systems Software Package, NTSYS-pc version 2.02k (copyright: Applied Biostatistics 1986-1998) (Rohlf, 1993) was used to compare the fingerprints of the mother plants with the standards in the form of a dendrogram prepared using this program. 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).

3.2.10.3 Paternity Analysis: Paternity analysis was done using the program FaMoz, a program written and maintained by Sophie Gerber, INRA-Recherches Forestieres-BP45, Laboratoire de Genetique et Amelioration des Arbres Forestiers, 33611 Gazinet Cedex, France. This software uses likelihood ratio to identify potential fathers (Chaix *et al.*, 2003). The genetic contribution of the mother tree is subtracted from the genotype of the embryo to obtain the contribution of the paternal parent. The exclusion probability for each locus was calculated. Exclusion probability is the average capability of a marker system to exclude a given relationship and is

determined by the genotypes of the individuals and the allele frequencies (Jamieson and Taylor, 1997).

Because any of the trees in the orchard could be a potential donor of pollen, the genotypes of all the different cultivars were included in the paternity analysis. For each cultivar, the genotype of the mother tree was included in order to identify cases of self-pollination. The allelic frequencies were calculated for each locus. Any missing alleles were represented with the value of –5 as required by the program.
A simulation was done to generate genotypes of a large number of offsprings (1000) from the allelelic frequencies of the parents present in the orchard. Another set of simulated offspring genotypes was generated from random genotypes of all possible parents. This second set of offsprings represented gene flow from outside the stand (Gerber *et al.*, 2000). The two simulations were represented graphically and the first crossing point of the two graphs was taken as the threshold of paternity. This point represented the balance of the occurrence of gene flow from outside the stand and that from inside the stand (Gerber *et al.*, 2000). This value of threshold of paternity was used in the test 'Fathers from inside/outside the stand' to obtain the most likely fathers for each embryo. When the embryos had more than one likely father, the LOD (log likelihood) score was used to determine the most likely one. The LOD score represented the ratio of the likelihood of one parent being the father compared with any other parent being the father. The parent with the highest LOD score was considered to be the most likely father.

3.2.10.4 Analysis of paternity analysis results: The number of embryos pollinated by a cultivar and the number of trees of that cultivar were compared using the simple regression analysis function of GenStat, 7th Edition.

Chapter Four

Cultivar Identification of Olive Trees used for the Study

4.1 Introduction:

Previous studies (Mekuria *et al.*, 1999; Barranco *et al.*, 2000; Cavagnaro *et al.*, 2001; Guerin *et al.*, 2002) have shown that confusion exists in the nomenclature of olive cultivars. In these studies, some cultivars with different names were found to be genotypically similar, while others with the same name but obtained from different sources were found to have different DNA fingerprints. Comparison of the DNA fingerprints of the cultivars used in the current study with those of standard cultivars (Guerin *et al.*, 2002) was important to verify the identity of the trees. This chapter describes the methodology and the results obtained after fingerprinting the trees from which fruits were collected, and the cultivar identification of representative trees of all the cultivars in the orchard that were potential pollen donors.

4.2 Materials:

Leaf samples from two to five trees of each of the 14 cultivars planted in the orchard were collected. DNA fingerprints were also analysed from trees in the orchard that appeared to be morphologically different from other trees reported to be of the same cultivar. This was to ensure that the genotypes of all the potential pollen donors were characterised for paternity analysis. The standards used for comparison of DNA fingerprints are shown in Table 4.1.

Table 4.1 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 (Guerin *et al.*, 2002)

Cultivar	Source of the standard
Barnea	The Volcani Centre, Bet-Dagan, Israel
Corregiola	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.
Manaki	*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.

* NOVA: National Olive Variety Assessment (Sweeny, 2003)

4.3 Methodology:

4.3.1 DNA extraction: DNA extraction was carried out as described in Section 3.2.5. The quality and concentration of DNA were measured as described in Section 3.2.5 and then diluted to 20 ng/µL for use in PCR amplification.

4.3.2 DNA amplification: The reaction mixture and the reaction cycles used for PCR amplification were as described in Section 3.2.7. Twenty six microsatellite primers (Table 4.2), previously used for olive (Sefc *et al.*, 2000; Cipriani *et al.*, 2002; de la Rosa *et al.*, 2002) were screened against the standard DNA samples of Barnea, Corregiola, Kalamata, Katsourella, Koroneiki, King Kalamata, Leccino, Manzanillo, Manaki, Mission, Pendolino, Sevillano, UC13A6 and Verdale (Table 4.1). The five best primer pairs with 5'-fluorescent labels on the forward primers were used for cultivar identification. These were: EMO2AJ416320, ssrOeUA-DCA3AJ279854, UDO99-006, UDO99-008 and UDO99-031 (Table 3.1). The fluorescent dyes are proprietary property of Applied Biosystems 850 Lincoln Centre Drive, Foster City, CA 94404, U.S.A.

4.3.3 Gel electrophoresis: For screening of primers, the PCR products were first observed after agarose gel electrophoresis as described in Section 3.2.8.1 to check for successful amplification of DNA. Subsequently the PCR products were separated by polyacrylamide gel electrophoresis (Section 3.2.8.2) to select the primers that produced satisfactory polymorphisms. For cultivar identification, PCR products obtained using selected primers (Table 3.1) were first observed in agarose gels to check for successful amplification and then separated by the ABI Prism 3700 DNA Analyser (Section 4.3.4).

Table 4.2 List of primers screened.

Reference	**Primers Tested**
de la Rosa *et al.* (2002)	EMO2 AJ416320
	EMO3 AJ416321
	EMO13 AJ416322
	EMO30 AJ416323
Cipriani *et al.* (2002)	UDO99-006
	UDO99-007
	UDO99-008
	UDO99-009
	UDO99-011
	UDO99-012
	UDO99-014
	UDO99-019
	UDO99-024
	UDO99-028
	UDO99-031
	UDO99-035
	UDO99-039
	UDO99-043
Sefc *et al.* (2000)	ssrOeUA-DCA1 AJ279853
	ssrOeUA-DCA3 AJ279854
	ssrOeUA-DCA4 AJ279855
	ssrOeUA-DCA5 AJ279856
	ssrOeUA-DCA10 AJ279860
	ssrOeUA-DCA14 AJ279863
	ssrOeUA-DCA15 AJ279864
	ssrOeUA-DCA16 AJ279865

4.3.4 ABI Prism 3700 DNA Analyser: PCR products were separated and analysed by capillary electrophoresis using the ABI Prism 3700 DNA Analyser (Section 3.2.9). Depending on the intensity of the bands observed through agarose gel electrophoresis, from 1 to 4 µL of the PCR product with the FAM, NED and HEX labelled primers were added to 100 µL of autoclaved milli Q water in each well of a 96 well PCR plate. Three µL of this solution was transferred to another 96 well plate and mixed with 5 µL of ROX-Formamide (6 µL ROX Genescan® 400MD, Applied Biosystems + 500 µL of Hi Di Formamide, Applied Biosystems) and applied to the ABI Prism 3700 DNA Analyser. The data obtained were analysed using the program Genescan version 3.5.1, 1989-2000 Applied Biosystems.

4.3.5 Data analysis: The presence or absence of alleles was recorded in Microsoft Excel and a dendrogram generated using the program NTSYS-pc, version 2.02k, 1986-1998, Applied Biostatistics (Section 3.2.10.2).

4.4 Results:

4.4.1 Screening of primers: Initial screening of microsatellite primers (Table 4.2) that had been previously used for fingerprinting in olive (Sefc *et al.*, 2000; Cipriani *et al.*, 2002; de la Rosa *et al.*, 2002) showed that while some primers had an appreciable level of polymorphism, others were not so polymorphic and in some cases no PCR products were detected. Based on this screening, five primers (ssrOeUA-DCA3AJ279854, EMO2AJ416320, UDO99-006, UDO99-008 and UDO99-031) were used for cultivar identification (Table 3.1).

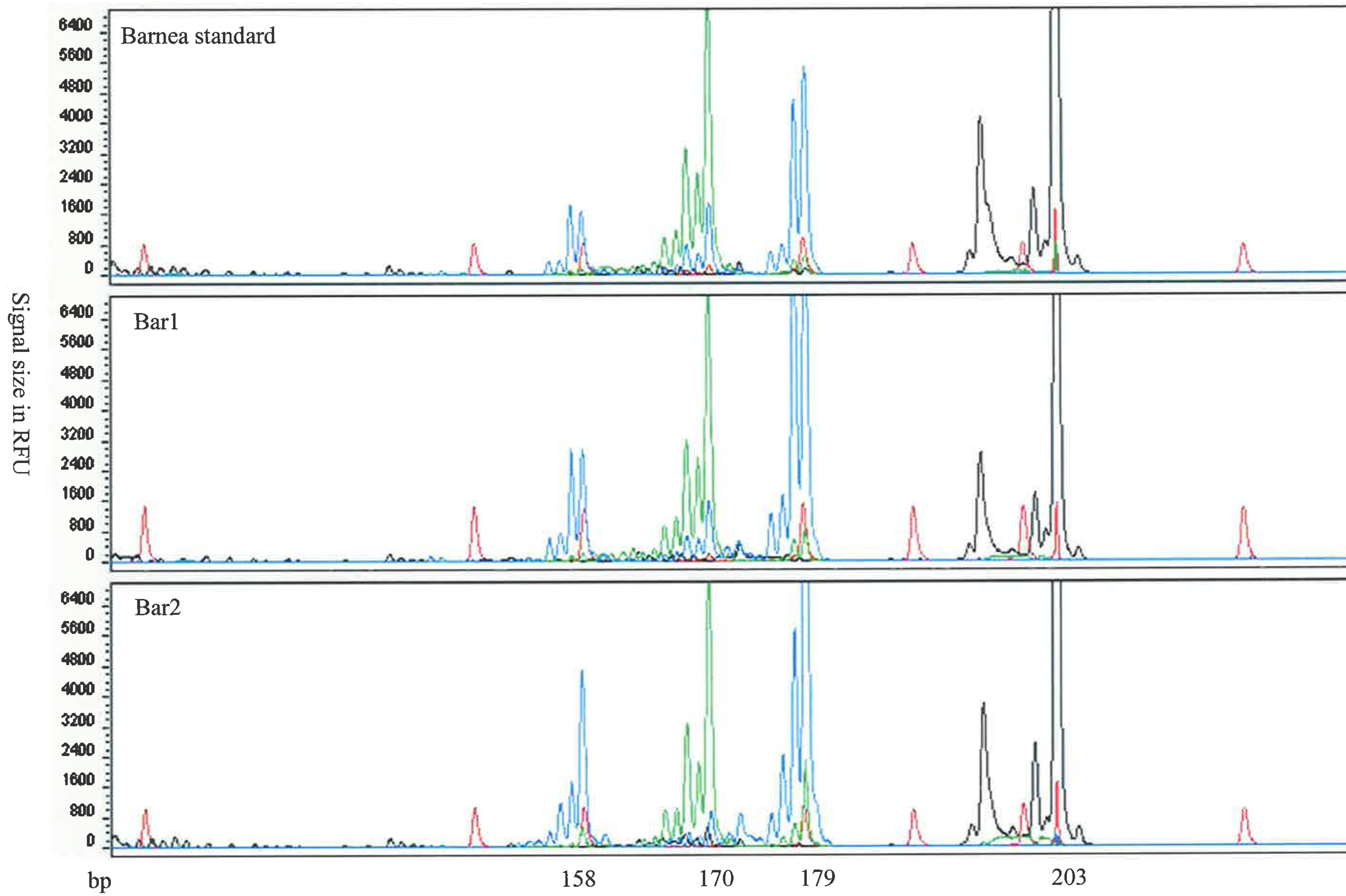
4.4.2 Cultivar identification: DNA fingerprinting uses information present in the genome of an organism for identification. Therefore it remains unaffected by environmental conditions otherwise likely to affect phenotypic characteristics. DNA fingerprinting using microsatellites has been used in several studies (Carriero *et al.*, 2002; Cipriani *et al.*, 2002) and has been proved to be an efficient method of cultivar identification in olives. The DNA fingerprinting data obtained in this study proved to be reproducible and the experiments were repeated to obtain at least two consistent observations.

The data obtained after separation of PCR products and analysis by Genescan showed that the fingerprints of most of the mother trees tested matched with the standards. Fig. 4.1 shows the results obtained from Genescan using the primers UDO99-006, UDO99-008, and EMO2 for the Barnea standard and for the selected Barnea trees, Bar1 and Bar2. The peaks obtained with the three primers in the test plants and the standard matched with each other.

One Corregiola plant showed a different fingerprint compared to the standard Corregiola DNA used and was referred to as atypical Corregiola (a-Cor). The cultivar referred to as Manaki by the grower did not match with the standard Manaki in the database and was referred to as atypical Manaki (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). Some trees identified by the grower as Kalamata had a different appearance and DNA fingerprint compared with the standard, and were called atypical Kalamata (a-Kal).

Fig 4.1 Genescan results showing comparison of Bar1 and Bar2 (Barnea trees) with the standard Barnea. The blue peaks at 158 and 179 bp were produced by the primer UDO99-006 which is labeled with FAM, the green peaks at 170 bp by the primer UDO99-008 which is labeled with HEX, and the black peaks at 203 bp by the primer EMO2 which is labeled with NED. The red peaks represent the internal size standards.

[RFU: Relative Fluorescence unit]


Barnea standard
Bar1
Bar2
Signal size in RFU
6400
5600
4800
4000
3200
2400
1600
800
0
bp
158
170
179
203

A dendrogram showing the relationship of the tested trees with the standards is shown in Fig. 4.2. The dendrogram showed that except for the atypical genotypes mentioned above, all the others matched with the standards.

4.5 Discussion:

DNA fingerprinting identifies differences in the genotype of organisms. Microsatellites are polymorphic repeat sequences present in the genome (Toth *et al.*, 2000). Microsatellites have a high mutation rate of 10^{-2} to 10^{-9} loci per generation (Li *et al.*, 2002) which is responsible for the high polymorphism of these markers. Being located in the non-coding regions of the genome, microsatellites survive the selection pressure and their polymorphism are not necessarily reflected in the agronomic characteristics of the plant (Saghai Maroof *et al.*, 1994).

Sexual reproduction leads to genetic variation in each generation. However, olives are clonally propagated to preserve their agronomic properties. Therefore it is unlikely that clonally propagated trees of a particular cultivar will show variation. In spite of having a high mutation rate, it has been found that leaf samples of trees of the same cultivar obtained from different parts of the world produce identical fingerprints with microsatellites (Jenny Guerin *pers. comm.*). Therefore we can conclude that the rate of mutation in microsatellite regions is very slow and therefore the technique can be efficiently used for cultivar identification in vegetatively propagated plants.

Fig. 4.2 Dendrogram showing comparison of the cultivars present at Milano Olives, Gumeracha, SA with the standard cultivars (Table 4.1)

Bar: standard Barnea.

Bar1 and Bar2: Barnea trees.

Cor: standard Corregiola.

Cor1, Cor2: Corregiola trees.

a-Cor: atypical Corregiola.

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 Manaki.

a-Ma1 and a-Ma2: atypical Manaki 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.

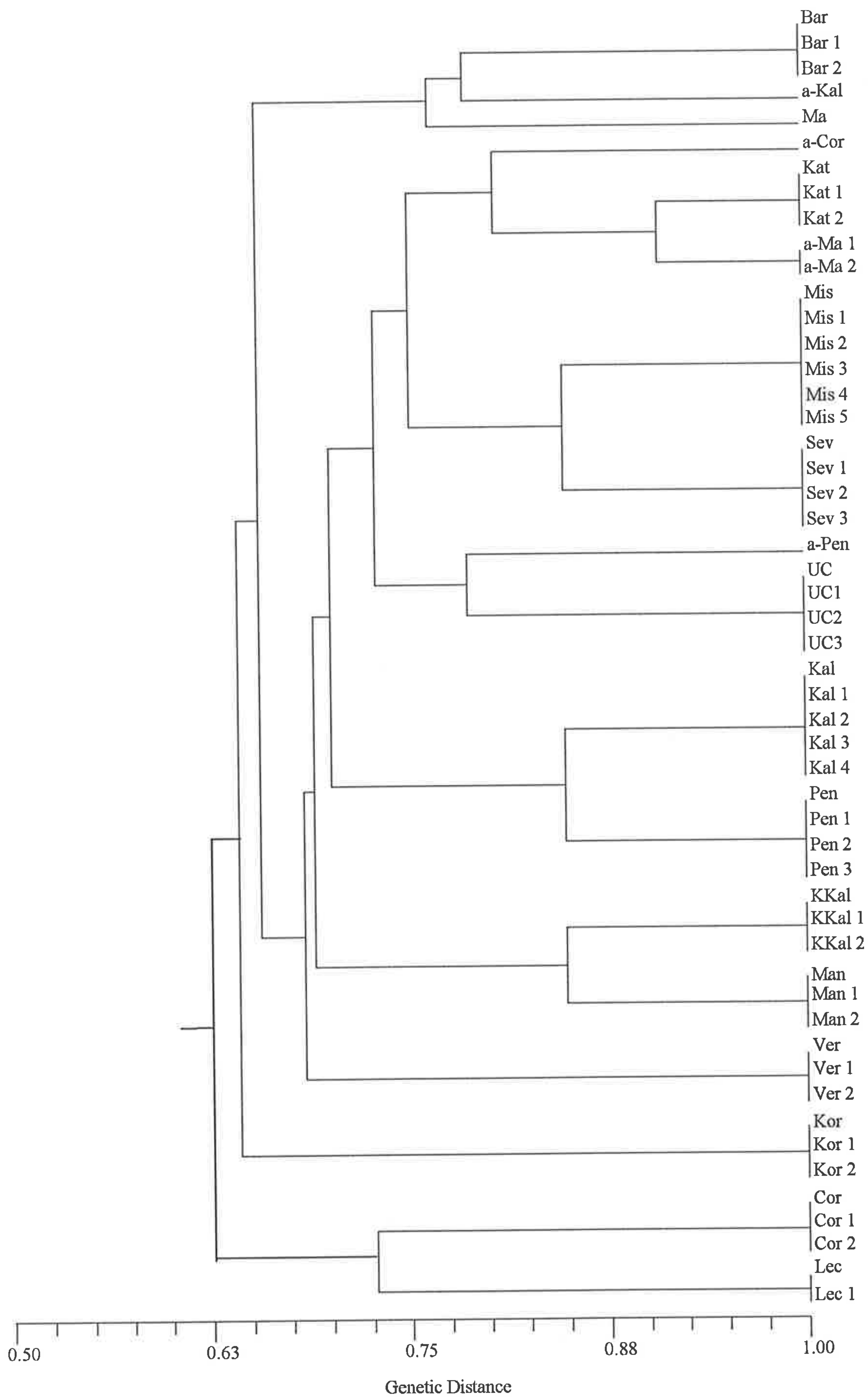
Sev1, Sev2, Sev3: Sevillano trees.

UC: standard UC13A6.

UC1, UC2, UC3: UC13A6 trees.

Ver: standard Verdale.

Ver1, Ver2: Verdale trees.


Bar
Bar 1
Bar 2
a-Kal
Ma
a-Cor
Kat
Kat 1
Kat 2
a-Ma 1
a-Ma 2
Mis
Mis 1
Mis 2
Mis 3
Mis 4
Mis 5
Sev
Sev 1
Sev 2
Sev 3
a-Pen
UC
UC1
UC2
UC3
Kal
Kal 1
Kal 2
Kal 3
Kal 4
Pen
Pen 1
Pen 2
Pen 3
KKal
KKal 1
KKal 2
Man
Man 1
Man 2
Ver
Ver 1
Ver 2
Kor
Kor 1
Kor 2
Cor
Cor 1
Cor 2
Lec
Lec 1
0.50
0.63
0.75
0.88
1.00
Genetic Distance

While using microsatellites, only a small portion (200-300 bp) of the total genome is studied. Therefore, any differences or similarities that are detected are limited to this portion of the DNA. However, the polymorphic nature of microsatellites makes them effective markers for DNA fingerprinting. RAPDs and ISSRs are able to scan larger portions of the DNA. These marker are not as effective as the codominant microsatellite markers for paternity analysis which is the aim of the project. When a difference is detected between the DNA fingerprints of two samples, it implies that the two samples differ in only that portion of the DNA and this difference may not be evident from the phenotypic character of the plants. At the same time, two samples having identical fingerprints are similar only in the portions of the DNA that is scanned. It is therefore possible that there may be differences in the portions of the DNA that are not being scanned. Somatic mutations in the region of the genome scanned may also be detected as differences in the genotype of the samples (Bowers *et al.*, 1993).

For this study, the DNA fingerprints of most of the cultivars matched the standards and their identities were confirmed. The atypical Corregiola, atypical Pendolino, atypical Manaki, and atypical Kalamata, which did not match the standards, also had different morphologies from those of the other trees of the same cultivar. The fingerprints of the atypical cultivars which did not match the standards were compared with the 95 samples in the University of Adelaide database (Guerin *et al.*, 2002). However, the fingerprints did not match with any of the cultivars present in the database.

Several authors have reported confusion in the nomenclature of olive cultivars. Bartolini *et al.* (1994) have reported the existance of 1200 cultivars around the world with over 3000 synonyms. Mekuria (2001) studied different accessions of some common cultivars and found that while some accessions matched with each other, others were genotypically different. While Manzanillo, Nevadillo Blanco, Picual and Kalamata accessions did not show any significant variability, accessions of Verdale and Corregiola obtained from different regions of Australia showed genetic differences.

Confusion regarding cultivar identity may be serious problem to the grower. Since olives have a long juvenile phase, it may take a few years before a mistake is identified. In this case, four genotypes were obtained which did not match with the standards and which could not be identified when compared with other samples. It is possible that these are cultivars not present in the database. However, since they have distinct genotypes, they will be considered as potential pollen donors in the orchard.

Confirmation of cultivar identity ensured that the conclusions made regarding the compatibility of the cultivars (Chapter Six) would be reliable as a future reference while planning new orchards to ensure that fruit yield is optimized. In addition all the genotypes present in the orchard must be taken into account as potential pollen donors.

Chapter Five

Floral Characteristics of Fourteen Olive Cultivars

5.1 Introduction:

Events at bloom time contribute significantly towards fruit yield in olive. For example, 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. Wu *et al.* (2002) showed that pollen viability and the relative proportions of staminate and complete flowers vary significantly between cultivars, and also that pollen viability varies between seasons. In addition, Orlandi *et al.* (2003) observed that both tree size and the average number of inflorescences per tree differed among cultivars. Therefore, the ultimate fruit yield in any given season depends to a large extent on the processes that occur during the flowering period.

This chapter reports the measurements that were made of the bloom time, percentage of complete flowers, and pollen vitality of cultivars of olives that were used to support the molecular studies into paternity analysis described in Chapter Six. These measurements were recorded during the bloom time from November to December in both 2002 and 2003.

5.2 Materials and Methods:

5.2.1 Trees: Olive trees of 14 cultivars growing at Milano Olives, Gumeracha, South Australia, were used for the study. The location and plan of the olive orchard have been described in Chapter 3 and a field map of the orchard showing the different cultivars is shown in Fig. 3.1. The cultivars studied were Barnea, Corregiola, Kalamata, Katsourella, King Kalamata, Koroneiki, Leccino, Mission, Manzanillo, atypical-Manaki, Pendolino, Sevillano, UC13A6, and Verdale and their identities were confirmed by comparison with the standards in the University of Adelaide Olive Group database (Chapter Four). Cultivars that did not match with the standards were referred to as 'atypical' (Section 4.4.2). One to five mother trees of each cultivar, depending on the number of trees present in the property, were selected and tagged. Inflorescences were collected from each of these trees and used to determine the percentage of complete flowers and the pollen vitality.

5.2.2 Weather data: The data for maximum and minimum temperatures, rainfall, and wind direction during the bloom period were obtained from the Bureau of Meteorology, Mt. Crawford Weather Station. The location of this weather station, compared to the research site at Gumeracha, is described in Chapter Three.

The wind roses, showing direction of wind, were obtained for the average wind direction in the morning (9 am) and afternoon (3 pm) during the bloom period. The wind speed and direction are measured by a number of means and the data are grouped by hour (observations between 7.30 am and 10.30 am labelled as 9 am, and between 2.30 pm and 4.30 pm labelled as 3 pm), and by speed. For the exact method used for estimation of wind speed and direction refer to the Australian Bureau of

Meteorology, Mt. Crawford station (http://www.bom.gov.au). The branches (petals) represent the direction from which the wind is blowing towards the centre. The length of the petals is proportional to the frequency of the wind and the thickness is proportional to the speed. The central circle represents periods of calm.

5.2.3 Bloom time: The selected trees of each cultivar were observed at intervals of three days, and the dates for start of bloom (10% flowers open), full bloom (80% flowers open), and end of bloom (80% flowers spent) were recorded.

5.2.4 Percentage of complete flowers: Five inflorescences from each tree were used to calculate the percentage of complete flowers. Complete flowers had well-developed stamens and a pistil, whereas, in staminate flowers, the pistil was either absent or rudimentary. The percentage of complete flowers was calculated using the formula:

Percentage of complete flowers = (Number of complete flowers / Total number of flowers) x 100.

5.2.5 Pollen vitality: Pollen from flowers of the inflorescences of each tree was collected. Mature undehisced anthers were removed with forceps, placed in Eppendorf tubes, and kept overnight at room temperature in a sealed plastic container containing silica gel. Pollen grains from the dehisced anthers were stained with fluorescein diacetate (2 mg/mL fluorescein diacetate in acetone) (Pinney and Polito, 1990), and after an interval of a few minutes, observed under a fluorescence microscope (Zeiss photomicroscope, Axiophot) at 520 nm, using an exciter filter of 395-440 nm. Five fields of observation were studied, and in each case, the numbers

of viable (showing fluorescence) and non-viable (no fluorescence) pollen grains were recorded. The percentage pollen vitality was calculated by the formula:

Pollen Vitality (%) = (Number of viable pollen grains / Total number of pollen grains) x 100.

During the 2002 season, pollen from the flowers of five inflorescences, collected from four sides and the centre of each tree, was collected in one tube and a sub-sample used for pollen vitality observations. To increase the efficiency of sub-sampling for statistical analysis in the 2003 season, pollen was again collected from inflorescences taken from the four sides and the centre of each tree, but in this case a sub-sample of each of the five pollen samples was tested separately for pollen vitality.

5.2.6 Data analysis: Standard deviations and 95% confidence intervals were calculated for the pollen vitality observations during the 2002 season using Smith's Statistical Package (Version 2.5, 2001, Gary Smith, Pomona College, Claremont, California 91711). The percentage of complete flowers and the pollen vitality data for the 2003 season were analysed using the General Linear Model of MINITAB (Release 13.20, Minitab Inc.) as an analysis of variance with subsamples.

5.3 Results:

5.3.1 Weather Data: The maximum and minimum temperatures and the total rainfall during the bloom period of 26 days during 2002 are shown in Table 5.1. It was observed in 2002 that there were two warm days with temperatures above 30°C as well as some cool days with maximum temperatures below 20°C. Most days in

2002 were dry with no rainfall. The most rain, 19 mm, was recorded on day 12 after the start of bloom of UC13A6, which was the first cultivar to start flowering.

The weather data for the bloom period in 2003 are shown in Table 5.2. The maximum temperature varied from 15.7°C on December 5, which was the lowest temperature during the period, to as high as 33.3°C on December 9. The minimum temperature ranged from 7.4°C on November 25 to 18.0°C on November 29. The period was mainly dry with only six days experiencing rainfall. The maximum rainfall experienced in a day was 1 mm on December 5, which corresponded with the start of bloom of Kalamata and Leccino.

Since olives are wind pollinated, it was important to know the direction of wind during the bloom period to determine whether there was adequate mixing of pollen. The wind roses for the bloom period of both the years of study (Fig. 5.1 and Fig. 5.2) show that relatively strong winds generally occurred from the east and west during the mornings, and from the west in the afternoons throughout the flowering period However, there were winds of lower strength blowing in other directions as well, thus ensuring good dispersal of pollen throughout the orchard. There were no periods of calm as can be seen from the shaded central circles.

Table 5.1 Temperature and Rainfall during Bloom Period of 2002

Date	Max.Temp.(°C)	Min. Temp. (°C)	Rainfall (mm)
15.11.02	22.2	7.0	0.0
16.11.02	28.7	10.3	0.0
17.11.02	31.8	19.2	0.0
18.11.02	26.2	8.6	0.0
19.11.02	23.2	11.5	0.0
20.11.02	16.4	9.1	1.0
21.11.02	23.9	5.7	0.0
22.11.02	25.2	11.1	0.0
23.11.02	32.3	14.9	0.0
24.11.02	24.1	17.7	0.2
25.11.02	19.9	12.7	4.0
26.11.02	15.5	8.9	19.0
27.11.02	17.8	8.4	0.4
28.11.02	24.3	8.4	0.0
29.11.02	20.7	11.2	0.0
30.11.02	18.7	6.6	0.0
1.12.02	26.7	7.1	0.0
2.12.02	25.0	17.1	0.0
3.12.02	16.8	7.7	0.0
4.12.02	18.5	12.2	0.0
5.12.02	18.2	14.5	8.4
6.12.02	15.5	6.8	4.0
7.12.02	22.4	5.3	0.0
8.12.02	20.2	13.6	0.0
9.12.02	14.3	6.1	0.0
10.12.02	16.1	5.8	0.2

Max. Temp. - Maximum Temperature in degrees Centigrade
Min. Temp. = Minimum Temperature in degrees Centigrade
Rainfall in millimetres

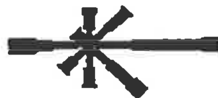
Table 5.2 Temperature and Rainfall during Bloom Period of 2003

Date	Max.Temp.(°C)	Min. Temp. (°C)	Rainfall (mm)
24.12.03	18.9	5.9	1
25.11.03	22.8	7.4	0.2
26.11.03	25.3	10.9	0.0
27.11.03	27.2	15.8	0.0
28.11.03	26.8	16.4	0.0
29.11.03	25.9	18.0	0.0
30.11.03	18.5	13.6	0.0
1.12.03	18.9	9.5	0.4
2.12.03	25.9	9.1	0.0
3.12.03	27.4	9.0	0.0
4.12.03	18.9	15.1	0.4
5.12.03	15.7	8.2	1.0
6.12.03	20.1	5.2	0.0
7.12.03	26.8	8.1	0.0
8.12.03	29.8	13.1	0.0
9.12.03	33.3	16.2	0.0
10.12.03	18.6	12.2	0.9
11.12.03	18.6	9.5	0.8
12.12.03	16.6	9.8	0.0
13.12.03	19.3	9.8	1.0

Max. Temp. = Maximum Temperature in degrees Centigrade.
Min. Temp. = Minimum Temperature in degrees Centigrade
Rainfall in millimetres

a) Wind direction at 9 am during November 2002.

b: Wind direction at 3 pm during November 2002



c) Wind direction at 9 am during December 2002.

d) Wind direction at 3 pm during December 2002

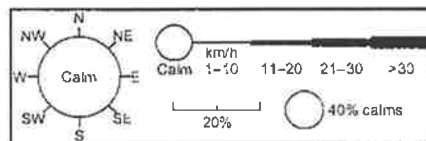


Fig. 5.1. Wind roses for November-December 2002.

a) Wind direction at 9 am during November 2003

b: Wind direction at 3 pm during November 2003

c) Wind direction at 9 am during December 2003

d) Wind direction at 3 pm during December 2003

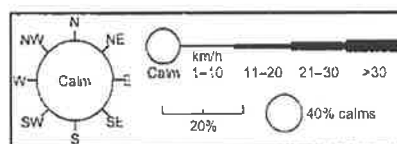


Fig. 5.2. Wind roses for November-December 2003

5.3.2 Bloom Time: Fig. 5.3 shows the different stages of bloom observed for each cultivar. The dates for start of bloom, full bloom, and end of bloom for each cultivar during 2002 are shown in Fig. 5.4 and the dates for 2003 are shown in Fig. 5.5. The rainfall and temperature maxima have also been indicated on the figures.

In 2002, the earliest cultivar to flower was UC13A6, which started on November 15, and the latest was Kalamata, which started on November 28. The temperature was highest (32°C) on November 23, which coincided with the start of flowering by Barnea, Corregiola, Koroneiki, atypical Manaki, Mission, Pendolino, Sevillano, and Verdale. The cultivars Manzanillo, atypical Pendolino, UC13A6, and one of the Verdale trees reached full bloom around this time. The wettest day of the period was November 26 and this corresponded with the full bloom stage of Barnea, Corregiola, Koroneiki, Katsourella, atypical Manaki, Manzanillo, Mission, Pendolino, Sevillano, and Verdale. There were some cool days from December 3-6 and this corresponded with the full bloom stage for Kalamata and atypical Corregiola. Except for UC13A6, Leccino, and Kalamata, the other cultivars reached full bloom around November 25, and extended to about November 28. Full bloom for UC13A6 occurred between November 20-24, for Leccino, about November 28, and for Kalamata, December 3. Generally, replicate trees from each cultivar reached full bloom within a few days of each other. The atypical Corregiola came to full bloom 8 days after the Corregiola trees.

Cultivars Corregiola and Verdale had fewer flowers on them than the other cultivars. This is probably because in 2002 many trees of these two cultivars were only around one year old as compared to other cultivars which were from 2 to 4.5 years old (refer

A B C D

Fig. 5.3 Stages of Bloom in Olive.

A: Balloon Stage, just before opening of flowers.

B: Start of Bloom (10% flowers open).

C: Full Bloom (80% flowers open).

D: End of Bloom (80% flowers spent).

Fig. 5.4 Bloom Time in Olive Cultivars at Gumeracha, SA. Observations made in 2002. T: Temperature (°C); R: Rainfall (mm).

Bar1 and Bar 2: Barnea trees.

a-Cor: atypical Corregiola tree.

Cor1, Cor2, Cor3: Corregiola trees.

Kal1, Kal2, Kal3, Kal4, Kal5: Kalamata trees.

Kor1, Kor2, Kor3, Kor4: Koroneiki trees.

Kat1, Kat2: Katsourella trees.

KKal1, KKal2, KKal3: King Kalamata trees.

Lec1, Lec2, Lec3, Lec4: Leccino trees.

a-Mk1, a-Mk2, a-Mk3, a-Mk4: atypical Manaki trees.

Man1, Man2, Man3: Manzanillo trees.

Mis1, Mis2, Mis3, Mis4, Mis5 : Mission trees.

Pen1, Pen2, Pen3: Pendolino trees.

a-Pen: atypical Pendolino.

Sev1: Sevillano tree.

UC1, UC2, UC3, UC4, UC5: UC13A6 trees.

Ver1, Ver2, Ver3, Ver4: Verdale trees.

	November, 2002																December.2002									
Date	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	1	2	3	4	5	6	7	8	9	10
T (°C)	22	29	32	26	23	16	24	25	32	24	20	16	18	24	21	19	27	25	17	19	18	16	22	20	14	16
R (mm)	0	0	0	0	0	1	0	0	0	0.2	4	19	0.4	0	0	0	0	0	0	0	8.4	4	0	0	0	0.2
Code																										
Bar1																										
Bar2																										
a-Cor																										
Cor1																										
Cor2																										
Cor3																										
Kal1																										
Kal2																										
Kal3																										
Kal4																										
Kal5																										
Kor1																										
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Man3																										
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a-Pen																										
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UC3																										
UC4																										
UC5																										
Ver1																										
Ver2																										
Ver3																										
Ver4																										

:Date of Full Bloom

to Table 6.1 for age of trees). As a result, the trees were smaller and had fewer flowers.

In 2003, flowering started 9 days later than in 2002 but was condensed into a shorter period. Manzanillo, atypical Manaki, UC13A6, and Verdale were the first cultivars to start flowering on November 25-26. Corregiola, atypical Corregiola, Koroneiki, Katsourella, King Kalamata, Mission, Pendolino, and atypical Pendolino started flowering around November 29-30 coinciding with a change in the temperature from 25.9°C to 18.5°C. Leccino and Kalamata were the last cultivars to flower on December 5. By this time, atypical Manaki, Manzanillo, Verdale, and some UC13A6 had already finished flowering. Except for Leccino and Kalamata, flowering of all the other cultivars overlapped each other. The bloom times of the replicate trees of each cultivar were generally synchronous. However, one tree of atypical Manaki flowered 4 days before the others and there were also differences in the flowering time of the replicate UC13A6 trees. The wettest days of the period were on December 5 and 13, with 1 mm of rainfall on both days. December 5 corresponded with the full bloom stage of Koroneiki and King Kalamata. Manzanillo, atypical Manaki, atypical Pendolino, UC13A6, and Verdale had finished flowering by this time, while Kalamata and Leccino had just started flowering. All flowering was completed by December 13.

The bloom period in 2003 started later than in 2002, but was shorter than the previous year by 7 days. Kalamata was among the cultivars that flowered last in both years. The bloom period of Leccino was around the same time as the other

Fig. 5.5 Bloom Time in Olive Cultivars at Gumeracha, SA. Observations made in 2003. T: Temperature (°C); T: Rainfall (mm).

Bar1 and Bar 2: Barnea trees.

Cor1, Cor2: Corregiola trees.

a-Cor: atypical Corregiola tree.

Kal1, Kal2, Kal3, Kal4, Kal5: Kalamata trees.

a-Kal: atypical Kalamata tree.

Kor1, Kor2, Kor3, Kor4: Koroneiki trees.

Kat1, Kat2: Katsourella trees.

KKal1, KKal2, KKal3: King Kalamata trees.

Lec1, Lec2, Lec3, Lec4: Leccino trees.

a-Mk1, a-Mk2, a-Mk3, a-Mk4: atypical Manaki trees.

Man1, Man2, Man3: Manzanillo trees.

Mis1, Mis2, Mis3, Mis4, Mis5 : Mission trees.

Pen1, Pen2, Pen3: Pendolino trees.

a-Pen: atypical Pendolino.

Sev1: Sevillano tree.

UC1, UC2, UC3, UC4, UC5: UC13A6 trees.

Ver1, Ver2, Ver3, Ver4: Verdale trees.

	November, 2003						December, 2003												
Date	25	26	27	28	29	30	1	2	3	4	5	6	7	8	9	10	11	12	13
T (°C)	22.8	25.3	27.2	26.8	25.9	18.5	18.9	25.9	27.4	18.9	15.7	20.1	26.8	29.8	33.3	18.6	18.6	16.6	19.3
R (mm)	0.2	0.0	0.0	0.0	0.0	0.0	0.4	0.0	0.0	0.4	1.0	0.0	0.0	0.0	0.0	0.9	0.8	0.0	1.0
Code																			
Bar1																			
Bar2																			
a-Cor																			
Cor1																			
Cor2																			
Kal1																			
Kal2																			
Kal3																			
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a-Mk1																			
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a-Pen																			
Sev1																			
UC1																			
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UC4																			
UC5																			
Ver1																			
Ver2																			
Ver3																			
Ver4																			

:Date of Full Bloom

cultivars in 2002, but it was later in 2003. There was more rainfall during 2002 season (36.2 mm) than during 2003 (4.7 mm). The bloom period of most of the cultivars overlapped during both years ensuring that pollen from all cultivars was available for pollination. However, since olives are wind pollinated, rainfall may have affected the movement of pollen.

5.3.3 Percentage of Complete Flowers: The percentage of complete flowers was calculated for each of the five inflorescences of each tree and the data were analysed using the General Linear Model of MINITAB Release 13.20 with sub-sampling. The raw data are shown in Appendix 1. The mean percentages of complete flowers in 2002 and the significance groups are shown in Fig. 5.6. Values ranged from 42.5% in King Kalamata to 92.5% in Leccino. Cultivars that were not significantly different from each other have the same letter over their bars. The results show that King Kalamata had the lowest percentage of complete flowers which was significantly lower than all other cultivars except Pendolino and Manzanillo, while, Leccino had the highest percentage of complete flowers which was significantly higher than all cultivars except atypical Manaki and Corregiola. The value in Corregiola significantly was higher than King Kalamata, Pendolino, Manzanillo, Kalamata, Verdale, Koroneiki, and Mission. There was no significant difference between Pendolino, Manzanillo, Kalamata, Verdale, Koroneiki, Mission, UC13A6, Barnea, and Katsourella.

In the 2003 season, inflorescences collected from four sides and the centre of each tree were again used to measure the percentage of complete flowers. The mean percentages of complete flowers and the significance groups are shown in Fig. 5.7.

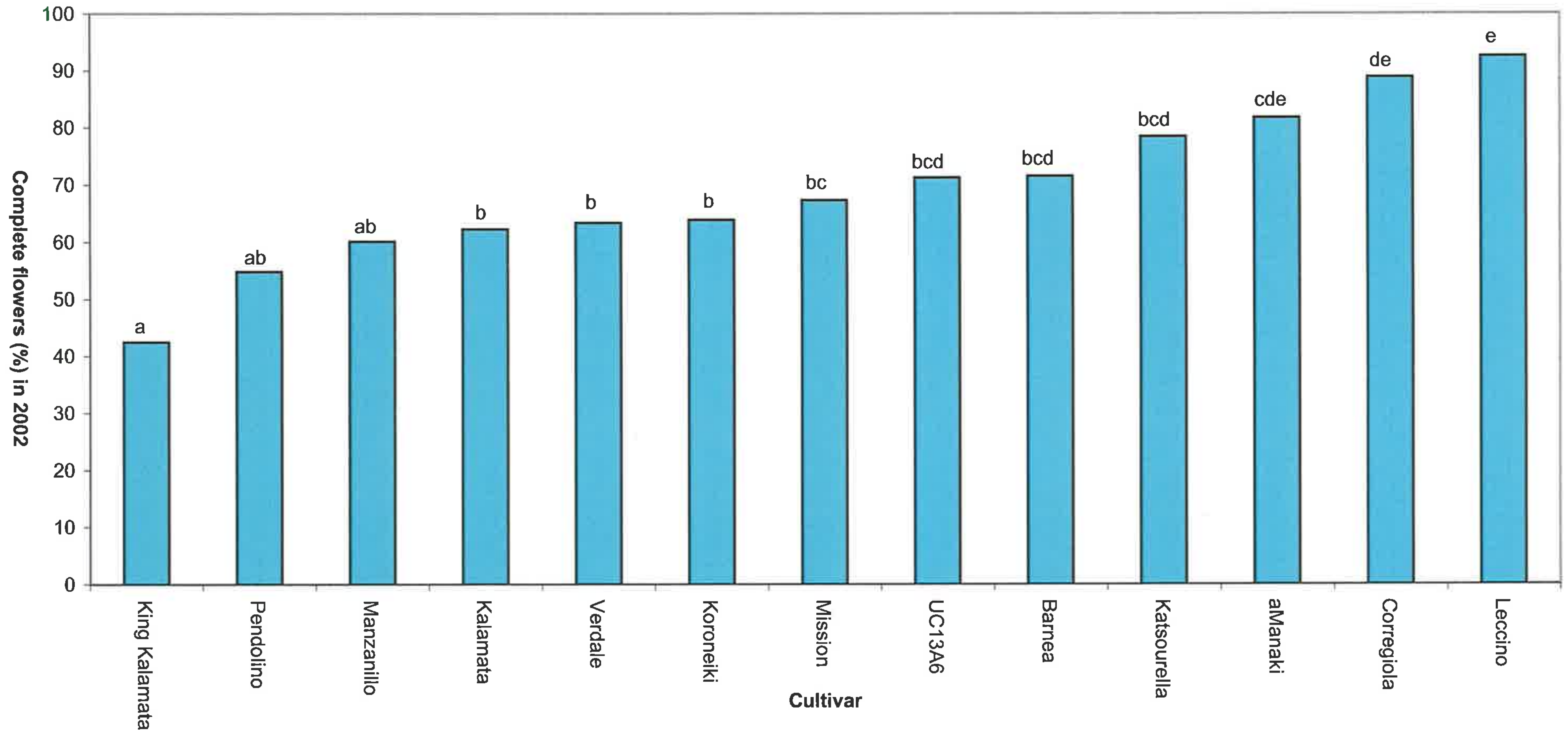

Fig 5.6 Percentage of complete flowers in the olive cultivars. Observations made in November-December 2002. The letters over the bars represent the significance groups. Cultivars having the same letter are not significantly different. (aManaki = atypical Manaki).

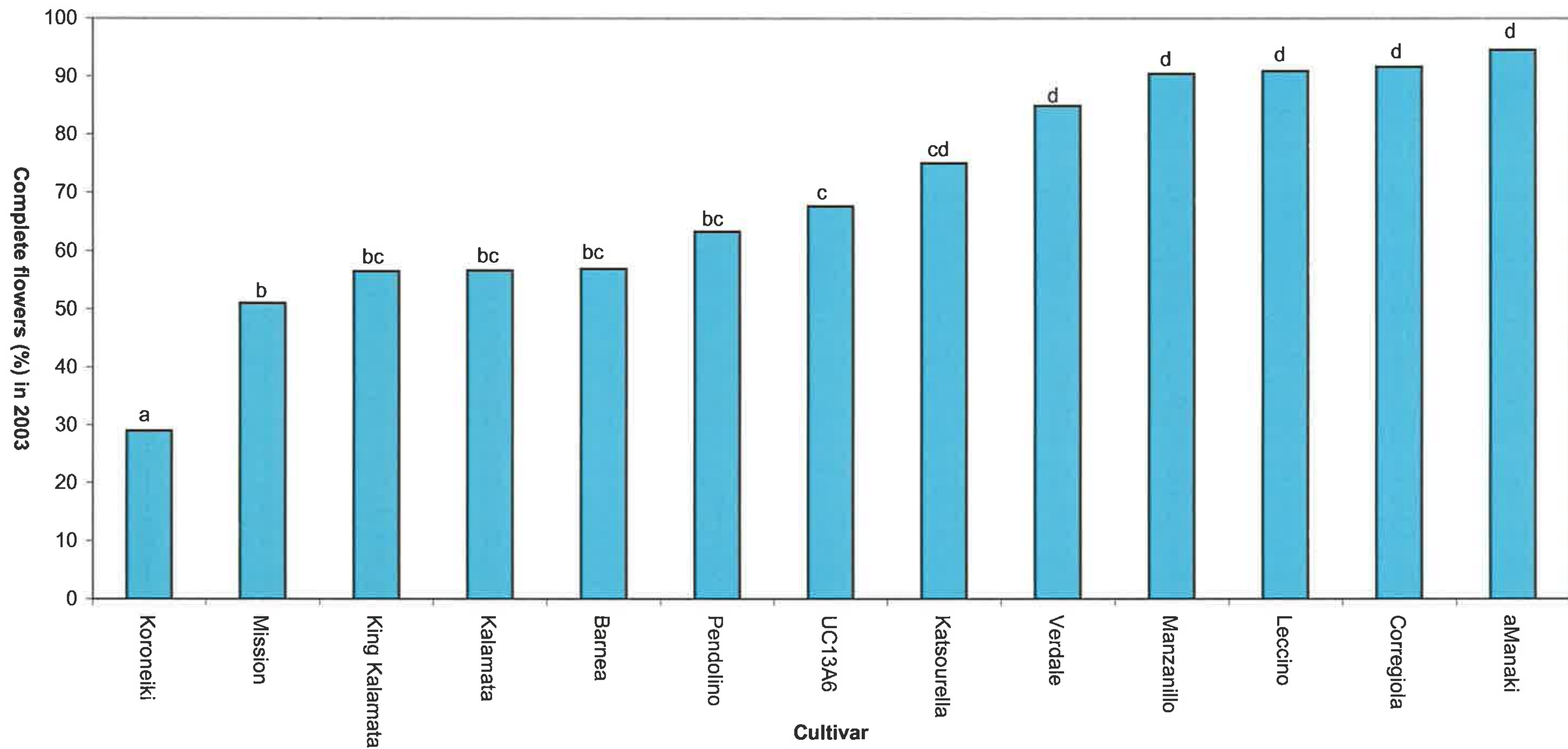

Fig 5.7 Percentage of complete flowers in the olive cultivars. Observations made in November-December 2003. The letters over the bars represent the significance groups. Cultivars having the same letter are not significantly different. (aManaki = atypical Manaki).

Values ranged from 29.0% in Koroneiki, which had a significantly lower percentage of complete flowers than all other cultivars, to 94.6% in atypical Manaki. The cultivars with the highest percentage of complete flowers included atypical Manaki, Corregiola, Leccino, Manzanillo, and Verdale. The percentage of complete flowers in all these cultivars were significantly higher than in Koroneiki, Mission, King Kalamata, Kalamata, Barnea, Pendolino, and UC13A6.

5.3.4 Pollen Vitality: Viable and non-viable pollen grains observed after fluorescein diacetate staining in 2003 are shown in Fig. 5.8. The numbers of viable and non-viable pollen grains were recorded for five inflorescences from each tree and the raw data are shown in Appendix 2. The percentages of pollen vitality for each cultivar in 2002 and 2003 are presented in Figures 5.9 and 5.10 respectively.

In 2002, the pollen vitality was recorded for pollen collected from five inflorescences of each tree and pooled together. Five fields of observation were recorded and the percentage pollen vitality was calculated based on the observations. The confidence intervals of the means were calculated in each case. The highest pollen vitality was recorded for Koroneiki (72.3%), and the lowest for King Kalamata (23.5%). Both Verdale and atypical Manaki had anthers that lacked pollen. Except for King Kalamata, Kalamata and Sevillano, pollen vitality of all the other cultivars ranged from 50.0 to 65.9%. Pollen vitality was 47.4% for Kalamata and 44.0% for Sevillano.

King Kalamata, which was found to have a low percentage of complete flowers, and hence more staminate flowers, was found to have low pollen vitality. On the other

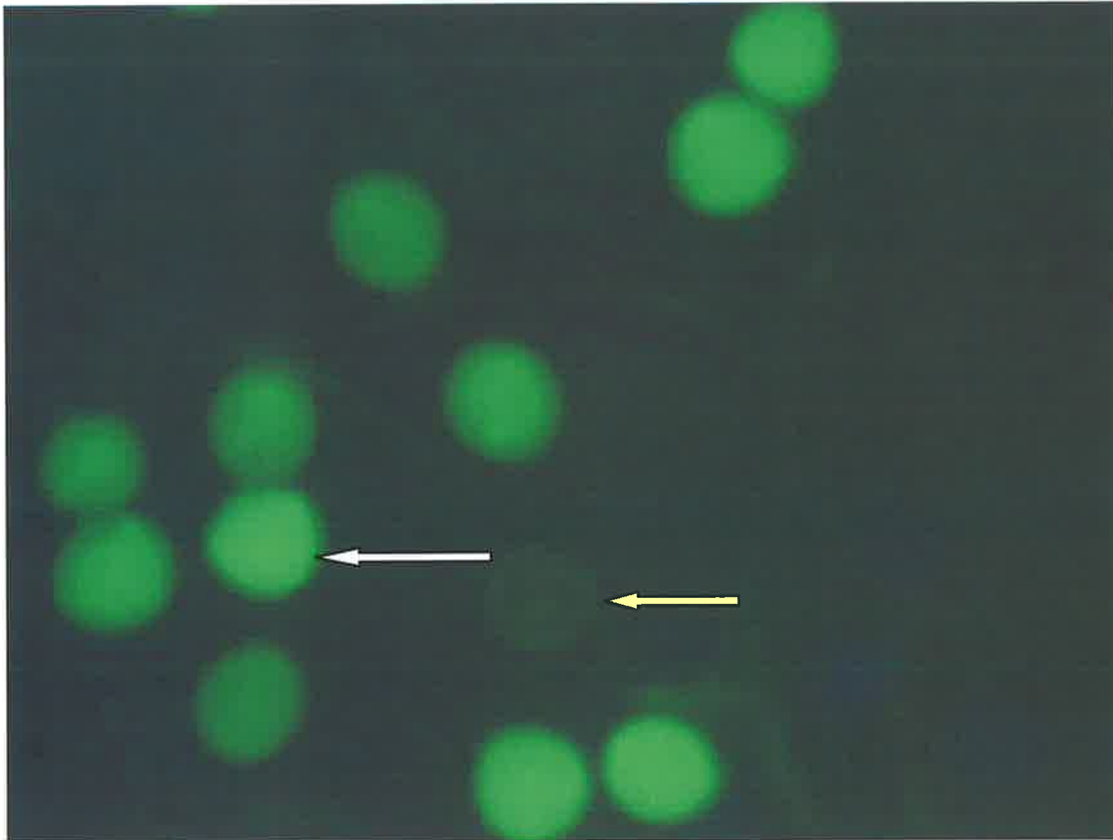

Fig. 5.8 Pollen from Leccino observed with fluorescein diacetate staining. Pollen grains showing bright green fluorescence (white arrow) are viable, while those without fluorescence (yellow arrow) are non-viable.

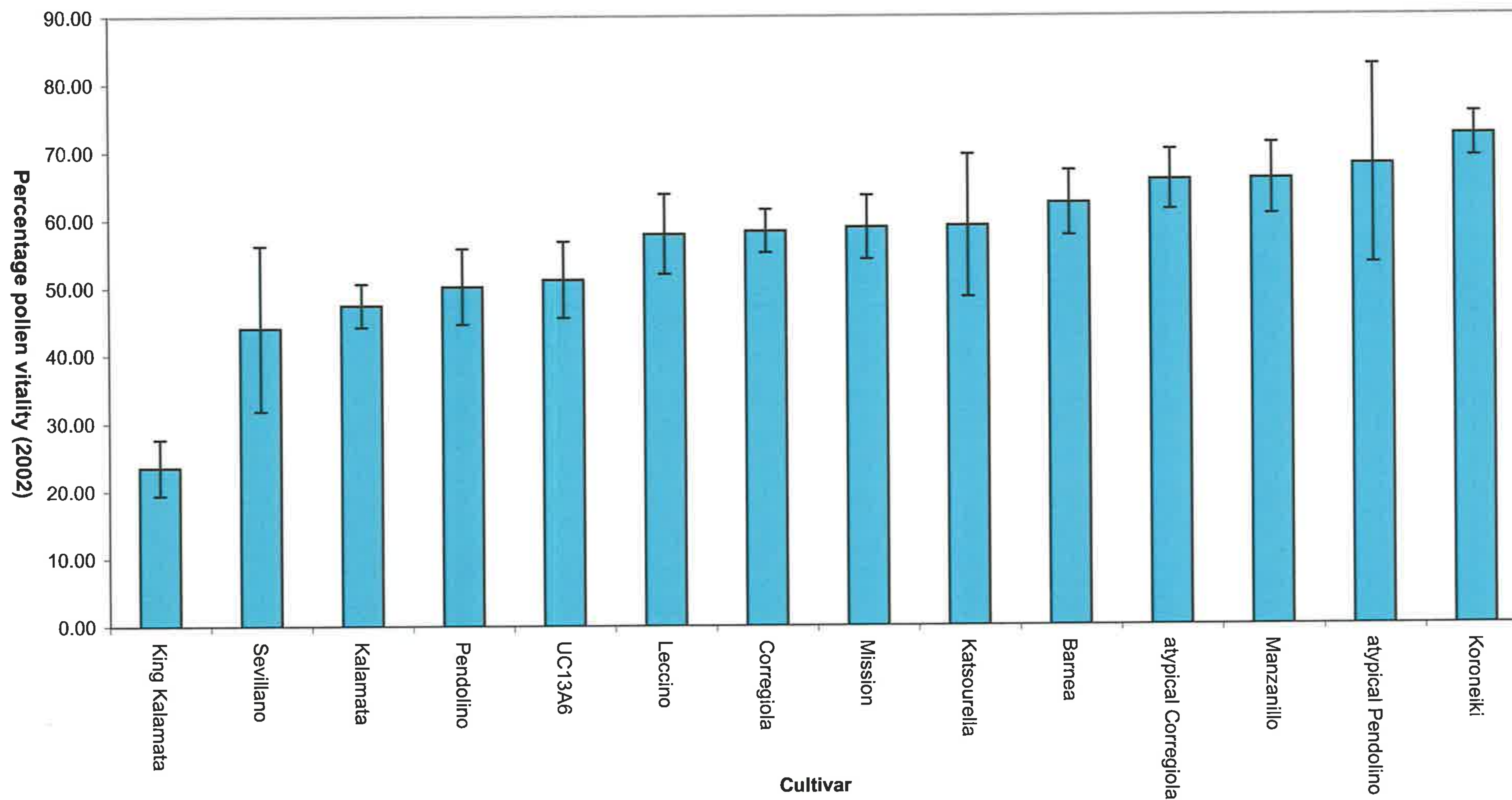

Fig 5.9 Percentage pollen vitality in the olive cultivars. Observations made in November-December 2002. Error bars show 95% confidence interval.

hand, Leccino and atypical Pendolino, which had a high percentage of complete flowers, also had comparatively high pollen vitality. There were no significant differences between trees of each cultivar (Appendix 2), although significant differences were observed between cultivars Fig 5.9.

In 2003, pollen samples were collected separately from inflorescences taken from four sides and the centre of each tree and used for vitality tests. After staining, the averages of five fields of view were recorded for each sample and the data were analysed using the General Linear Model of MINITAB with sub-sampling.

The pollen vitality ranged from 19.7% in UC13A6 to 65.5% in Leccino. UC13A6 and King Kalamata, which had significantly lower pollen vitalities compared to all other cultivars. Leccino had significantly higher pollen vitality than all other cultivars. There was no significant difference in the percentage pollen vitalities of Sevillano, Barnea, Pendolino, Kalamata, and Corregiola. Verdale and atypical Manaki lacked pollen in both years, indicating that they are unlikely to be major pollen donors. However, this observation was based on only five inflorescences per tree and it is possible that some flowers on the trees may have had viable pollen. Leccino, Koroneiki, Manzanillo, and Mission had high pollen vitalities ranging from 46.6% to 65.5%.

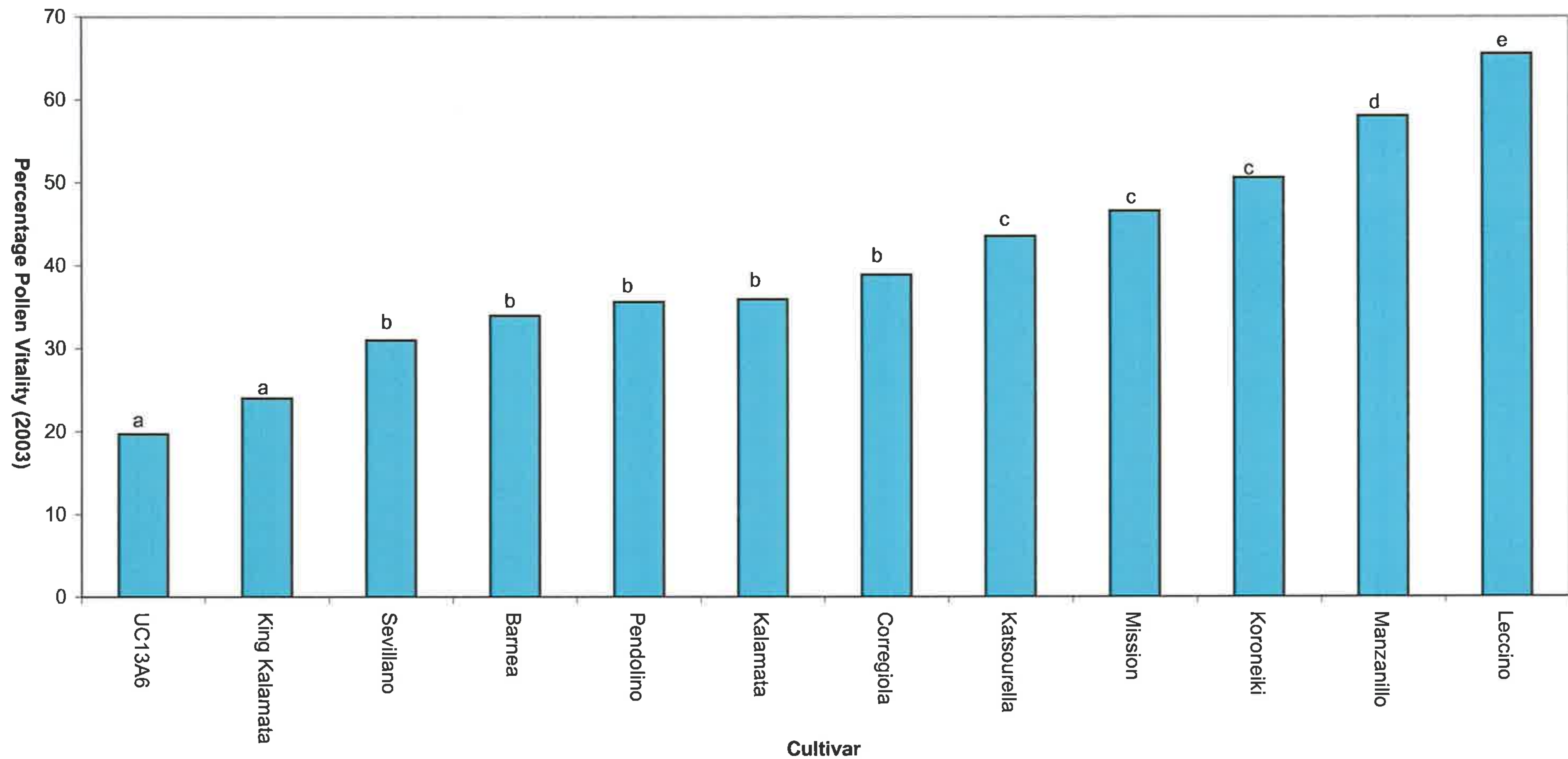

Fig 5.10 Percentage pollen vitality in the olive cultivars. Observations made in November-December 2003. The letters over the bars represent the significance groups. Cultivars having the same letter are not significantly different.

5.4 Discussion:

The data for bloom time, percentage of complete flowers, and pollen vitality generally show consistency among the selected trees of the same cultivar. However, there were considerable differences in the percentages of complete flowers and pollen vitality values between cultivars.

Based on the dates for bloom time in 2002, all cultivars may be considered as potential pollen donors for all other cultivars, although some, such as Kalamata and UC13A6, overlapped for only a short time, and atypical Corregiola flowered after some other cultivars were spent. In 2003, Kalamata and Leccino were the last cultivars to flower, and this occurred after atypical Manaki, Manzanillo, UC13A6, and Verdale were spent. All of the cultivars came into bloom at a later date during the second year as compared to the first year, but they remained in bloom for a shorter period. As a result the flowers were spent by about the same time. While in 2002, UC13A6 flowered much earlier than the other cultivars, in the following year, it started flowering along with the cultivars Verdale and atypical Manaki. Atypical Corregiola had the longest bloom period of 13 days in 2003, while in the previous year it was in bloom only for 9 days. The bloom period of replicate trees of a particular cultivar showed more consistency in the bloom time during the first year as compared to the second year. In 2003, replicate trees differed in their flowering dates by 1-3 days in some cultivars.

Previous studies have shown that trees of the same cultivar grown in similar environmental conditions bloom at the same time (Dal Pero Bertini, 1960), even though there may be some variation between years (Griggs *et al.*, 1975; Ghrisi *et al.*,

1999). Some of this variation may be a result of climatic conditions (Griggs *et al.*, 1975; Lavee *et al.*, 2002). Griggs *et al.* (1975) observed that cooler weather delayed flowering and when the weather conditions became warmer, all the cultivars studied by them started flowering. Induction of flowering occurs during the previous year as a consequence of winter chilling and this chilling requirement may vary depending on the cultivar. The average minimum and maximum temperatures during 2002 were 10.3°C and 21.7°C respectively compared to those during 2003, which were 10.3°C and 22.8°C. Therefore the temperatures during the bloom period were similar during the two years of study. High temperatures and relative humidity have been found to be unconducive to fruit set (Androulakis and Loupassaki, 1990). Olives are wind pollinated and therefore drier conditions are likely to cause more effective pollen dispersal. Since there was less rainfall during the second year of the trial, this may have allowed more efficient movement of pollen between trees. However, the rainfall was only a few millimetres and so any major effect on pollen dispersal is unlikely.

All cultivars with viable pollen possess the ability to act as pollen donors. However, it was observed that the pollen vitality varied significantly among cultivars. In particular, Verdale and atypical Manaki were found to be male sterile in both 2002 and 2003. Therefore, based on the sample of flowers that were tested, it appears unlikely that these two cultivars would act as major pollen donors. However, there may have been some viable pollen either in the unsampled flowers or later in the season that could have contributed to pollination. Studies conducted by Wu (2002) showed that the cultivar SA Verdale (called Verdale in the present study) was male sterile for most of the bloom period during the year 2000, but towards the end of the

bloom period it was found to bear some viable pollen with a percentage viability of 5.0%. The occurrence of male sterility in Verdale in some seasons in South Australia, but not in others (S. Wu, pers. comm.), suggests that the effect is due to an interaction of particular environmental conditions on the genotype of this cultivar. Further work is needed to explain this phenomenon including sampling flowers over the whole flowering period.

However, marked variations in pollen vitality were found to occur between some cultivars, such as UC13A6, Barnea, King Kalamata, and Koroneiki, and in general, the pollen vitality of all cultivars, except for Leccino and King Kalamata, decreased during the second year. More information is needed on the possible environmental conditions that contribute to the variability of pollen vitality from year to year. Also, cultivars that show high variability in pollen vitality from year to year need to be identified, and research carried out to determine whether this plasticity affects their usefulness as pollen donors.

The cultivars Leccino, Katsourella, atypical Manaki, and Corregiola consistently had high percentages of complete flowers in both years, whereas the percentages decreased in the case of Barnea, Mission, Koroneiki, and Kalamata, and increased in the case of Manzanillo. Water stress experienced by the tree during the period of early flower development has been found to be a cause of pistil abortion (Hartmann and Panetsos, 1961). However, in the present study, the grower supplemented natural rainfall by artificial irrigation (Section 3.1.1) and so it is unlikely that the trees would have experienced water stress. Another factor that may have influenced the differences in morphological characters between the two seasons is the age of the

tree. Since trees of different cultivars varied in age from 1 to 4.5 years, it is likely to have affected the variation in bloom period, percentage pollen vitality and complete flowers among the years. Also, many trees of cultivars Corregiola and Verdale were only about a year old during the first year of study. So it is possible that differences in morphological characters will be observed as they grow older (Garcia *et al.*, 2000). Verdale showed significant increase in percentage of complete flowers in the second year, while the value was high for both years in case of Corregiola. However, Corregiola showed a decrease in pollen vitality during the second year. It is possible that some cultivars have a more sensitive environment x genotype interaction, or phenotypic plasticity, than others that appear to be more environmentally stable, and further research is needed to clarify this. This observation highlights the need to identify the environmental conditions suited to different cultivars, and to derive recommendations for growers who are contemplating new orchards.

This study compared a number of characteristics of olive cultivars between two seasons. It was found that bloom time, percentage of complete flowers, and pollen vitality are reasonably consistent within a particular cultivar in a particular season. However, the variations in the bloom period, percentage complete flowers, and pollen vitality between the cultivars and between years emphasises the value of recording both sets of data to assess potential fecundity. In addition, the observations extend the information available for maximising yield in olive by enabling the selection of cultivars with overlapping bloom time. However, it is likely that bloom time varies from year to year, and therefore these observations need to be carried out over several more seasons in order to develop clear guidelines for the establishment

of new orchards by olive growers. Paternity analysis of the embryos may show whether these variations in the bloom time, percentage of complete flowers and pollen vitality affect fruit set in these plants.

Chapter Six

Analysis of Fruits: Identification of Pollen Donors

6.1 Introduction:

Previous studies by Wu *et al.*, (2002) showed that while certain cultivars are cross-compatible, others are incompatible with each other. Also, Fontanazza *et al.* (1980) and Lavee *et al.* (2002) observed that even self fertile cultivars gave higher yields with cross-pollination. However, these studies were based on artificial transfer of pollen from one cultivar to another, and may not represent pollen behaviour in a mixed orchard under natural pollination conditions.

This chapter describes a study conducted in the olive orchard at Gumeracha (Section 3.1.1) and the results obtained through paternity analysis to identify pollen donors for embryos. The genotype of each embryo was compared with that of the mother tree and with that of all the potential pollen donors to obtain the most likely father based on segregation of the particular alleles that were used as markers (Thompson and Meagher, 1987).

6.2 Material:

Olive fruits collected from the two selected mother trees of five cultivars (Fig 3.1) were used for the study. The fruits were collected approximately 15 weeks after full bloom when the embryos had reached maturity so that the triploid endosperm was completely absorbed (Reusch, 2000). Approximately 15 fruits were collected from each of the north, south, east, and west side of each mother tree (about 60 fruits per

tree) to ensure random sampling across the canopy, packed into paper bags so that the fruits from each side were kept separate, and stored at 4°C. Ten fruits were randomly sub-sampled from each bag and used for DNA extraction to give a total of 40 fruits analysed per tree. The extra fruits were collected to allow for the possible occurrence of parthenocarpic fruits, which do not have embryos.

All the cultivars present in the orchard were regarded as potential pollen donors. The number of trees of each cultivar varied from 1, in the case of atypical Corregiola, to 526 in the case of Mission. The trees had been planted at different times and the approximate tree age during the first year of study varied from 1 year to 4.5 years. The number, age, and location of the cultivars in the property are presented in Table 6.1.

6.3 Methodology:

The fleshy pericarp of each fruit was removed and the embryo was dissected out from inside the stone (Fig 6.1). DNA was extracted from the embryos according to the procedure described in section 3.2.6. One micro-litre of the DNA obtained in TE buffer solution was used for PCR as described in section 3.2.7. The primers EMO2, UDO99-006, UDO99-008, UDO99-031, UDO99-024, ssrOeUA-DCA3, ssrOeUA-DCA4, and ssrOeUA-DCA14 were used for fingerprinting the embryos. The sequences of the primers, along with the annealing temperature, and the number and range of alleles scored are shown in Table 3.1. Samples of the PCR products were observed on 1.75% (w/v) agarose gels to check that amplification had occurred prior to separation using the ABI Prism 3700 DNA Analyser (section 3.2.9). The data obtained were analysed using the program Genescan version 3.5.1 (P.E. Biosystems,

Table 6.1 Cultivars used in the study, age of trees (at November 2002), location in the orchard, and number of trees present.

Cultivar	Tree Age (yrs) Nov. 2002	Location Valve No. Refer Fig 3.1	Number of trees*
Barnea	2.1	V7	2
Corregiola	1.0	V4/5	20
Corregiola	2.3	V6	49
Corregiola	4.5	Trial Plot	21
atypical Corregiola	1.0	V4/5	1**
Kalamata	2.8	V3	13
Kalamata	4.5	V7+V8	215
atypical Kalamata	4.5	V7	4**
Koroneiki	4.4	V4/5	249
Katsourella	2.1	V7	5
King Kalamata	3.9	V7	26
Leccino	4.5	Trial Plot	11
atypical Manaki	4.4	V4/5	17
Manzanillo	2.8	V3	22
Mission	3.9	V1 + V2	526
Pendolino	4.5	V7+V8	16
atypical Pendolino	2.8	V3	18**
Sevillano	2.1	V7	3
UC13A6	3.8	V3	232
Verdale	1.3	V4/5 + V6	42

*Only sample trees of each cultivar were identified by DNA fingerprinting. It is possible that additional atypical trees were present.
** Only trees identified through DNA fingerprinting are listed. It is possible that there were more trees of the same genotype in the orchard.

Portion of fleshy pericarp
to show the stone.

Stone opened to obtain seed.

Seed separated from stone.

Fig 6.1 Olive fruit cut open to show the stone and seed. The seed is dissected to obtain embryo which is used for microsatellite analysis

850 Lincoln Centre Drive, Foster City, California 94404, U.S.A.) (Section 3.2.9). The peaks present after amplification by each primer were scored, and recorded on a Microsoft® Excel sheet in the form of a binary matrix indicating presence or absence of a particular allele. The PCRs were repeated at least twice to ensure that consistent PCR products were obtained.

The genotypes of the mother trees and the embryos were scored and recorded in the form of a text file and used for paternity analysis by the software program FaMoz (Dr Sophie Gerber, INRA-Recherches Forestieres-BP45, Laboratoire de Genetique et Amelioration des Arbres Forestiers, 33611 Gazinet Cedex, France, 2003) (Section 3.2.10.3). The allele frequencies in the potential pollen donors were also included, and the most likely father was determined for each embryo. Prior to paternity testing, a simulation was done using 1000 progeny to calculate the threshold of paternity. Inclusion of the threshold for paternity ensured that only genotypes having a LOD score higher than this value would be considered as likely fathers. When more than one genotype was obtained as likely fathers, then the genotype with the highest LOD (log likelihood) score was selected.

Relationship between number of trees and number of embryos pollinated was explored using the simple regression analysis function of GenStat, 7th Edition.

6.4 Results:

6.4.1 Markers used for paternity analysis:

The eight microsatellite primers that were used for the analysis were tested on the progeny of a cross between Frantoio x Kalamata, and the amplification products

were found to segregate with a ratio of 1:1:1:1. The segregation of primers in the progeny was analysed using the Chi Square test. The number of alleles amplified per primer ranged from four in EMO2 to eight in ssrOeUA-DCA4. The average number of alleles per primer was 6.4, and the cumulative exclusion probability of the eight primers was 0.998. This indicates that the primer set used for the study had a high probability of excluding all unlikely fathers. During the paternity analysis, individual primers were removed successively from the data set to observe the effect on identification of potential fathers. The least effect was observed after removal of EMO2, which had the lowest individual exclusion probability and only four alleles. However, the removal of primers led to an increasing number of parents being identified as potential fathers indicating that the information provided by the complete set of eight primers increased the level of discrimination.

6.4.2 Results of paternity analysis:

6.4.2.1 Barnea: Paternity analysis was carried out on fruits collected from two Barnea trees located in Valve 7 on the same row. The immediate neighbours were King Kalamata, Sevillano, Katsourella, Kalamata, and Pendolino. However, since the wind direction data showed that adequate mixing of pollen would have occurred (Section 5.3.1), it was assumed that pollen from all cultivars had some probability of reaching the flowers of Barnea.

6.4.2.1.1 Barnea results from first year of study (2002-2003): Flowering commenced on November 22, the trees reached full bloom on November 25, and flowering continued until November 30. The temperatures at each of these stages were 25°C, 20°C, and 19°C respectively. The day after the start of bloom

(November 23) was warm with maximum temperature of 32°C. There were a few rainy days around the time of full bloom, with a maximum of 19 mm during one period of 24 hours on November 26. This bloom period overlapped with all other cultivars except for atypical Corregiola. The stage of full bloom coincided with the bloom period of Corregiola, Koroneiki, Katsourella, King Kalamata, atypical Manaki, Manzanillo, Mission, Pendolino, atypical Pendolino, Sevillano, UC13A6, and Verdale. The pollen vitality was 62.4% indicating that there was sufficient viable pollen available for self-fertilisation, and the percentage of complete flowers was 71.5% showing that there was a large proportion of female flowers for fruit set.

Barnea Tree 1 (Year 1): This tree had relatively few fruits, so 32 from the north and 8 from the east were studied. After paternity analysis of the embryos collected from this tree, pollen donors were identified for 34 out of 40 embryos. The threshold for paternity was 0.02 and the LOD scores ranged from 2.57 to 9.71. For ten of the embryos, the pollen donors were unequivocal, being Kalamata (3), Sevillano (2), Katsourella (2), Verdale (2), and atypical Pendolino (1). The remaining embryos were most likely pollinated by Pendolino (15), Mission (3), Leccino (2), Sevillano (2), Kalamata (1), and atypical Kalamata (1) (Table 6.1). Pollen donors for the remaining six embryos could not be determined.

Among the embryos collected from the east, two were pollinated by Leccino, three by Pendolino, one by Mission, one by atypical Pendolino, and one was unidentified. Among the embryos analysed from the north, twelve were pollinated by Pendolino, four by Kalamata, two by Mission, four by Sevillano, two by Katsourella, two by Verdale, one by atypical Kalamata, and for five the pollen donors were unidentified.

Cultivars Sevillano, Katsourella, and Verdale were located to the north of the Barnea tree.

Barnea Tree 2 (Year 1): Ten fruits were collected from each side, north, south, east, and west of the second Barnea tree. Paternity analysis identified potential pollen donors for 28 out of the 40 embryos analysed. The threshold for paternity was 0.02 and the LOD scores ranged from 1.10 to 4.29. A single father was identified in the case of 8 embryos, which included Verdale (2), Sevillano (4), Kalamata (1), and atypical Kalamata (1). In the case of the remaining embryos, the most likely pollen donors were Pendolino (8), Kalamata (4), Corregiola (3), Katsourella (1), atypical Pendolino (1), Leccino (1), Sevillano (1), and atypical Kalamata (1) (Table 6.2).

Fruits from the north side of the tree were pollinated by Pendolino (1), Verdale (1), Katsourella (1), Sevillano (1), atypical Pendolino (1) and for five the pollen donors were unidentified. Cultivars located towards the north of the mother tree were Katsourella, Sevillano, Verdale, and Leccino. Fruits from the south were pollinated by Pendolino (2), Kalamata (2), atypical Kalamata (1), Leccino (1), Corregiola (1), and for three the pollen donors were unidentified. Pendolino and Corregiola were located towards the south of the mother tree. Fruits from the east were pollinated by Pendolino (3), Corregiola (2), Verdale (1), Kalamata (1), Sevillano (1) and for two the pollen donors were unidentified. Only Verdale was towards the east of the tree. In the west, the fruits were pollinated by Sevillano (3), Kalamata (2), Pendolino (2), atypical Kalamata (1), and for two the pollen donor was unidentified. Only Kalamata was planted to the west of the tree.

Among the embryos tested from both the trees, Pendolino pollinated 29%, Kalamata and Sevillano each pollinated 11%, Verdale pollinated 5%, Mission, Corregiola, Leccino, Katsourella and atypical Kalamata 4% each, atypical Pendolino pollinated 3%, and 23% were pollinated by unidentified donors. These results are represented in Table 6.2. Cultivars King Kalamata, Koroneiki, Manzanillo, UC13A6, atypical Corregiola, and atypical Manaki did not pollinate any of the embryos that were analysed. No selfing was observed in the embryos studied.

6.4.2.1.2 Barnea results from second year of study (2003-2004): During the second year, flowering started on November 27 and extended until December 7. The maximum temperatures were around 26-27°C at the beginning and end of bloom. However, temperatures went down to 18.9°C just before full bloom, returning to 26-27°C at the time of full bloom (December 2-3). The coldest day of the period was on December 5 with maximum temperature 15.7°C. There was little rain, amounting to 0.4 mm, on December 1 and 4.

Full bloom coincided with full bloom stage in Corregiola, Katsourella, Mission, Pendolino, and UC13A6. However, bloom period of Barnea overlapped with all the cultivars, including Kalamata, atypical Kalamata, and Leccino, which flowered late. There were 56.9% complete flowers and pollen vitality was 33.9%.

Barnea Tree 1 (Year 2): Pollen donors were identified for all of the 40 embryos tested from this tree. Out of these, there was only one possible pollen donor for 13 embryos, including Kalamata (5), Pendolino (3), Mission (2), Verdale (2), and Corregiola (1). Among the remaining embryos, 9 were pollinated by Mission, 3 by

Kalamata, 5 by King Kalamata, 4 by UC13A6, 2 by Sevillano, 3 by Verdale, and 1 by Pendolino. The threshold for paternity was 0.06 and the LOD scores ranged from 0.62 to 7.51. These results are presented in Table 6.2.

The embryos collected from the northern side of the tree were pollinated by Mission (4), King Kalamata (2), Kalamata (1), UC13A6 (1), Sevillano (1), and Verdale (1). Out of these cultivars, Sevillano, King Kalamata, and Kalamata were located close by towards the north. Embryos collected from the south were pollinated by King Kalamata (1), Mission (3), Kalamata (4), and UC13A6 (2). Kalamata, Mission, and UC13A6 were among the cultivars located towards the north, although Mission and UC13A6 were located at some distance. Towards the east, the main pollen donors were Mission (2), Pendolino (2), Kalamata (2), UC13A6 (1), Corregiola (1), King Kalamata (1), and Verdale (1). Only Corregiola and Verdale were planted towards the east of the Barnea trees. Embryos collected from the west were mainly pollinated by Verdale (3), Mission (2), Pendolino (2), King Kalamata (1), Kalamata (1), and Sevillano (1). Only the cultivar Kalamata was located towards the west of the Barnea trees, although there were Verdale, Pendolino, Katsourella, and Sevillano trees in the proximity.

Barnea Tree 2 (Year 2): Among the forty embryos analysed from this tree, pollen donors were identified for 35 of them (Table 6.2). Pollen donors were unequivocal for 7 of the embryos and these were UC13A6 (3), Pendolino (1), King Kalamata (1), Kalamata (1), and Verdale (1). The most likely pollen donors for the remaining embryos were Mission (16), Sevillano (4), King Kalamata (2), Verdale (3), UC13A6

(2), and Pendolino (1). The threshold for paternity was 0.06 and the LOD scores ranged from 0.41 to7.55.

Ten fruits were collected from each side of the tree. The embryos collected from the north were pollinated by UC13A6 (2), Sevillano (2), Mission (3), Pendolino (1), and for two the pollen donors were unidentified. Sevillano was the only cultivar among these which was located towards the north. In the south, the main pollen donors were Verdale (3), Mission (3), King Kalamata (1), Pendolino (1), Kalamata (1) and for one the pollen donor was unidentified. King Kalamata and Pendolino were among the cultivars planted towards the south of the Barnea trees. The main pollen donors for the fruits collected from the east were Mission (5), King Kalamata (1), Sevillano (1), UC13A6 (1), Verdale (1), and for one the pollen donor was unidentified. Out of these cultivars, King Kalamata and Verdale were among the neighbours towards the east. The pollen donors towards the west were Mission (5), UC13A6 (2), King Kalamata (1), Sevillano (1), and for one the pollen donor was unidentified. None of these cultivars were planted towards the west of the Barnea tree, although King Kalamata and Sevillano were close neighbours.

Based on the results obtained from analysis of 80 fruits collected from the two Barnea trees, Mission was the main pollen donor in 2004 pollinating 34% of the embryos. The other pollen donors were UC13A6, Kalamata and Verdale pollinated 11% of the embryos each, King Kalamata pollinated 10% of the embryos, Sevillano and Pendolino pollinated 8% of the embryos, and Corregiola pollinated 1% of the embryos, and 6% of the embryos were pollinated by unknown genotypes. Leccino, Katsourella, Koroneiki, Manzanillo, atypical Kalamata, atypical Corregiola, atypical

Pendolino, atypical Corregiola, and atypical Manaki did not pollinate and of the embryos studied in 2004.

Table 6.2. Results from paternity analysis of Barnea embryos.

Pollen donor	Number of embryos				Total		Percentage Embryos	
	Tree1		Tree2					
	2003	2004	2003	2004	2003	2004	2003	2004
Pendolino	15	4	8	2	23	6	29	8
Kalamata	4	8	5	1	9	9	11	11
Sevillano	4	2	5	4	9	6	11	8
Mission	3	11	0	16	3	27	4	34
Corregiola	0	1	3	0	3	1	4	1
Verdale	2	5	2	4	4	9	5	11
Leccino	2	0	1	0	3	0	4	0
Katsourella	2	0	1	0	3	0	4	0
atypical Kalamata	1	0	2	0	3	0	4	0
atypical Pendolino	1	0	1	0	2	0	3	0
King Kalamata	0	5	0	3	0	8	0	10
UC13A6	0	4	0	5	0	9	0	11
Unknown	6	0	12	5	18	5	23	6

6.4.2.1.3 Barnea embryos with unidentified pollen donors: Pollen donors for 18 embryos in 2003 and 5 embryos in 2004 could not be identified. When analysed using FaMoz, the software concluded that there was no likely father inside the pool of potential paternal genotype provided. This indicated that these embryos were fathered by a genotype which was either present in the stand but was not identified as different, or whose wind-borne pollen was carried from outside the study site. No feral olive trees were observed near the site, although presence of such trees inside the neighbouring properties cannot be ruled out. There is also an olive orchard at a distance of about 200 m from the study site. The grower of this site claimed to have the same cultivars in his orchard, although the trees were not fingerprinted. The fingerprints of the embryos whose fathers could not be identified were visually analysed to determine if their paternal genotype could be identified. It was observed that the paternal alleles were contributed by more than one genotype with the alleles presented in Table 6.3 in any combination. Since there were more than one unidentified pollinating cultivar, the genotype of the pollen donor could not be determined. However, the paternal alleles in the unidentified embryos from both the years were similar.

6.4.2.1.4 Conclusions from analysis of Barnea embryos over two seasons: Paternity analysis of Barnea embryos over two seasons showed that while some pollen donors pollinated Barnea consistently, there were others which were sporadic. Cultivars Pendolino, Kalamata, Sevillano, Mission, Corregiola, and Verdale were among the pollen donors in both the years. On the other hand Leccino, Kalamata, atypical Kalamata, Katsourella, and atypical Pendolino fathered Barnea embryos

Table 6.3 Combination of alleles in the unknown genotypes pollinating Barnea embryos.

Primer	Combination of alleles (in bp) in pollen donor
ssrOeUA-DCA3	238, 241, 252, null
EMO2	203, 212, null
UDO99-006	146, 167, null
UDO99-008	157, 161, 166, null
UDO99-031	140, 151, and either 108 or 110
ssrOeUA-DCA4	131, 137, 155, null
ssrOeUA-DCA14	172, 179, 187, null
UDO99-024	166, 181, 186, and either 188 or 194

only during the first year, while King Kalamata and UC13A6 did the same only during the second year.

The absence of Leccino as the pollen donor during the second year may be explained by the fact that it flowered late during the second year and so the period of overlap of bloom time of the cultivars was shorter. Kalamata was also a late flowering cultivar in both the years. However, Kalamata was located near the Barnea trees and also had greater number of trees than Leccino. This could be the reason that Leccino pollinated some Barnea embryos during the first year, although none during the second year. During the second year, atypical Kalamata started flowering when Barnea had almost reached end of its bloom period. However, even though the bloom period of atypical Pendolino and Katsourella corresponded with that of

Barnea, these cultivars successfully pollinated Barnea during the first year, and not in the second year. King Kalamata, which was planted close to the Barnea trees, had low pollen vitality in both years, although it pollinated 10% of the embryos during the second year. UC13A6 was observed to have a pollen vitality of 52% in the first year, and 20% in the second year. However, it pollinated 11% of the Barnea embryos during the second year and none during the first year.

There were significant differences in the proportion of embryos fathered by individual cultivars in the two years. Pendolino was the major pollinating cultivar in the first year having pollinated 29% of the embryos. However, in the following year, Pendolino pollinated only 8% of the embryos. The major pollinating cultivar in 2004 was Mission which pollinated 34% of the embryos, even though it had pollinated only 4% of the embryos in the previous year. Verdale pollinated 11% of the embryos during the second year, as compared to 5% during 2003, while Corregiola pollinated 4% of the embryos in 2003 and 1% in 2004. Kalamata and Sevillano performed similarly in both years. There were also 18 embryos with unidentified fathers in 2004, as compared to 5 in 2003. Visual analysis of the genotypes of these embryos indicated that there was more than one unidentified pollen donor involved. One or more of these unidentified pollen donors is thought to have made a higher contribution to pollination during the first year of study. The cultivars Koroneiki, Manzanillo, atypical Manaki, and atypical Corregiola did not pollinate any of the embryos studied in either year. No selfing was observed in the Barnea embryos tested.

6.4.2.2 Corregiola: The two Corregiola trees selected for paternity analysis were located in valve 6. Both the trees were surrounded by other Corregiola trees. Trees of the cultivar King Kalamata were also located next to the first tree.

6.4.2.2.1 Corregiola results from first year of study (2002-2003): The bloom period in Corregiola overlapped with all other cultivars except atypical Corregiola which started flowering after Corregiola was spent. Bloom period started on November 23 along with start of bloom in Koroneiki, atypical Manaki, Mission, and Pendolino. The day of start of bloom was relatively warm with maximum temperature of 32°C. Gradually the temperatures cooled down and were below 20°C at the time of full bloom. Full bloom occurred between November 25 and 27 in the different trees tested. The period of full bloom coincided with rainfall and there was 19 mm of rainfall on November 26. Full bloom in Corregiola coincided with full bloom in Barnea, Koroneiki, Katsourella, atypical Manaki, Manzanillo, Mission, Pendolino, Sevillano, atypical Pendolino, and UC13A6, Verdale. The flowers were spent on November 30. Around 88.8% of the flowers were complete and pollen vitality in was 58.3%.

Corregiola Tree 1 (Year 1): Of the forty embryos tested from this tree, pollen donors for 32 embryos were identified (Table 6.4). Among them, single pollen donors were identified for 19 embryos. These included Kalamata (10), atypical Manaki (8), and Leccino (1). The most likely pollen donors for the remaining embryos were Mission (4), Pendolino (3), Sevillano (2), Kalamata (1), Barnea (1), atypical Pendolino (1), and atypical Manaki (1). The threshold for paternity was 0.02 and the LOD scores for the best fathers ranged from 0.34 to 7.42.

Pollen donors towards the north were atypical Manaki (4), Kalamata (2), Pendolino (1), Mission (1) and two were unidentified. Kalamata and Pendolino were planted to the north of this tree, although at some distance. Towards the south, the flowers were pollinated by Kalamata (4), atypical Manaki (1), Mission (1), Pendolino (1), atypical Pendolino (1), and the remaining two were unidentified. Towards the east, the pollen donors were Kalamata (4), Mission (2), Pendolino (1), atypical Manaki (1) and two unidentified. In the west the pollen donors were atypical Manaki (3), Kalamata (1), Sevillano (2), Barnea (1), Leccino (1) and two were unidentified. Out of these cultivars, only Kalamata was located to the west of the tree, although Sevillano, atypical Manaki, and Barnea were located nearby.

Corregiola Tree 2 (Year 1): For the second Corregiola tree, pollen donors were identified for 36 embryos out of the 40 embryos tested (Table 6.4). Five of them had a single identified pollen donor. These included Kalamata (2) and atypical Manaki (3). The remaining embryos were most likely pollinated by Mission (19), Kalamata (3), Sevillano (2), Manzanillo (2), Verdale (2), Pendolino (1), atypical Pendolino (1), and Katsourella (1). The threshold for paternity was 0.02 and the LOD scores ranged from 1.40 to 8.78.

The embryos collected from the north of the tree were pollinated by Mission (5), Kalamata (3), Manzanillo (1), and Verdale (1), out of which Verdale was located at some distance towards the north. Towards the south, the embryos were pollinated by Mission (5), Manzanillo (1), Katsourella (1), atypical Manaki (1), atypical Pendolino (1), and for one the pollen donor was unidentified. In the east, the pollen donors were Mission (3), atypical Manaki (2), Verdale (1), Pendolino (1), Sevillano (1), and

for two the pollen donor was unidentified. Atypical Manaki was located towards the east of this tree. Towards the west, embryos were pollinated by Mission (6), Kalamata (2), Sevillano (1), and for one the pollen donor was unidentified. Kalamata was planted towards the west.

Based on the results obtained from embryos collected from both trees, it was observed that Mission pollinated 29% of the embryos, Kalamata pollinated 20% of the embryos, atypical Manaki pollinated 15% of the embryos, Pendolino and Sevillano pollinated 5% of the embryos, atypical Pendolino, Manzanillo, and Verdale pollinated 3% of the embryos, Barnea, Katsourella, and Leccino pollinated 1% of the embryos, and pollen donors for 15% of the embryos could not be analysed. No self-pollination was observed and the cultivars atypical Corregiola, King Kalamata, Koroneiki, UC13A6, and atypical Kalamata were not found to be pollen donors for any of the embryos tested.

6.4.2.2.2 Corregiola results from second year of study (2003-2004): Bloom period in Corregiola started on November 29 and extended till December 7. Full bloom was on December 2-3 and coincided with the full bloom period of cultivars Barnea, Katsourella, Mission, Pendolino, and UC13A6. Full bloom in Corregiola corresponded with the final stages of bloom in Verdale, while the end of bloom in Corregiola corresponded with full bloom in Kalamata and Leccino, and start of bloom in atypical Kalamata. Maximum temperatures were in the range 25-27°C at the start, full, and end of bloom. However, there were some cooler days during the bloom period when the temperatures went down to 18-20°C. The coldest day of the period was December 5 when the maximum temperature was 15.7°C. There were

three wet days during this bloom period. December 2 and 4 had 0.4 mm rainfall, while December 5 experienced 1 mm of rainfall, which was the maximum during the bloom period. Corregiola had 91.7% complete flowers and pollen vitality was 38.9%.

Corregiola Tree 1 (Year 2): Paternity analysis was used to identify pollen donors for forty embryos of which only 25 could be identified (Table 6.4). Of these embryos, 14 had only one likely father. These likely fathers were Kalamata (7) and atypical Manaki (7). The most likely fathers for the remaining embryos were Mission (8), Pendolino (2), and Sevillano (1). The threshold for paternity was 0.02 and the LOD scores ranged from 0.25 to 8.62.

Ten embryos were analysed from north, south, east, and west of the tree. The pollen donors for the embryos collected from the north were Mission (2), Kalamata (1), Sevillano (1), and Pendolino (1). Pollen donors for five embryos could not be determined. Cultivars Sevillano and Pendolino were located towards the north of this tree at some distance. The embryos collected from the south were fathered by atypical Manaki (3), and Kalamata (2), while for five of them donors were unidentified. Towards the east, the pollen donors were Kalamata (3), atypical Manaki (3), Mission (1), and for three of them donors were unidentified. Of these pollen donors, only atypical Manaki was located towards the east, although at some distance. The pollen donors towards the west were Mission (5), Kalamata (1), Pendolino (1), and atypical Manaki (1). Pollen donors for two embryos collected from this side of the tree could not be identified. Kalamata and Pendolino were among the cultivars planted towards the west of this tree.

Corregiola Tree 2 (Year 2): Forty embryos were analysed from this tree and pollen donors for 31 embryos could be identified (Table 6.4). There was only one likely pollen donor for 16 embryos and these were atypical Manaki (8), Kalamata (6), Verdale (1), and Leccino (1). The most likely pollen donors for the remaining embryos were Mission (14) and Sevillano (1). The threshold for paternity was 0.02 and the LOD scores ranged from 0.10 to 7.42.

Of the ten embryos collected from the north of the tree, atypical Manaki pollinated three, Kalamata pollinated two, and Mission, Sevillano and Verdale pollinated one each. Pollen donors for two embryos were unidentified. Sevillano and Verdale were among the cultivars planted towards the north of this tree. The pollen donors for the embryos collected from the south were Mission (7), and atypical Manaki (2), and for one the donor was unidentified. Towards the east, the embryos were pollinated by Mission (4), Kalamata (2), atypical Manaki (1), and for three the donors were unidentified. Out of these cultivars, Mission and atypical Manaki were planted towards the east, although they were not immediate neighbours. Embryos collected from the west were pollinated by atypical Manaki (2), Kalamata (2), Mission (2), Leccino (1), and for three the donors were unidentified. Only the cultivar Kalamata was planted towards the west of this tree.

Paternity analysis of embryos collected from the two Corregiola trees indicated that Mission was the main pollen donor and this cultivar pollinated 28% of the embryos. Kalamata and atypical Manaki were also significant pollen donors pollinating 16% and 19% of the embryos respectively. Pendolino and Sevillano pollinated 3% of the embryos each, and Leccino and Verdale pollinated 1% of the embryos each. Pollen

donors for 30% of the embryos could not be identified. Cultivars Barnea, Katsourella, King Kalamata, Koroneiki, Manzanillo, UC13A6, atypical Pendolino, atypical Corregiola, and atypical Kalamata did not pollinate any of the embryos this year. No self-pollination was observed.

Table 6.4. Results from paternity analysis of Corregiola embryos.

Pollen donor	Number of embryos				Total		Percentage Embryos	
	Tree1		Tree2					
	2003	2004	2003	2004	2003	2004	2003	2004
Kalamata	11	7	5	6	16	13	20	16
atypical Manaki	9	7	3	8	12	15	15	19
Mission	4	8	19	14	23	22	29	28
Pendolino	3	2	1	0	4	2	5	3
Sevillano	2	1	2	1	4	2	5	3
Barnea	1	0	0	0	1	0	1	0
atypical Pendolino	1	0	1	0	2	0	3	0
Leccino	1	0	0	1	1	1	1	1
Manzanillo	0	0	2	0	2	0	3	0
Verdale	0	0	2	1	2	1	3	1
Katsourella	0	0	1	0	1	0	1	0
Unknown	8	15	4	9	12	24	15	30

6.4.2.2.3 Corregiola embryos with unidentified fathers: Pollen donors could not be identified for 12 embryos in 2003 and 24 embryos in 2004. Visual analysis of the genotypes of these embryos (Section 6.4.2.1.3) showed similar paternal alleles in embryos from both the years. Based on the paternal contribution of alleles in these genotypes, it was concluded that the embryos were pollinated by more than one unidentified cultivar with the allele combination presented in Table 6.5.

Table 6.5 Combination of alleles in the unknown genotypes pollinating Corregiola embryos.

Primer	Combination of alleles (in bp) in pollen donor
EMO2	203, null
UDO99-006	146, 167, 179, null
UDO99-008	155, 157, 166
ssrOeUA-DCA3	238, 247, 252
UDO99-031	108, 110, 151
ssrOeUA-DCA4	131, 164, null
ssrOeUA-DCA14	169, 182
UDO99-024	166, 177, 186, null

6.4.2.2.4 Conclusions from analysis of Corregiola embryos over two seasons: Paternity analysis results obtained from two years of study show fairly consistent results with regard to the main pollen donors for Corregiola. Mission was the main pollen donor in both the years, pollinating 29% embryos in 2003 and 28% embryos in 2004. Kalamata and atypical Manaki were also significant pollen donors.

Kalamata pollinated 20% of the embryos in 2003 and 16% in 2004. Atypical Manaki pollinated 15% of the embryos in 2003 and 19% in 2004. Pendolino, Sevillano, Leccino, and Verdale also pollinated some embryos in both years.

However, Katsourella, atypical Pendolino, Manzanillo, and Barnea pollinated Corregiola embryos only during the first year. No self-pollination was observed in the embryos studied and the cultivars King Kalamata, Koroneiki, UC13A6, atypical Corregiola, and atypical Kalamata did not pollinate any embryos in either year.

Some inconsistencies were observed in the pollen donors of embryos collected from Tree 1. Barnea, atypical Pendolino, Manzanillo, and Katsourella were identified as pollen donors only during the first year, while Verdale was found to pollinate embryos from only the second tree. However, the main pollen donors, Mission, Kalamata, and atypical Manaki remained fairly consistent. These three cultivars were the main pollen donors for both the trees. Sevillano and Verdale pollinated some embryos in both years. The cultivars Koroneiki, King Kalamata, atypical Kalamata, UC13A6, and atypical Corregiola did not pollinate any of the embryos tested, even though Koroneiki and King Kalamata had the advantage of proximal location. No self-pollination was observed in any of the embryos tested.

6.4.2.3: Koroneiki: Koroneiki trees were planted in Valve 4/5. One of the selected trees was located towards the interior of the valve where the immediate neighbours were other Koroneiki trees and atypical Manaki trees. The second selected tree was located towards the outer end of the valve where the immediate neighbours were Verdale, Corregiola, and Koroneiki.

6.4.2.3.1: Koroneiki results from first year of study (2002-2003): The Koroneiki trees started flowering on November 23 which was a warm day with maximum temperature 32°C. The flowers reached full bloom on November 27 and were spent by November 30. There was rainfall on November 24, 25, 26, and 27. Full bloom of Koroneiki corresponded with full bloom in Barnea, Corregiola, Katsourella, atypical Manaki, Manzanillo, Mission, and Pendolino. Bloom period overlapped with all other cultivars except atypical Corregiola, which started flowering one day after the flowers of Koroneiki were spent. Pollen vitality was 72.3% and percentage of complete flowers was 64%.

Koroneiki Tree 1 (Year 1): Paternity analysis identified fathers for 29 of 40 embryos (Table 6.6). Out of these, only one genotype was identified as the likely father in the case of 14 embryos. The remaining had multiple likely fathers, so the one with the maximum LOD score was selected as the most likely father. The threshold for paternity was 0.01 and the LOD scores ranged from 0.17 to 6.06. The genotypes that were found to have pollinated the embryos from this tree included Mission (9), Pendolino (4), atypical Manaki (5), Barnea (3), Leccino (3), Manzanillo (1), Verdale (1), King Kalamata (1), Katsourella (1), and Kalamata (1). Among the embryos for which only one possible pollen donor was identified, 5 were pollinated by atypical Manaki, 3 by Leccino, 2 by Barnea, and 1 each by Mission, King Kalamata, Katsourella, and Kalamata. No likely father was identified for 11 embryos. The tree was surrounded by other Koroneiki trees and atypical Manaki trees.

The fruits collected from the north of the tree were pollinated by Mission (1), Pendolino (2), atypical Manaki (3), Barnea (1), and for three the donors were unidentified. Fruits towards the south were pollinated by Mission (3), Barnea (1), Manzanillo (1), atypical Manaki (1), Verdale (1), and for three the donors were unidentified. The Mission trees were located towards the south of this tree. In the east, the pollen donors were Mission (4), Barnea (1), Leccino (1), King Kalamata (1), Katsourella (1), Pendolino (1) and for one the donor was unidentified. In the west, the embryos were pollinated by Kalamata (1), Leccino (2), Pendolino (1), Mission (1) atypical Manaki (1) and for four the donors were unidentified. Kalamata and Pendolino trees were located towards the west at some distance.

Koroneiki Tree 2 (Year 1): In case of the second Koroneiki tree, paternity analysis identified fathers for 34 of the 40 embryos analysed (Table 6.6). Among them, 26 were pollinated by Mission, 4 by Kalamata, 2 by Pendolino, and 1 each by atypical Kalamata and atypical Pendolino. Ten embryos had only one identified father and the pollen donors were Kalamata (4), Mission (5), Pendolino (1). The other embryos were pollinated by Mission (21), Pendolino (1), atypical Kalamata (1), and atypical Pendolino (1). The threshold for paternity was 0.01 and the LOD scores ranged from 0.35 to 8.65

The embryos analysed from the north showed that Mission was the main pollen donor. Mission pollinated 7 embryos, Pendolino pollinated 2 embryos and Kalamata pollinated 1 embryo. The pollen donors towards the south included Mission (7), Kalamata (1), atypical Kalamata (1) and for one the donor was unidentified. The immediate neighbouring trees towards the north and south were Koroneiki trees. In

the east, Mission pollinated 6 embryos, Kalamata pollinated 1 embryo, atypical Pendolino pollinated 1 embryo, and for two the donors were unidentified. In the west Mission pollinated 6 embryos, Kalamata pollinated one, and for three the donors were unidentified. There were some Verdale trees towards the west and mainly Koroneiki trees towards the east.

Among the embryos analysed, Mission pollinated 44%, Pendolino 8%, Kalamata, and atypical Manaki 6%, Barnea and Leccino 4%, and Manzanillo, Verdale, King Kalamata, Katsourella, atypical Kalamata, and atypical Pendolino pollinated 1%. Cultivars Sevillano, Corregiola, UC13A6, and atypical Corregiola were not present among the pollen donors identified. No likely father could be identified for 21% of the embryos. No self-pollination was observed in the embryos studied.

6.4.2.3.2: Koroneiki results from second year of study (2003-2004): During the second year, Koroneiki trees started to flower on November 29 and remained in bloom till December 8. Full bloom was on December 4 and coincided with the full bloom stage in atypical Corregiola, King Kalamata, Pendolino, and Sevillano. The cultivars atypical Manaki, Manzanillo, UC13A6, and Verdale had almost finished flowering when Koroneiki reached full bloom stage, while the cultivars Kalamata, atypical Kalamata, and Leccino did not start flowering. Bloom period of Koroneiki overlapped with all the cultivars to some extent. Maximum temperatures were around 18°C at the start of bloom and during full bloom, and around 30°C at the end of bloom. The coldest day of the period was December 5 with a maximum temperature of 15.7°C. There was some rainfall amounting to 0.4 mm on the day of

full bloom and 1 mm rainfall on the next day. There was also some rain on December 1. Pollen vitality was 50.6% and 29.0% of the flowers were complete.

Koroneiki Tree1 (Year 2): Out of the forty embryos studied from this tree, pollen donors for 34 were identified (Table 6.6). Ten embryos had only one likely father, being Kalamata (5), UC13A6 (3), atypical Pendolino (1), and atypical Manaki (1). The remaining embryos were pollinated by Mission (11), UC13A6 (3), atypical Manaki (2), Pendolino (2), Katsourella (2), Kalamata (1), Sevillano (1), atypical Kalamata (1), and Verdale (1). Pollen donors for six embryos could not be identified. The threshold for paternity was 0.01 and the LOD scores ranged from 0.20 to 9.44.

Ten embryos were studied from the north, south, east, and west of the tree. Among the embryos collected from the north, the pollen donors identified were Mission (3), Kalamata (1), atypical Pendolino (1), atypical Manaki (1), UC13A6 (1), and for three the donors were unidentified. Only the cultivar atypical Manaki was located towards the north, interspersed between the Koroneiki trees. Embryos collected from the south were pollinated by Mission (4), Pendolino (2), Kalamata (1), Sevillano (1), atypical Kalamata (1), and one was unidentified. The valves 1 and 2 containing all the Mission trees were towards the south of this tree. The pollen donors in the east were UC13A6 (3), Mission (3), Verdale (1), atypical Manaki (1), Katsourella (1), and one unidentified. The embryos collected from the west were pollinated by Kalamata (4), UC13A6 (2), Mission (1), atypical Manaki (1), Katsourella (1), and for one the donor was unidentified. Since this tree was near the outer edge of the

Koroneiki planting, it was near the Kalamata and UC13A6 valves which were towards the west.

Koroneiki Tree 2 (Year 2): Pollen donors were identified for 37 out of the forty embryos tested from this tree (Table 6.6). Ten of these pollen donors were unequivocal, being UC13A6 (8), Katsourella (1), and Kalamata (1). The remaining embryos were most likely pollinated by Mission (22), UC13A6 (4), and Sevillano (1). Pollen donors for 3 embryos could not be identified. The threshold for paternity was 0.01 and the LOD scores ranged from 1.05 to 10.88.

Ten embryos were analysed from the north, south, east, and west of the tree. The pollen donors towards the north were Mission (4), UC13A6 (4), Sevillano (1), and for one the donor was unidentified. The pollen donors in the south were Mission (7), UC13A6 (1), and Katsourella (1), and for one the donor was unidentified. The valves with the Mission trees were towards the south of the Koroneiki trees. Towards the east, the pollen donors were Mission (5), UC13A6 (4), and Kalamata (1). The pollen donors towards the west were Mission (6), UC13A6 (3), and unidentified (1).

The results obtained from analysis of 80 embryos collected from two Koroneiki trees indicated that Mission was the main pollen donor and pollinated 41% of the embryos UC13A6 pollinated 23% of the embryos, Kalamata pollinated 9% of the embryos, atypical Manaki and Katsourella pollinated 4% each, Sevillano and Pendolino pollinated 3% each, and Verdale, atypical Kalamata, and atypical Pendolino each pollinated 1% of the embryos. Pollen donors for 11% of the embryos could not be

identified. The cultivars Barnea, Corregiola, King Kalamata, Leccino, Manzanillo, and atypical Corregiola did not pollinate any of the embryos analysed. No self-pollination was observed.

Table 6.6 Results from paternity analysis of Koroneiki embryos.

Pollen donor	Number of embryos				Total		Percentage Embryos	
	Tree1		Tree2					
	2003	2004	2003	2004	2003	2004	2003	2004
Mission	9	11	26	22	35	33	44	41
atypical Manaki	5	3	0	0	5	3	6	4
Pendolino	4	2	2	0	6	2	8	3
Barnea	3	0	0	0	3	0	4	0
Leccino	3	0	0	0	3	0	4	0
Manzanillo	1	0	0	0	1	0	1	0
Verdale	1	1	0	0	1	1	1	1
King Kalamata	1	0	0	0	1	0	1	0
Katsourella	1	2	0	1	1	3	1	4
Kalamata	1	6	4	1	5	7	6	9
atypical Kalamata	0	1	1	0	1	1	1	1
atypical Pendolino	0	1	1	0	1	1	1	1
UC13A6	0	6	0	12	0	18	0	23
Sevillano	0	1	0	1	0	2	0	3
Unknown	11	6	6	3	17	9	21	11

6.4.2.3.3 Koroneiki embryos with unidentified fathers: Pollen donors for 21% of the embryos in 2003 and 11% of the embryos in 2004 could not be identified. Visual analysis of the fingerprints (Section 6.4.2.1.3) was able to identify the paternal alleles in the genotype of each embryo and it is proposed that these alleles (Table 6.7) are present in any combination in the unidentified pollen donors of these embryos.

Table 6.7 Combination of alleles in the unknown genotypes pollinating Koroneiki.

Primer	Combination of alleles (in bp) in pollen donor
EMO2	203, 212, null
UDO99-006	146, 167, null
UDO99-008	163, 166, null.
ssrOeUA-DCA3	230, 247, 252, null
UDO99-031	138, 140, 146, null
ssrOeUA-DCA4	155, 164, null
ssrOeUA-DCA14	169, 178, 182, 186
UDO99-024	166, 186, 188, null

6.4.2.3.4 Conclusions from analysis of Koroneiki embryos over two seasons: Data obtained from analysis of embryos collected from two trees over two seasons showed that Mission was the main pollen donor in both the years, pollinating 44% of the embryos in 2003 and 41% of the embryos in 2004. Pendolino, Kalamata, atypical Manaki, Verdale, Katsourella, atypical Kalamata, and atypical Pendolino

pollinated some embryos in both the years. However, Barnea, Leccino, Manzanillo, and King Kalamata pollinated Koroneiki embryos only during the first year. On the other hand UC13A6 was a significant pollen donor, pollinating 23% of the embryos in 2004, and none in 2003. Sevillano also pollinated some embryos in 2004, though not in 2003. The cultivars Corregiola and atypical Corregiola did not pollinate any of the Koroneiki embryos studied. There was no selfing in any of the embryos studied over the two years and no self-pollination was observed.

6.4.2.4: Kalamata:

The Kalamata trees were located in valves 7 and 8. The Kalamata tree in valve 8 was surrounded by other Kalamata trees, while Pendolino, King Kalamata, and UC13A6 were the closest neighbouring cultivars for the tree in Valve 7.

6.4.2.4.1 Kalamata results from first year of study (2002-2003): Bloom time observations of the cultivar showed that the trees came into bloom on November 28 when the temperature was 24°C, and were spent by December 7. Full bloom was on December 3 when the temperature was 17°C. There was some rainfall on December 5 and 6. Start of bloom corresponded with the full bloom stage of King Kalamata, Leccino, Mission, and atypical Manaki. Full bloom corresponded with start of bloom in atypical Corregiola and full bloom stage in King Kalamata. When Kalamata reached full bloom, the cultivars Barnea, Corregiola, Koroneiki, Katsourella, atypical Manaki, Manzanillo, Mission, Pendolino, atypical Pendolino, Sevillano, UC13A6, and Verdale were spent. Pollen vitality was 47.4% and percentage of complete flowers 62.3%.

Kalamata Tree 1 (Year 1): Paternity analysis identified pollen donors for 35 out of 40 embryos studied (Table 6.8) and a single father could be identified for 29 of them. Among these, 27 were pollinated by Koroneiki, 1 by Verdale, and 1 by Corregiola. Among the remaining embryos, King Kalamata pollinated 2, and Leccino, Koroneiki, Mission, Sevillano, and atypical Pendolino pollinated 1 each. Pollen donors for five embryos could not be identified. The threshold for paternity was 0.01 and the LOD scores ranged from 0.03 to 8.76.

Among the fruits collected from the north, 4 were pollinated by Koroneiki, 1 by Verdale, 1 by Corregiola, 1 by Sevillano, 1 by King Kalamata and for two the donors were unidentified. Pollen donors on the south side were Koroneiki (8), atypical Pendolino (1) and for one the donor was unidentified. In the east, embryos were pollinated by Koroneiki (8), Leccino (1) and for one the donor was unidentified, while towards the west, the embryos were pollinated by Koroneiki (8), King Kalamata (1) and for one the donor was unidentified. Based on the location of the tree, the immediate neighbours were other Kalamata trees. However Pendolino, and King Kalamata trees were located nearby towards the east, and UC13A6 was towards the south. Leccino, Katsourella, Sevillano and Barnea trees were located towards the north, but at some distance.

Kalamata Tree 2 (Year 1): In case of the second tree, pollen donors for 37 embryos out of 40 tested were identified (Table 6.8) and 32 of them had a single identified pollen donor. The embryos for which a single pollen donor was identified included Koroneiki (18), Corregiola (6), atypical Manaki (4), Mission (1), atypical Pendolino (1), and Sevillano (1). The remaining embryos were pollinated by Corregiola (2),

Koroneiki (1), atypical Manaki (1), UC13A6 (1), and atypical Corregiola (1). Likely fathers could not be identified for 3 embryos. The threshold for paternity was 0.01 and the LOD scores ranged from 0.12 to 9.52.

The pollen donors on the north side were Koroneiki (5), atypical Manaki (3), UC13A6 (1) and for one the donor was unidentified. Towards the south they were pollinated by Koroneiki (4), Corregiola (3), and Mission, atypical Manaki and atypical Corregiola (1 each). The pollen donors in the east were Koroneiki (4), Corregiola (3), and atypical Manaki, Mission, Sevillano (1 each). In the west the pollen donors were Koroneiki (6), Corregiola (2) and two were unidentified. This tree was centrally located in valve 8 with other Kalamata trees as immediate neighbours.

Based on the results obtained, it was observed that Koroneiki pollinated 59% of embryos, Corregiola pollinated 11%, atypical Manaki pollinated 6%, King Kalamata, Sevillano, and Mission pollinated 3%, and Verdale, atypical Pendolino, Leccino, UC13A6, and atypical Corregiola pollinated 1% of embryos while cultivars Barnea, Katsourella, Kalamata, Manzanillo, atypical Kalamata, Pendolino, and UC13A6 did not pollinate any of the embryos studied. Pollen donors could not be identified for 10% of the embryos.

6.4.2.4.2 Kalamata results from second year of study (2003-2004): During the second year, the bloom period started on December 5 and ended on December 11, with full bloom on December 7. Maximum temperatures were low (15.7°C) at the start of bloom, but increased to 26.8°C on the day of full bloom, and reached 33.3°C

on December 9, before cooling down to 18.6°C at the end of bloom. The period was mainly dry, with some rainfall experienced only on the day of start of bloom and end of bloom. Start of bloom in Kalamata overlapped with the bloom period of all other cultivars except Manzanillo, atypical Kalamata, atypical Manaki, and Verdale. When Kalamata reached the full bloom stage, Barnea, Corregiola, Katsourella, Pendolino, atypical Pendolino, UC13A6, and Verdale had reached the end of bloom, while atypical Kalamata had just started flowering. Full bloom in Kalamata coincided with full bloom in Leccino. Kalamata had 56.6% complete flowers, and 35.9% pollen vitality.

Kalamata Tree 1 (Year 2): Forty embryos were studied from this tree, but the pollen donors for 34 of them could be identified. Of these, 27 had only one likely pollen donor, being Koroneiki (21), Corregiola (5), and atypical Manaki (1). The most likely pollen donors for the remaining embryos were Koroneiki (4), Corregiola (1), atypical Manaki (1), and UC13A6 (1). The threshold for paternity was 0.02 and the LOD scores ranged from 0.16 to 9.45.

The embryos collected from the north were pollinated by Koroneiki (7), Corregiola (1), and for two the donors were unidentified. Towards the south, the pollen donors were Corregiola (3), Koroneiki (3), UC13A6 (1), atypical Manaki (1), and for two the donors were unidentified. The UC13A6 trees were located towards the south of this tree. The pollen donors in the east were Koroneiki (8), Corregiola (1), and atypical Manaki (1). All the three cultivars were located at some distance to the east of the tree. The pollen donors in the west were Koroneiki (7), Corregiola (1), and two were unidentified.

Kalamata Tree 2 (Year 2): Pollen donors were identified for 32 of the 40 embryos identified from this tree. The pollen donors for 21 embryos were unequivocal, and these were Koroneiki (10), Corregiola (10), and atypical Manaki (1). The pollen donors for the remaining embryos were Koroneiki (5), Corregiola (1), Mission (1), atypical Pendolino (1), UC13A6 (1), Pendolino (1), and Leccino (1). The threshold for paternity was 0.02 and the LOD scores ranged from 0.39 to 8.63

The pollen donors for the embryos collected from the north were Koroneiki (3), Corregiola (3), Mission (1), atypical Pendolino (1), UC13A6 (1) and one was unidentified. The pollen donors in the south were Koroneiki (6), Corregiola (2), and two were unidentified. Towards the east, the pollen donors were Koroneiki (4), atypical Manaki (1), Corregiola (1), Pendolino (1), and three were unidentified. The Koroneiki, atypical Manaki, and Corregiola trees were located towards the east, though at some distance. The pollen donors for the embryos collected from the west were Corregiola (5), Koroneiki (2), Leccino (1), and two were unidentified.

The results obtained from paternity analysis of both the Kalamata trees show that Koroneiki was the main pollen donor and pollinated 50% of the embryos. Corregiola was also a significant pollen donor and pollinated 21% of the embryos. The other pollen donors were atypical Manaki (4%), UC13A6 (3%), and Pendolino, Leccino, atypical Pendolino, and Mission (1% each). Pollen donors for 18% of the embryos could not be identified. No self-pollination was observed and the cultivars Barnea, Katsourella, King Kalamata, Manzanillo, Sevillano, Verdale, atypical Kalamata, and atypical Corregiola did not contribute any pollen to the embryos.

Table 6.8 Results from paternity analysis of Kalamata embryos.

Pollen donor	Number of embryos				Total		Percentage Embryos	
	Tree1		Tree2					
	2003	2004	2003	2004	2003	2004	2003	2004
Koroneiki	28	25	19	15	47	40	59	50
King Kalamata	2	0	0	0	2	0	3	0
Corregiola	1	6	8	11	9	17	11	21
Verdale	1	0	0	0	1	0	1	0
Sevillano	1	0	1	0	2	0	3	0
Leccino	1	0	0	1	1	1	1	1
atypical Pendolino	1	0	0	1	1	1	1	1
atypical Manaki	0	2	5	1	5	3	6	4
Mission	0	0	2	1	2	1	3	1
UC13A6	0	1	1	1	1	2	1	3
atypicalCorregiola	0	0	1	0	1	0	1	0
Pendolino	0	0	0	1	0	1	0	1
Unknown	5	6	3	8	8	14	10	18

6.4.2.4.3 Kalamata embryos with unidentified fathers: Pollen donors for 10% of the embryos in 2003 and 18% of the embryos in 2004 could not be identified. The genotypes of the embryos with unidentified pollen donors showed that the paternal

contribution of alleles were similar in both the years. In some embryos, two paternal alleles were observed in the genotype. This is probably because of the segregation of these two alleles with the same chromosome. Such an occurrence was observed in the case of UDO99-031 alleles 110 and 138, 110 and 146, and 110 and 142, and in the case of ssrOeUA-DCA14 alleles 178 and 182, and 182 and 188. The paternal contribution of alleles (Table 6.9) indicate the presence of more than one unidentified genotype acting as pollen donors for these embryos.

Table 6.9 Combination of alleles in the unknown genotypes pollinating Kalamata.

Primer	Combination of alleles (in bp) in pollen donor
EMO2	207, 215, null
UDO99-006	146, 171, 179, null
UDO99-008	157, 163, and either of the maternal alleles 161 or 166
ssrOeUA-DCA3	236, 238, 241, 244, 247
UDO99-031	110, 142, 146, 151, (110-146), (110-138)
ssrOeUA-DCA4	131, 137
ssrOeUA-DCA14	178, 182, 188, (172-182), 182-188)
UDO99-024	181, 188, null

6.4.2.4.4 Conclusions from analysis of Kalamata embryos over two seasons: The results of paternity analysis of Kalamata embryos over two seasons, indicated that Koroneiki was the main pollen donor in both the years, pollinating 59% of the embryos in 2003 and 50% of the embryos in 2004. Corregiola pollinated 11% of the

embryos in 2003, and 21% in 2004. Cultivars King Kalamata, Verdale, and atypical Corregiola pollinated some embryos in 2003, and none in 2004. Full bloom stage of one King Kalamata tree that was recorded coincided with Kalamata in 2003. However, in 2004 King Kalamata was nearing the end of bloom when Kalamata reached full bloom. Verdale was still in bloom when Kalamata started flowering in 2003, however, in 2004, Verdale had reached end of bloom period when Kalamata started flowering. In the case of atypical Corregiola, the bloom period overlapped with Kalamata in both years, although atypical Corregiola pollinated one Kalamata tree in 2003 and none in 2004. Cultivars Barnea, Katsourella, Manzanillo, and atypical Kalamata did not pollinate any of the Kalamata embryos studied. No self-pollination was observed.

The main pollen donor was Koroneiki in both the years despite the fact that most of the flowers were spent at the time Kalamata was in full bloom. However, cultivars King Kalamata, Verdale, Sevillano, Leccino, and atypical Pendolino pollinated some embryos from this tree only during the first year, while cultivars UC13A6 and atypical Manaki pollinated some embryos only during the second year. In the case of the second tree, Koroneiki and Corregiola were the main pollen donors in both the years, although, as in the case of the first tree, Corregiola pollinated more embryos in the second year. Pendolino, atypical Pendolino, and Leccino pollinated some embryos only during the second year, while Sevillano and atypical Corregiola pollinated some embryos only during the first year.

6.4.3.5 Mission:

The two Mission trees selected for the study were located in Valves 1 and 2. They were both situated towards the centre of the valve and had only Mission trees as their immediate neighbours.

6.4.2.5.1 Mission results from first year of study: One of the trees came into bloom on November 20, while the remaining started flowering on November 23. Maximum temperature on November 20 was 16°C, increasing to 32°C on November 23. Start of bloom in Mission coincided with start of bloom in Barnea, Corregiola, Koroneiki, atypical Manaki, Sevillano, and Pendolino. Full bloom stage spanned from November 25 to 28 in the trees studied. During this time the days were cool with temperatures ranging from 16°C to 24°C. The day with maximum rainfall was November 26 with 19 mm rainfall. Full bloom in Mission coincided with the full bloom stage in Barnea, Corregiola, Koroneiki, Katsourella, atypical Manaki, Manzanillo, Pendolino, UC13A6, Sevillano, and Verdale. The flowers were spent by the December 1. Flowering in Mission overlapped with that of all other cultivars except atypical Corregiola which started flowering the day after Mission was spent. Pollen vitality was 58.9% and 62.3% of the flowers were complete.

Mission Tree 1 (Year 1): Of the 40 embryos studied, pollen donors for 22 embryos could be identified (Table 6.10). In all cases, only one potential pollen donor was identified. Koroneiki was the major pollen donor which pollinated 21 embryos, while selfing was observed in the case of one embryo. The self-pollinated embryo was among the fruits collected from the south of the tree. Pollen donors could not

be identified for 18 embryos. The threshold for paternity was 0.02 and the LOD scores ranged from 0.18 to 2.66.

Mission Tree 2 (Year 1): In the case of the second Mission tree, pollen donors were identified for 25 embryos out of the 40 studied (Table 6.10). Of these, 22 embryos were pollinated by Koroneiki, 2 by Corregiola, and 1 by Katsourella. Single pollen donors were identified for 24 embryos. The embryos pollinated by Corregiola and Katsourella were collected from the western side of the tree. Koroneiki pollinated all the remaining embryos for which pollen donors could be identified. Pollen donors could not be identified for 15 embryos. The threshold for paternity was 0.02 and the LOD scores ranged from 0.16 to 3.53.

The Koroneiki trees were present in valve 4/5 which was located to the north of the Mission trees. The Koroneiki trees were the main pollen donors pollinating 54% of the embryos tested. Corregiola pollinated 3%, and Katsourella pollinated 1% of the embryos. There was also one self-pollination (1% of the embryos). None of the remaining 13 cultivars contributed any pollen to the Mission fruits tested. Pollen donors for 41% of the embryos could not be identified.

6.4.2.5.2 Mission results from second year of study: During the second year, bloom period started on November 29 and extended till December 9, with full bloom on December 2 and 3. Maximum temperatures were around 26-27°C at the time of full bloom, but there were cool days with temperature as low as 15.7°C and warm days with temperature as high as 33.3°C at the end of bloom. There was 0.4 mm rainfall on December 1 and 4 and 1 mm rainfall on December 5. Full bloom

coincided with full bloom in Barnea, Corregiola, Katsourella, Pendolino, Sevillano, and UC13A6, and overlapped with bloom period of all other cultivars except Leccino, Kalamata, and atypical Kalamata. However, the bloom period of Mission overlapped with the bloom period of all the cultivars. Mission had 51.0% complete flowers and pollen vitality was 46.6%.

Mission Tree 1 (Year 2): Pollen donors were identified for 26 of the 40 embryos studied from this tree (Table 6.10). Out of these, single pollen donors were identified for 24 embryos, being Koroneiki (22), Mission (1), and Barnea (1). The most likely pollen donor for the remaining two embryos was Corregiola. The threshold for paternity was 0.01 and the LOD scores ranged from 0.18 to 2.50.

The single occurrence of self-pollination was observed in an embryo collected from the northern side of the tree. The embryos pollinated by Corregiola were collected from the north and west (1 each), and the one pollinated by Barnea was collected from the south. Pollen donors could not be identified for two embryos from the north, four from the south, three from the east, and five from the west.

Mission Tree 2 (Year 2): Pollen donors were identified for 19 of the 40 embryos studied from this tree (Table 6.10). Only one likely pollen donor was identified for all the embryos, being Koroneiki (17) and Corregiola (2). The threshold for paternity was 0.01 and the LOD scores ranged from 0.21 to 2.83.

The embryos pollinated by Corregiola were collected from the western side of the tree. Pollen donors for six embryos from the north, four from the south, six from the

east, and five from the west could not be identified. The pollen donor for all the remaining embryos was Koroneiki.

The data obtained from analysis of 80 embryos from two trees indicated that Koroneiki was the main pollen donor and pollinated 49% of the embryos. Corregiola pollinated 5% of the embryos and Barnea pollinated 1% of the embryos. Self-pollination was observed in 1% of the embryos. Pollen donors for 44% of the embryos could not be identified.

Table 6.10 Results from paternity analysis of Mission embryos.

Pollen donor	Number of embryos				Total		Percentage Embryos	
	Tree1		Tree2					
	2003	2004	2003	2004	2003	2004	2003	2004
Koroneiki	21	22	22	17	43	39	54	49
Mission	1	1	0	0	1	1	1	1
Corregiola	0	2	2	2	2	4	3	5
Katsourella	0	0	1	0	1	0	1	0
Barnea	0	1	0	0	0	1	0	1
Unknown	18	14	15	21	33	35	41	44

6.4.2.5.3 Mission embryos with unidentified fathers: Pollen donors could not be identified for 41% of the embryos in 2003 and 44% of the embryos in 2004.

Genotypes of these embryos showed that the paternal alleles in both the years were similar (Section 6.4.2.1.3). It is proposed that the pollen donors for these embryos were individuals having the alleles shown in Table 6.11 in any combination. In some cases, two paternal alleles were observed in the genotype, and this is probably due to two alleles segregating to the same chromosome. Such an occurrence was observed in UDO99-008 alleles 161 and 163.

Table 6.11 Combination of alleles in the unknown genotypes pollinating Mission.

Primer	Combination of alleles (in bp) in pollen donor
EMO2	212, 215, null
UDO99-006	171, 179, null, and either of the maternal alleles.
UDO99-008	161, 163, (161-163)
ssrOeUA-DCA3	241, 244, 247, and either of the maternal alleles.
UDO99-031	138, 142, 146, and either of the maternal alleles.
ssrOeUA-DCA4	131, 152
ssrOeUA-DCA14	148, 169, 178, 182, null
UDO99-024	181, 188, null, and either of the maternal alleles.

6.4.2.5.4 Conclusions from analysis of Mission embryos over two seasons: Koroneiki was observed to be the main pollen donor for Mission in both the years and pollinated 54% of the embryos in 2003, and 49% in 2004. There was one instance of self-pollination in both the years. Corregiola pollinated some embryos in both the years, Katsourella pollinated 1% of the embryos in 2003, and Barnea

pollinated 1% of the embryos in 2004. The highest number of unidentified pollen donors was recorded for Mission indicating that these trees were near by or highly compatible with the Mission cultivar.

6.4.3 Statistical analysis of number of embryos pollinated by a cultivar compared with tree number:

A simple linear regression between number of embryos pollinated by a cultivar and the number of trees of that cultivar was performed for each year separately. The data for the number pollinated and number of trees was transformed on the log-scale prior to analysis in order to normalise the data.

In a regression analysis, the null hypothesis was that there was no relationship between number pollinated and the number of trees or alternatively the slope was equal to zero. The alternative hypothesis was that there was a relationship, assumed linear, between the two variables and the slope was non-zero. The assumptions of regression analysis were that the data was normally distributed with constant variance and that the observations were independent and identically distributed, therefore cultivars had the same distribution. Diagnostic plots such as residual versus fitted values examine these assumptions.

It can be concluded from the regression analysis for 2002 observations that there is a significant positive relationship between Pollen Number (log +1) and Number of Trees (log) ($p=0.028$, $R^2=23.5\%$). The predictive equation is given by:

$$\text{Log (Number Pollinated} + 1) = 1.390 + 0.31 * \log(\text{Number of Trees})$$

The fitted model is presented graphically in Figure 6.2

The plot of the residuals versus fitted values for this analysis identified one observation with a large residual associated with cultivar UC13A6. The low R^2 (or percentage of variance accounted for) is most likely associated with this observation. The analysis was repeated with this observation removed. The significance of the regression analysis was not changed with this observation removed ($p<0.001$, R^2 = 54%).

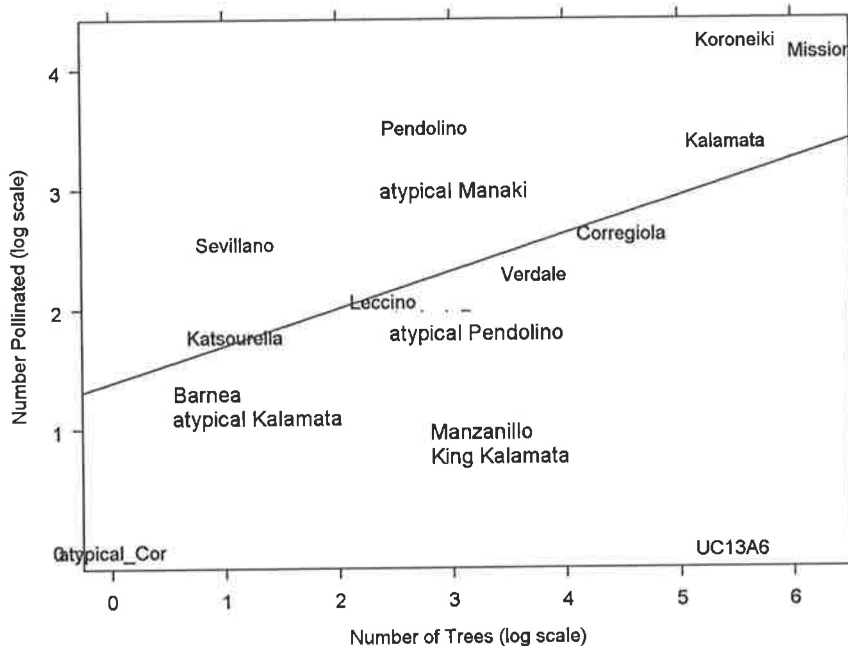

Fig 6.2 Fitted line from 2002 regression analysis of number of embryos pollinated (log scale) verses number of trees (log scale).

It can be concluded from the regression analysis for 2003 observations that there is a significant positive relationship between number of embryos pollinated and number of trees ($p<0.001$, R^2=64.1). The predictive equation is given by:

$$\text{Log(Number Pollinated + 1)} = 0.251 + 0.613*\text{log(Number of Trees)}$$

The fitted model is presented graphically in Figure 6.3

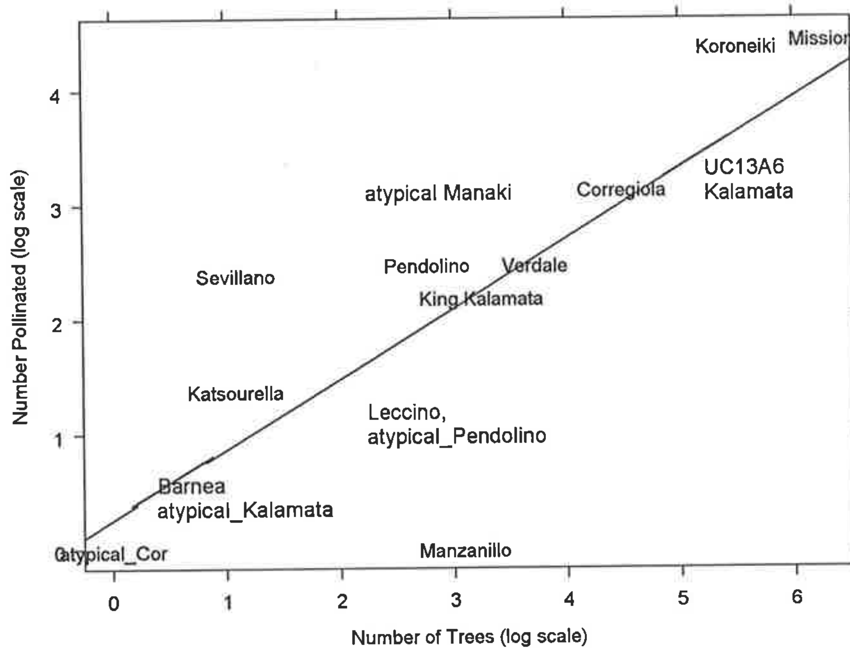

Figure 6.3: Fitted line from 2003 regression analysis of number of embryos pollinated (log scale) verses number of trees (log scale).

6.5 Discussion:

6.5.1 Molecular markers and paternity analysis:

The usefulness of microsatellites in testing paternity in olives has been demonstrated by de la Rosa *et al.* (2004). In their study, they used four microsatellite markers to identify the paternal parent of olive seedlings in a breeding program. They found that in spite of covering inflorescences with bags at the time of anthesis, there was considerable contamination by foreign pollen, thus showing that controlled pollination experiments can lead to false results. This indicates that a more definitive method of identifying the pollen donor is required. de la Rosa *et al.* (2004) compared the genotypes of the seedlings with that of the potential parents and identified the maternal and paternal contribution of alleles. A similar procedure was

used here to identify the pollen donors after natural pollination in a mixed olive orchard. Paternity analysis is a more definitive method of identifying pollen donors since alleles from the paternal parent are traced in the progeny to determine the identity of the parent.

Isozymes have been used for determining pollen donors in almonds (Jackson and Clarke, 1991) and avocados (Sulaiman *et al.*, 2004). In the study conducted by Jackson and Clarke (1991), there were only four potential donors and they could be distinguished based on the isozyme patterns at eight loci. Similarly, the study conducted by Sulaiman *et al.* (2004) considered only six potential pollen donors. However, in some cases, the pollen donor could not be distinguished based on the isozyme polymorphisms, indicating that isozymes have a low level of polymorphism and are not suitable when a large number of potential pollen donors were involved. In the present study, there were seventeen genotypes which were potential pollen donors in the orchard. Therefore, a molecular marker having a high level of polymorphism was used. Microsatellites have been shown to be highly polymorphic in olives (Cipriani *et al.*, 2002; de la Rosa *et al.*, 2004; Sefc *et al.*, 2000). The set of eight microsatellite markers used for paternity analysis had a high exclusion probability (0.998) indicating that they could adequately distinguish among the pollinating genotypes.

Chaix *et al.* (2003) used microsatellite markers to estimate gene flow in *Eucalyptus*. They used six microsatellite markers having a cumulative exclusion probability of 0.997 to determine the outcrossing rate for 724 offsprings collected from 30 trees. They found a 96.7% outcrossing rate and concluded that wind pollination led to long

distance pollen flow. Similar studies have been done on oak (Dow and Ashley, 1998; Streiff *et al.*, 1999) and rhododendron (Kameyama *et al.*, 2002). The aim of all these studies was to determine the population structure and gene flow in a natural population. The present study however aimed at identifying the compatible pollen donors in a commercial olive orchard which had been planted with clonally propagated cuttings.

6.5.2 Compatible olive cultivars identified:

The results obtained from the analysis of 80 embryos from two trees are shown in Table 6.12. The results clearly point out the necessity of having compatible pollen donors in an olive orchard. Only one case of self-pollination was observed in each year in the case of Mission. This indicates that cross fertilisation is preferred when pollen from other cultivars are available. It has been observed by Cuevas *et al.* (2001) that even those cultivars that are traditionally considered to be self-compatible, benefit from cross-pollination. Mission has been found to have a very low fruit set following self-pollination (Griggs *et al.*, 1975). Griggs *et al.* (1975) also found Mission to be cross-compatible with Manzanillo, while Mission has been found to be incompatible with Manzanilla de Sevilla (Cuevas *et al.*, 2001) and with Manzanillo (Cuevas and Polito, 1997). While it is not known whether the cultivars used in the above studies are genotypically same as the Manzanillo used in this study, it is interesting to note that Manzanillo did not pollinate any of the Mission embryos studied in spite of its proximal location. This does not however conclude that none of the Mission embryos were actually pollinated by Manzanillo, since the results are based on only a sample of fruits collected from two trees. The main

Table 6.12 Results from paternity analysis of embryos over two years

Pollen donor:	Bar	Cor	Kor	Kal	Mis	Kat	KKal	Lec	Pen	a-Mk	Man	Sev	UC13A6	Ver	a-Cor	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
Corregiola	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

Bar: Barnea; Cor: Corregiola, Kor: Koroneiki; Kal: Kalamata; Mis:Mission; Kat: Katsourella; Kkal: King Kalamata; Lec: Leccino; Pen: Pendolino; a-Mk: atypical Manaki; Man: Manzanillo; Sev: Sevillano; Ver: Verdale; a-Cor: atypical Corregiola; a-Kal: atypical Kalamata; a-Pen: atypical Pendolino.

pollen donors for Mission found in this study were Koroneiki and Corregiola (Table 6.13).

Studies conducted by Lavee *et al.* (2002) on 36 cultivars of olives over a period of twelve years have shown that the compatibility relationship between two cultivars depends on the combination and reciprocal combinations did not necessarily give similar results. They have also shown that reproductive characteristics vary from year to year and hence this study was conducted over two years. In their studies, and also in studies by Lavee (1998), Koroneiki emerged as a self-compatible cultivar, although Androulakis and Loupassaki (1990) found Koroneiki to be only partially self fertile. However, the present study showed that none of the Koroneiki embryos tested were pollinated by self-pollen. On the contrary, the cultivars Mission, UC13A6, Kalamata, atypical Manaki, and Pendolino emerged as the main pollen donors for Koroneiki (Table 6.13), with Mission pollinating 43% of the Koroneiki embryos tested.

Lavee *et al.* (2002) found that Koroneiki tested as pollen donor for Barnea had small to medium effect. When Barnea was pollinated by self-pollen, it gave an average of 12.4 fruits per 100 inflorescences on an average over 5 years. In contrast, as many as 63.1 fruits were obtained when pollinated with Manzanillo pollen. Picholine was found to be the best pollen donor for Barnea. The results obtained in this study are in partial accordance to the finding by Lavee *et al.* (2002) in that Barnea was self-incompatible and Koroneiki was not a good pollen donor for Barnea. However, none of the embryos tested were pollinated by Manzanillo. The main pollen donors for

Barnea were Mission, Pendolino, Kalamata, Sevillano, Verdale, UC13A6, and King Kalamata (Table 6.13).

Kalamata has been observed to be self incompatible and cross-compatible to Frantoio (Bini, 1984; Wu *et al.*, 2000; Wu *et al.*, 2002). Although the cultivar Frantoio was not present in the study site, Corregiola, which is genetically similar to Frantoio (Guerin *et al.*, 2002), was observed to be a significant pollen donor for Kalamata embryos. Wu *et al.* (2000) and Lavee *et al.* (2002) also found Kalamata to be partially cross-compatible with Manzanillo, Barnea, and Verdale. In the present study, Manzanillo or Barnea did not pollinate any of the Kalamata embryos studied, although Verdale was found to pollinate one Kalamata embryo. However, only two Barnea trees were present in the orchard which limited the amount of pollen produced by this cultivar.

Pollen donors could not be identified for a significant proportion of the embryos tested. It is proposed that these embryos were pollinated by pollen from unidentified cultivars in the orchard or from pollen carried by wind from trees outside the orchard. Since olives are wind pollinated, it is not possible to regulate the pollen load reaching the flowers when conducting a study under natural conditions. Visual analysis of the microsatellite markers in these embryos revealed a similar pattern of paternal alleles in both the years.

There were also some instances of possible incompatibility relationships, as some cultivars were not identified as fathers of the embryos tested. However, the results are based on only the sample of embryos studied, and it is possible that other

embryos in the same tree or in other trees were pollinated by these cultivars. It should also be noted that factors such as wind strength and direction, tree age, pollen fertility or flowering time may have prevented or reduced pollination by some cultivars. Table 6.13 summarises the possible compatible and incompatible cultivars identified in the study and Table 6.14 summarises the self and cross-compatible combinations revealed in this study.

Table 6.13 Possible compatibility and incompatibility groups in identified

Mother Tree	Compatible with	Incompatible with
Barnea	Mission (30/160)	Barnea (0/160)
	Pendolino (29/160)	atypical Corregiola (0/160)
	Kalamata (18/160)	Manzanillo (0/160)
	Sevillano (15/160)	atypical Manaki (0/160)
	Verdale (13/160)	Koroneiki (0/160)
	UC13A6 (9/160)	
	King Kalamata (8/160)	
	Corregiola (4/160)	
	atypical Kalamata	
	Leccino (3/160)	
	Katsourella (3/160)	
	atypical Pendolino	
Corregiola	Mission (45/160)	Corregiola (0/160)
	Kalamata (29/160)	atypical Corregiola (0/160)
	atypical Manaki	atypical Kalamata (0/160)
	Pendolino (6/160)	King Kalamata (0/160)
	Sevillano (6/160)	UC13A6 (0/160)
	Verdale (3/160)	Koroneiki (0/160)
	atypical Pendolino	
	Manzanillo (2/160)	
	Leccino (2/160)	
	Katsourella (1/160)	
Koroneiki	Mission (68/160)	Koroneiki (0/160)
	UC13A6 (18/160)	Corregiola (0/160)
	Kalamata (12/160)	atypical Corregiola (0/160)
	atypical Manaki (8/160)	
	Pendolino (8/160)	
	Katsourella (4/160)	
	Leccino (3/160)	
	Barnea (3/160)	
	atypical Kalamata	

Table 6.13 (contd.)

Mother Tree	Compatible with	Incompatible with
Koroneiki (contd.)	Sevillano (2/160) Verdale (2/160) atypical Pendolino Manzanillo (1/160) King Kalamata (1/160)	
Kalamata	atypical Manaki (8/160) King Kalamata (2/160) Mission (3/160) UC13A6 (3/160) atypical Pendolino Leccino (2/160) Pendolino (2/160) Sevillano (2/160) Verdale (1/160) atypical Corregiola	atypical Kalamata (0/160) Katsourella (0/160) Manzanillo (0/160)
Mission	Koroneiki (82/160) Corregiola (6/160) Mission (2/160) Katsourella (1/160) Barnea (1/160)	Kalamata (0/160) atypical Corregiola (0/160) atypical Kalamata (0/160) King Kalamata (0/160) Leccino (0/160) atypical Manaki (0/160) Manzanillo (0/160) Pendolino (0/160) atypical Pendolino (0/160) Sevillano (0/160) UC13A6 (0/160) Verdale (0/160)

6.5.3 Possible reasons for difference in compatibility relationships:

The paternity analysis studies were accompanied by observations on the weather conditions and morphological characteristics of the flower. Consideration of the environmental and morphological characters indicates that there may be several factors affecting compatibility relationships in the orchard.

Table 6.14 Selfed and crossed embryos of the five cultivars studied.

Pollen donor:	Barnea	Corregiola	Koroneiki	Kalamata	Mission
Mother Tree					
Barnea	0	4	0	18	30
Corregiola	1	0	0	29	45
Koroneiki	3	0	0	12	68
Kalamata	0	26	87	0	3
Mission	1	6	82	0	2

6.5.3.1 Tree age: Olive trees are known to have a juvenile phase and although they may start bearing fruits at an early age, they have been observed to reach their full yield potential at their twelfth to twentieth year (Hartmann and Opitz, 1966). This suggests that the morphological features of the trees are likely to undergo changes during the early years of their growth. The trees in the study site varied in age from 1 yr to 4.5 yrs. Some of the Corregiola and Verdale trees were the youngest in the orchard. In addition, these trees were quite small, as compared to other trees during the first year of study. The number of flowers borne by these trees reduced the availability of pollen for pollinating other flowers. It was observed that Corregiola trees were larger and had more flowers during the second year. This cultivar was found to pollinate nearly double the number of embryos during the second year as compared to the first year (Table 6.15). Such a difference was however not observed in the case of Verdale.

6.5.3.2 Tree number: The trees in the study site were planted in the form of separate blocks (valves) and the number of trees of each cultivar varied from 1, in the case of atypical Corregiola to 526 in the case of Mission (Table 6.1). The cultivars with a large number of trees are likely to be more effective in pollinating other cultivars since they have more flowers bearing pollen and hence contribute a greater proportion to the pollen load in the air. It was observed that the cultivars which pollinated the maximum number of embryos included Mission and Koroneiki which were also among the cultivars with the maximum number of trees. Table 6.13 shows the number of embryos pollinated by each cultivar in the two years studied. The relationship between the number of trees of a cultivar and the number of embryos pollinated by it were compared (Section 6.4.3). It can be concluded that in both 2002 and 2003 there is a linear relationship between the number of trees and the number pollinated. However, it should be noted that some cultivars did not produce the same number of pollinated embryos from one year to the next and UC13A6 is an example of this.

6.5.3.3 Wind direction and temperature: The wind direction data showed that adequate mixing and pollen movement could be expected. However, the study site was located on a slope and there remains a possibility of wind currents which may have prevented or facilitated the movement of pollen from certain cultivars. Cuevas *et al.* (1994) found that the most favorable temperature was 25°C, and temperatures below 20°C and above 30°C did not favour pollen tube growth. Hartmann and Opitz (1966) reported that the response to temperature depended on the cultivar. The maximum temperatures recorded during the bloom period of the two seasons showed that even though the temperatures remained in the mid-twenties, there were some

cooler days with temperatures as low as 16°C and as high as 33°C. Such unfavorable temperatures may retard the growth of pollen tubes and prevent pollination.

Table 6.15 Numbers of embryos pollinated by the cultivars in the two years.

Cultivar	Number of embryos pollinated in 2003	Number of embryos pollinated in 2004	Total
Barnea	4	1	5
Corregiola	14	22	36
Kalamata	30	29	59
Koroneiki	90	79	169
Mission	64	84	148
King Kalamata	3	8	11
Katsourella	6	3	9
Manzanillo	3	0	3
atypical Manaki	22	21	43
Leccino	8	2	10
Sevillano	15	10	25
atypical Kalamata	4	1	5
atypical Corregiola	1	0	1
atypical Pendolino	6	2	8
Pendolino	33	11	44
Verdale	8	11	19
UC13A6	1	29	30
Unknown	88	87	175

6.5.3.4 Rainfall: Since olive is wind pollinated, rainfall may reduce pollen movement. The bloom period of 2002 had a few rainy days when wind pollination may have been reduced. Even though the rainfall was only a few millimetres on most days, there was one day in 2002 with as much as 19 mm rainfall and this corresponded with the full bloom stage of most of the cultivars studied (Fig 5.2).

6.5.3.5 Position in the orchard: In some cases, the position of the pollen donor had an obvious effect on pollination. This was observed in the case of Corregiola where one of the trees was located close to the Kalamata trees and was mainly pollinated by Kalamata during the first year. The second tree was located near the Mission trees and was mainly pollinated by Mission. This however was not the case during the second year, probably due to the brief overlap in the bloom period of Kalamata and Corregiola. Corregiola is clearly compatible with both Kalamata and Mission, but it is possible that the location of the mother tree closer to either Kalamata or Mission gave the pollen from that cultivar a better chance to reach the flowers and effect successful pollination.

The results also show that pollen is successfully transported around trees as pollen donors were not always adjacent to the flowers they pollinated. Thus there was no apparent effect of the direction of canopy.

6.5.3.6 Pollen vitality: The cultivars atypical Manaki and Verdale were found to lack pollen in both the years of study. However, atypical Manaki was found to pollinate Corregiola, Kalamata and Koroneiki, and Verdale was found to pollinate Barnea, Corregiola, Kalamata, and Koroneiki. Some pollen may have been present

in the flowers that were not sampled, or some viable pollen may have been produced later in the bloom period. King Kalamata had the lowest pollen vitality among the cultivars tested and also made little impact as a pollen donor. Paternity analysis results showed that the 26 King Kalamata trees fathered only 11 embryos over the two years. It is possible that the pollen from King Kalamata trees competed poorly with pollen of other cultivars. Pollen vitality observations carried out in different studies have been found to vary. Cuevas *et al.* (2001) observed that the pollen of Gordal Sevillana was highly viable. However, Fernandez-Escobar *et al.* (1983) had noted that the pollen of the same cultivar was of poor quality. Therefore it was important to measure the pollen vitality of the cultivars used for the study in both the years.

6.5.4 Atypical trees: DNA fingerprinting results (Chapter 4) showed that there were genotypic differences among the trees that were considered to be Corregiola, Kalamata, and Pendolino. Such differences could have resulted if the atypical genotypes had originated from a seed of the parental tree, instead of being vegetatively propagated. A seedling would be expected to share the same S-allele as the parent and hence would be incompatible to the parent, and also show similar incompatibility reaction as the parent. However, they do not appear to be closely related based on DNA fingerprinting (Fig 4.2). In the present study, atypical Corregiola did not pollinate any Corregiola embryos, suggesting that they may share the same S-alleles. However, Corregiola was compatible to Barnea and Mission, while atypical Corregiola was not. Both the genotypes were found to be compatible to Koroneiki and Kalamata. Atypical Kalamata was found to be incompatible to Kalamata, and also shared similar compatibility as Kalamata with Koroneiki, Barnea

and Mission. However, Kalamata was found to be compatible to Corregiola, while atypical Kalamata was not. Pendolino and atypical Pendolino showed similar compatibility reaction, being compatible to all the cultivars tested, except Mission. This could possibly be due to the presence of the same incompatibility allele in the two genotypes.

6.5.5 Sporophytic/Gametophytic/Late-acting control of incompatibility: The identification of the gametophytic and sporophytic systems may be achieved by examining compatibility groups determined in diallel matrix crosses (Lewis 1994). Simple patterns with no reciprocal differences are observed in gametophytic incompatibility systems whereas nonreciprocal incompatibilities were frequently observed in sporophytic systems. While the paternity analysis study was not a diallel cross, the reciprocity of the compatibilities has been examined for the five cultivars studied (Table 6.14). The results obtained in the study were used to place the cultivars into possible compatibility groups as follows. The numbers in parenthesis indicate the number of observations made. For example only two out of the 160 Mission embryos tested were self pollinated, and, in the case of Barnea and Corregiola, 4 Corregiola embryos, out of the 160 tested, were pollinated by Barnea and 1 Barnea embryo, out of the 160 tested, was pollinated by Corregiola.

1. Self-compatible cultivars: Mission (very low: 2/160)
2. Self-incompatible cultivars: Barnea (0/160)
   Corregiola (0/160)
   Kalamata (0/160)
   Koroneiki (0/160)

3. Cross-compatible cultivars: Barnea and Corregiola (4/160 and 1/160)

   Corregiola and Kalamata 29/160 and 26/160)

   Corregiola and Mission (45/160 and 6/160)

   Kalamata and Koroneiki 87/160 and 12/160)

   Koroneiki and Mission (68/160 and 82/160)

   Barnea and Mission (30/160 and 1/160)

4. Possible non-reciprocal compatible cultivars:

   Barnea and Kalamata (18/160 and 0/160)

   Kalamata and Mission (3/160 and 0/160)

   Koroneiki with Barnea (3/160 and 0/160)

5. Possible cross-incompatible cultivars: Corregiola and Koroneiki (0/160)

Among the possible non-reciprocal combinations, only 1% of the Kalamata embryos were pollinated by Mission. Similarly only 1% of the Koroneiki embryos were pollinated by Barnea. These observations do not conclusively indicate that Kalamata is compatible to Mission pollen or that Koroneiki is compatible to Barnea pollen. There were 526 Mission trees in the orchard and these trees were also located in proximal location to Kalamata. Therefore availability of Mission pollen could not have been a limitation. However, in case of Barnea, there were only two trees in the orchard. In spite of proximal location of the Barnea trees to the Koroneiki trees, the low proportion of Barnea pollen in the pollen load may have resulted in fewer numbers of Koroneiki embryos being pollinated by Koroneiki.

Around 11% of the Barnea embryos were pollinated by Kalamata. Therefore it was concluded that Barnea was compatible to Kalamata embryos. However, again, the

low proportion of Barnea pollen in the pollen load could have been the reason that none of the Kalamata embryos tested were pollinated my Barnea. It is also possible that the Barnea fathered Kalamata embryos were not present among the fruits sampled, but were present in the orchard.

Such low values were also observed in the case of reciprocal compatibility between Barnea and Corregiola. However, in this case, the value was low in both directions. Therefore, if the combination is considered to be incompatible because of the low level of observation, the incompatibility relation would be reciprocal. However, 30 Barnea embryos were fathered by Mission, whereas only 1 Mission embryo was fathered by Barnea. This could again be attributed to the fewer number of Barnea trees present in the orchard. The compatible and incompatible groups identified in this study needs to be confirmed through artificial cross-pollination.

In general, the crosses were reciprocal between Corregiola and Kalamata, Corregiola and Mission, Kalamata and Koroneiki, Koroneiki and Mission, and Barnea and Mission. Corregiola and Koroneiki were reciprocal in their inability to fertilise each other and Barnea and Corregiola each contributed a low number of fruits to the other. The other non-reciprocal cross identified was based on 3 Kalamata fruits being fathered by Mission, but none in the other direction, and 3 Koroneiki fruits that were fathered by Barnea and no Barnea fruits fathered by Koroneiki. These embryos only represent less than 2% of embryos studied from each tree and may be due to a 'leakiness' in the incompatibility system which has been observed in other species. In a study conducted in almond (Ballester *et al.*, 1998), 0-0.5% fruit set was considered to be self incompatible, 1-6% as partially self-incompatible, and more

than 6% as self-compatible. Kaufmane and Rumpunen (2002) observed 1-11% fruit set in Japanese quince cultivars which show self-incompatibility. Therefore classification of a cultivar as self-compatible of self-incompatible will vary depending on the level of fruit set that is required to give a commercially significant yield.

The results obtained from paternity analysis in this study may be confirmed using hand pollinations and genotyping of the resulting embryos to verify paternity. More evidence is required to state whether the incompatibility system in olive is under gametophytic or sporophytic control. However, these results, coupled with the observations that pollen germinates on a wet stigma and penetrates into the style (Wu *et al.*, 2002), point to a gametophytic type system.

A third type of incompatibility system, the late-acting self-incompatibility system, has been described by Seavey and Bawa (1986) and Sedgley (1994). In this system, rejection of incompatible pollen occurs either in the ovary before they reach the ovule, or before fertilization, or after zygote formation. Olives are known to have high level of fruit drop (Marco, 1977; Marco *et al.*, 1990; Martin, 1990; Ghrisi *et al.*, 1999; Cuevas *et al.*, 2001). Although this phenomenon is commonly attributed to nutritional effects of crop load (Martin, 1990; Radi *et al.*, 1990), the possibility of late acting self-incompatibility operating in olives has not been investigated.

Observations made by Cuevas and Polito (1997); Cuevas *et al.* (2001); and Wu *et al.* (2002) showed that when the pollen of an incompatible olive cultivar reaches the stigma, growth of the pollen tube was stopped when it had only penetrated a few

layers of the stigma cells. However, they also observed that even though in some cases there was a high rate of fertilisation of Manzanillo with Sevillano, occasionally it was not reflected in high fruit set. They proposed that the incompatibility system may be both prezygotic and post zygotic. Wu *et al.* (2002) observed that the number of pollen tubes decreased from stigma, to upper style, to lower style until usually one or two pollen tubes were observed in the lower style. Studies on olives by Ateyyeh *et al.* (2000) showed that in self-pollinated flowers, the pollen grains germinated, but could not grow through the style, while in cross pollinated flowers, the pollen tubes grew through the transmitting stylar tissue. Thus they concluded that incompatibility in olives occurs at the stigmatic level and it is gametophytic. Further research is required to understand the incompatibility system operating in olives in order to be able to conclusively identify self and cross-compatible cultivars.

6.6 Conclusion:

The study reported in this chapter suggests that there is a genetically controlled compatibility mechanism operating among the cultivars. Pollen donors for the embryos were not necessarily immediate neighbours of the trees, but were located in different parts of the orchard. The cultivars showed a higher tendency to be fertilized by some cultivars as compared to the others. There was no overall effect of temperature and rainfall on pollination. In some cases tree age, tree number, and pollen vitality may have affected the potential of a pollen donor. The most significant observation was that except for two Mission embryos, all other embryos analysed showed cross-pollination, indicating that this is the preferred method under natural conditions in a mixed orchard.

Chapter Seven

General Discussion

The aim of this study was to identify the pollen donors of five olive cultivars and analyse the data alongside the variations in bloom period, pollen vitality, percentage of complete flowers, and weather conditions (temperature, rainfall, and wind direction). The approach used here examines compatibility relationship among olives under natural field conditions in a mixed orchard to obtain an accurate assessment of cultivar interactions by testing the paternity of fruits that have grown to maturity. These methods have not been reported previously to identify pollen donors for olive cultivars.

Such a study was made possible with the availability of codominant molecular markers, microsatellites, which could be conveniently used for paternity analysis. Microsatellite markers have been developed in olives and shown to be efficient for distinguishing cultivars (Sefc *et al.*, 2000; Carriero *et al.*, 2002; Cipriani *et al.*, 2002; de la Rosa *et al.*, 2002), as well as for identifying the paternal parent (de la Rosa *et al.*, 2004).

It is important to establish the genetic identity of a cultivar before studies are conducted on olive trees. A database was established at the University of Adelaide (Guerin *et al.*, 2002) to facilitate identification of olive samples by comparing them with standard samples sourced from different parts of the world. The olive trees used in this study were DNA fingerprinted using five microsatellite markers and compared

with the standard cultivars in the database (Chapter 4). It was observed that most of the cultivars matched the standards, although there were some, atypical Manaki, atypical Corregiola, atypical Pendolino, and atypical Kalamata, whose fingerprints could not be matched with any of the samples in the database. DNA fingerprinting of the trees prior to morphological and molecular observations ensured that any conclusions that would be made following further study would be reliable and characteristic of the cultivar. Besides, identification of atypical genotypes ensured that these genotypes could also be included as potential fathers. Such a situation may often be encountered in an olive orchard since there is a lot of confusion about the cultivar names and genetic identity (Sweeney, 2003).

The events that occur at bloom time are crucial for the ultimate fruit set in the trees. To ensure availability of pollen from potential pollen donors, the bloom time of the cultivars need to overlap. Since the stigma of olive flowers retain receptivity for only 3 to 5 days after the flowers open (Griggs *et al.*, 1975), it is essential that viable and compatible pollen reach the stigma during this time to effect fertilisation. A recent study conducted by Cuevas and Polito (2004) proposed that staminate flowers serve to increase the pollen output in flowers, and that there is no significant difference in the number and viability of the pollen in staminate and hermaphrodite flowers. However, Ateyyeh *et al.* (2000) observed that the ratio of complete and staminate flowers varied in different years, but were characteristic of each cultivar. Lavee and Datt (1978) could not find any significant relationship between the mean number of percentage flowers per tree and fruit set, and concluded that flower number was in excess of the number necessary to form fruits. It is also true that if

most of the flowers on the tree were staminate, it would reduce the fruit set in the tree, since only the complete flowers can set fruit.

Variation in bloom time dates of cultivars between years highlighted the importance of recording the dates over several years before any generalised idea can be developed regarding choice of cultivars with overlapping bloom period to ensure pollen availability. Again, pollen vitality and percentage complete flowers were observed to vary significantly between years and between cultivars. This pointed out the importance of planting more than one pollen donor in an orchard to allow for such variations.

Samples of Verdale and atypical Manaki lacked pollen in both the years. However, since both cultivars were found to pollinate some embryos in both years, the trees must have had a low amount of viable pollen. These cultivars would not be recommended as the sole pollen donor in an orchard as this low amount of pollen is not dependable for a commercially valuable fruit yield. These cultivars may have borne viable pollen later on in the season or in small quantities in unsampled flowers. Further work needs to be done to record pollen vitality in these two cultivars over several seasons and at different times during the bloom period.

Mission and Koroneiki were the cultivars that pollinated the highest number of embryos in both the years and they were also observed to have high pollen vitality. On the other hand, even though cultivars Manzanillo and Leccino had high pollen vitality, they may have been disadvantaged by the lower number of trees present in the orchard. Observations of the percentage of complete flowers showed all cultivars

had some complete flowers to ensure fruit set and the lowest percentage was observed in the case of King Kalamata in 2002 and Koroneiki in 2003.

The choice of cultivars in a mixed orchard is important. Often growers are not aware of the compatibility relationships and they choose cultivars based on market demand and hence may plant incompatible cultivars in their orchards (Cuevas and Polito, 1997). This is the first study of paternity analysis to identify the most efficient pollen donors for five important olive cultivars (Barnea, Corregiola, Kalamata, Koroneiki, and Mission). The very low number of self-pollinations observed indicated that cross-pollination occurs preferentially when pollen from other cultivars is available. Barnea was found to be compatible with several cultivars, although the most important pollen donors were Mission and Pendolino. Corregiola had three major pollen donors, Mission, Kalamata, and atypical Manaki. The main pollen donors for Kalamata were Koroneiki and Corregiola. Koroneiki was mainly pollinated by Mission, and to some extent by UC13A6 during the second year. Koroneiki appeared to be the main pollen donor for Mission. These observations indicate preferences for pollen for certain cultivars. Some cultivars such as UC13A6 and Corregiola, performed better during the second year, than during the first. Corregiola trees were among the youngest in the orchard and so it is possible, that they were still immature during the first year. However, the difference in the performance of UC13A6 trees could not be attributed to any particular morphological factor. It appears that cultivars may differ in their ability to pollinate in different years. This again highlights the importance of having multiple pollen donors in an orchard to provide for poor performance of any particular cultivar in a year.

The mechanism of self-incompatibility in olives is not understood, although it has been found that pollen tubes fail to grow through the style in the case of an incompatible reaction (Wu *et al.*, 2002). Extensive research conducted in other angiosperm families have shown a gametophytic self-incompatibility system operates in Solanaceae, Schrophulariaceae, and Papaveraceae (Franklin-Tong and Franklin, 2003), while a sporophytic system of incompatibility operates in Brassicaceae (Hiscock and McInnis, 2003). Since gametophytic self-incompatibility is regulated by the haploid genome, it is unaffected by dominance-recessive relationships of the alleles of genes regulating self-incompatibility, and as a result the incompatibility relationships are expected to be reciprocal (Lewis, 1994). On the other hand, sporophytic incompatibility is regulated by the diploid genome of the sporophytic tissue and is therefore influenced by the dominance-recessive relationship of the incompatibility alleles. The study conducted over here was not planned according to a diallel cross. However, analysis of reciprocity of the compatibility relationships showed Barnea and Kalamata, Koroneiki and Barnea, and Kalamata and Mission, did not show reciprocity. Again, these observations may have been affected by the presence of only two Barnea trees in the orchard which must have contributed only a low proportion to the pollen load. Several cases of reciprocal compatibility and possible incompatibility were observed and these observations point toward a gametophytic control of incompatibility in olive. Further research is required to identify the factors involved in recognition of olive pollen as compatible or incompatible. The cultivars identified as compatible or incompatible in this study may be used for artificial cross-pollination followed by paternity analysis of the embryos to gain an understanding of the mechanism of self-incompatibility in olive.

In conclusion, this study identified the compatible pollen donors for five commercially important olive cultivars. The study was conducted under natural conditions and therefore any adverse effects of artifical pollination were avoided. In addition, this method traced the paternal alleles in the embryo in order to identify the pollen donor. Therefore this method is much more reliable than the traditional methods of measurement of yield or observation of pollen tube growth. The simplicity of the method makes it an useful tool for future studies on identification of pollen donors for other olive cultivars, or for other crops. The results obtained will be a valuable source of information when planning a new olive orchard, or when interplanting a monocultivar olive orchard with pollen donors. The compatible and incompatible cultivars identified can be used for further research to gain insight into the self-incompatibility system operating in olives.

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Appendix 1

1a: Percentage of complete flowers in the olive cultivars at Milano Olives, Gumeracha, 2002-2003.

1b: Percentage of complete flowers in the olive cultivars at Milano Olives, Gumeracha, 2003-2004.

Appendix 1a

Percentage of Complete Flowers in the Olive Cultivars at Milano Olives, Gumeracha, 2002-2003

	Direction of canopy																	
Tree	N			S			E			W			C			Tot. M	Tot. C	%Comp. (Mean)
	M	Comp.	% Comp.	M	Comp.	%Comp.	M	Comp.	%Comp.	M	Comp.	%Comp.	M	Comp.	%Comp.			
Bar1	4	17	80.95	3	24	88.89	3	19	86.36	0	26	100.00	2	19	90.48	12	105	**89.74**
Bar2	10	9	47.37	8	19	70.37	11	13	54.17	12	17	58.62	13	8	38.10	54	66	**55.00**
Cor1	7	11	61.11	4	8	66.67	3	12	80.00	3	15	83.33	0	12	100.00	17	58	**77.33**
Cor2	0	15	100.00	0	25	100.00	1	19	95.00	1	22	95.65	0	20	100.00	2	101	**98.06**
a-Cor	2	20	90.91	1	13	92.86	1	22	95.65	2	17	89.47	1	15	93.75	7	87	**92.55**
Kal1	8	13	61.90	14	10	41.67	10	17	62.96	7	19	73.08	24	8	25.00	63	67	**51.54**
Kal2	9	16	64.00	8	27	77.14	5	26	83.87	10	14	58.33	16	13	44.83	48	96	**66.67**
Kal3	22	12	35.29	20	13	39.39	14	16	53.33	16	16	50.00	9	18	66.67	81	75	**48.08**
Kal4	15	17	53.13	3	27	90.00	3	28	90.32	21	9	30.00	10	23	69.70	52	104	**66.67**
Kal5	6	24	80.00	7	21	75.00	3	22	88.00	4	19	82.61	15	24	61.54	35	110	**75.86**
Kor1	12	14	53.85	7	18	72.00	21	4	16.00	6	17	73.91	16	12	42.86	62	65	**51.18**
Kor2	1	25	96.15	7	17	70.83	0	29	100.00	2	16	88.89	15	2	11.76	25	89	**78.07**
Kor3	1	23	95.83	10	13	56.52	7	14	66.67	7	26	78.79	11	19	63.33	36	95	**72.52**
Kor4	11	13	54.17	12	19	61.29	10	12	54.55	18	9	33.33	2	15	88.24	53	68	**56.20**
Kat1	1	11	91.67	5	12	70.59	3	9	75.00	0	14	100.00	13	12	48.00	22	58	**72.50**
Kat2	4	8	66.67	1	11	91.67	6	13	68.42	2	12	85.71	2	13	86.67	15	57	**79.17**
KKal1	10	11	52.38	16	6	27.27	13	7	35.00	9	8	47.06	10	10	50.00	58	42	**42.00**
KKal2	13	9	40.91	11	7	38.89	8	7	46.67	12	9	42.86	15	6	28.57	59	38	**39.18**
KKal3	9	7	43.75	13	9	40.91	18	4	18.18	10	9	47.37	5	18	78.26	55	47	**46.08**

Tree	Direction of canopy															Tot. M	Tot. C	%Comp. (Mean)
	N			S			E			W			C					
	M	Comp.	% Comp.	M	Comp.	%Comp.	M	Comp.	%Comp.	M	Comp.	%Comp.	M	Comp.	%Comp.			
Lec1	3	10	76.92	2	15	88.24	2	17	89.47	5	17	77.27	1	20	95.24	13	79	**85.87**
Lec2	1	18	94.74	1	24	96.00	0	15	100.00	2	16	88.89	0	34	100.00	4	107	**96.40**
Lec3	0	23	100.00	2	20	90.91	3	16	84.21	3	20	86.96	1	26	96.30	9	105	**92.11**
Lec4	0	29	100.00	0	13	100.00	2	17	89.47	0	22	100.00	1	19	95.00	3	100	**97.09**
a-Mk1	4	18	81.82	4	25	86.21	1	29	96.67	0	19	100.00	1	20	95.24	10	111	**91.74**
a-Mk2	3	6	66.67	3	13	81.25	3	10	76.92	4	15	78.95	2	14	87.50	15	58	**79.45**
a-Mk3	2	15	88.24	4	16	80.00	3	12	80.00	3	14	82.35	2	13	86.67	14	70	**83.33**
a-Mk4	2	12	85.71	4	14	77.78	2	20	90.91	6	7	53.85	11	15	57.69	25	68	**73.12**
Man1	12	2	14.29	4	10	71.43	12	8	40.00	6	17	73.91	14	15	51.72	48	52	**52.00**
Man2	5	17	77.27	5	15	75.00	6	11	64.71	4	12	75.00	7	9	56.25	27	64	**70.33**
Man3	6	9	60.00	9	11	55.00	5	10	66.67	8	4	33.33	2	14	87.50	30	48	**61.54**
Mis1	9	13	59.09	8	16	66.67	7	13	65.00	1	17	94.44	5	11	68.75	30	70	**70.00**
Mis2	5	16	76.19	6	11	64.71	10	12	54.55	0	15	100.00	8	18	69.23	29	72	**71.29**
Mis3	3	15	83.33	1	14	93.33	3	17	85.00	6	14	70.00	6	10	62.50	19	70	**78.65**
Mis4	4	11	73.33	10	7	41.18	4	20	83.33	5	16	76.19	9	15	62.50	32	69	**68.32**
Mis5	12	7	36.84	6	9	60.00	7	10	58.82	13	6	31.58	9	8	47.06	47	40	**45.98**
Pen1	22	23	51.11	2	18	90.00	17	2	10.53	11	21	65.63	0	38	100.00	52	102	**66.23**
Pen2	15	23	60.53	17	14	45.16	26	14	35.00	24	11	31.43	26	12	31.58	108	74	**40.66**
Pen3	11	19	63.33	22	15	40.54	8	20	71.43	2	16	88.89	20	12	37.50	63	82	**56.55**
a-Pen	0	21	100.00	0	13	100.00	0	16	100.00	2	17	89.47	0	18	100.00	2	85	**97.70**
Sev1	8	8	50.00	4	11	73.33	7	12	63.16	18	4	18.18	13	4	23.53	50	39	**43.82**

	Direction of canopy																	
Tree	**N**			**S**			**E**			**W**			**C**			**Tot. M**	**Tot. C**	**%Comp.**
	M	**Comp.**	**% Comp.**	**M**	**Comp.**	**%Comp.**	**M**	**Comp.**	**%Comp.**	**M**	**Comp.**	**%Comp.**	**M**	**Comp.**	**%Comp.**			**(Mean)**
UC1	11	10	47.62	6	21	77.78	8	13	61.90	3	21	87.50	2	11	84.62	30	76	**71.70**
UC2	6	8	57.14	0	12	100.00	3	19	86.36	8	14	63.64	1	18	94.74	18	71	**79.78**
UC3	6	17	73.91	1	24	96.00	1	14	93.33	8	12	60.00	2	17	89.47	18	84	**82.35**
UC4	11	12	52.17	7	8	53.33	12	11	47.83	8	12	60.00	12	18	60.00	50	61	**54.95**
UC5	9	20	68.97	6	14	70.00	4	10	71.43	9	18	66.67	6	8	57.14	34	70	**67.31**
Ver1	3	16	84.21	0	9	100.00	3	11	78.57	4	11	73.33	11	13	54.17	21	60	**74.07**
Ver2	22	6	21.43	14	6	30.00	12	12	50.00	16	15	48.39	5	10	66.67	69	49	**41.53**
Ver3	10	13	56.52	15	11	42.31	5	11	68.75	6	18	75.00	6	21	77.78	42	74	**63.79**
Ver4	7	9	56.25	7	8	53.33	4	12	75.00	7	19	73.08	3	16	84.21	28	64	**69.57**

M = Male or staminate flower; Comp = Complete flower; % Comp. = Percentage of complete flowers; Tot. M = Total male flowers;
Tot. C. = Total Complete flowers
Bar: Barnea; Cor: Corregiola; a-Cor: Corregiola; Kal: Kalamata; Kor: Koroneiki; Kat: Katsourella; KKal: King Kalamata; Lec: Leccino;
a-Mk: atypical Manaki; Man: Manzanillo; Mis: Mission; Pen: Pendolino; a-Pen: atypical Pendolino; Sev: Sevillano; UC: UC13A6; Ver: Verdale.

Appendix 1b

Percentage Complete flowers in the olive cultivars at Milano Olives, Gumeracha, 2003-2004

Tree	Direction of canopy																	
	N			S			E			W			C			Tot. M	Tot. C	%Comp.
	M	Comp.	% Comp.	M	Comp.	% Comp.	M	Comp.	% Comp.	M	Comp.	% Comp.	M	Comp.	% Comp.			(Mean)
Bar1	0	20	**100.00**	11	13	**54.17**	9	7	**43.75**	12	12	**50.00**	3	13	**81.25**	35	65	**65.00**
Bar2	9	10	**52.63**	10	6	**37.50**	13	11	**45.83**	12	9	**42.86**	7	11	**61.11**	51	47	**47.96**
Cor1	2	21	**91.30**	0	14	**100.00**	1	16	**94.12**	0	21	**100.00**	1	11	**91.67**	4	83	**95.40**
Cor2	2	15	**88.24**	1	21	**95.45**	2	23	**92.00**	4	10	**71.43**	1	12	**92.31**	10	81	**89.01**
a-Cor1	1	16	**94.12**	5	12	**70.59**	2	10	**83.33**	1	11	**91.67**	2	11	**84.62**	11	60	**84.51**
Kal1	9	11	**43.75**	15	4	**21.05**	5	20	**80.00**	14	8	**36.36**	9	6	**40.00**	52	49	**48.51**
Kal2	19	7	**40.63**	8	12	**60.00**	9	10	**52.63**	6	10	**62.50**	5	8	**61.54**	47	47	**50.00**
Kal3	1	13	**92.86**	10	13	**56.52**	16	4	**20.00**	11	19	**63.33**	14	20	**58.82**	52	69	**57.02**
Kal4	3	14	**82.35**	4	9	**69.23**	12	7	**36.84**	7	18	**72.00**	14	11	**44.00**	40	59	**59.60**
Kal5	8	16	**66.67**	11	12	**52.17**	5	14	**73.68**	6	9	**60.00**	5	11	**68.75**	35	62	**63.92**
a-Kal	3	17	**85.00**	3	11	**78.57**	11	14	**56.00**	8	10	**55.56**	19	9	**32.14**	44	61	**58.10**
Kor1	5	13	**72.22**	9	3	**25.00**	16	5	**23.81**	9	2	**18.18**	13	4	**23.53**	52	27	**34.18**
Kor2	17	2	**10.53**	10	7	**41.18**	5	7	**58.33**	9	2	**18.18**	15	4	**21.05**	56	22	**28.21**
Kor3	29	1	**3.33**	20	0	**0.00**	19	3	**13.64**	17	8	**32.00**	18	3	**14.29**	103	15	**12.71**
Kor4	6	9	**60.00**	10	3	**23.08**	8	9	**52.94**	6	7	**53.85**	11	2	**15.38**	41	30	**42.25**
Kat1	3	8	**72.73**	6	12	**66.67**	4	11	**73.33**	2	9	**81.82**	3	9	**75.00**	18	49	**73.13**
Kat2	8	8	**50.00**	3	12	**80.00**	0	15	**100.00**	5	7	**58.33**	1	12	**92.31**	17	54	**76.06**

	N			S			E			W			C			Tot. M	Tot. C	%Comp.
	M	Comp.	% Comp.	M	Comp.	% Comp.	M	Comp.	% Comp.	M	Comp.	% Comp.	M	Comp.	% Comp.			(Mean)
KKal1	5	14	**73.68**	8	12	**60.00**	9	13	**59.09**	4	15	**78.95**	11	15	**57.69**	37	69	**65.09**
KKal2	6	17	**73.91**	11	4	**26.67**	1	18	**94.74**	19	9	**32.14**	11	7	**38.89**	48	55	**53.40**
KKal3	13	5	**27.78**	14	8	**36.36**	8	12	**60.00**	9	10	**52.63**	5	15	**75.00**	49	50	**50.51**
Lec1	1	20	**95.24**	1	19	**95.00**	1	26	**96.30**	1	12	**92.31**	2	13	**86.67**	6	90	**93.75**
Lec2	1	14	**93.33**	2	18	**90.00**	1	16	**94.12**	1	21	**95.45**	3	20	**86.96**	8	89	**91.75**
Lec3	0	20	**100.00**	5	16	**76.19**	2	12	**85.71**	6	18	**75.00**	1	21	**95.45**	14	87	**86.14**
Lec4	0	12	**100.00**	0	25	**100.00**	8	16	**66.67**	0	20	**100.00**	2	29	**93.55**	10	102	**91.07**
a-Mk1	0	11	**100.00**	0	8	**100.00**	0	17	**100.00**	0	14	**100.00**	2	10	**83.33**	2	60	**96.77**
a-Mk2	1	13	**92.86**	3	18	**85.71**	1	19	**95.00**	0	13	**100.00**	1	13	**92.86**	6	76	**92.68**
a-Mk3	1	9	**90.00**	0	8	**100.00**	2	17	**89.47**	0	12	**100.00**	3	11	**78.57**	6	57	**90.48**
a-Mk4	0	14	**100.00**	1	9	**90.00**	1	17	**94.44**	0	9	**100.00**	0	9	**100.00**	2	58	**96.67**
Man1	1	7	**87.50**	0	12	**100.00**	0	15	**100.00**	1	15	**93.75**	0	10	**100.00**	2	59	**96.72**
Man2	0	18	**100.00**	0	17	**100.00**	2	9	**81.82**	4	11	**73.33**	2	7	**77.78**	8	62	**88.57**
Man3	1	10	**90.91**	2	10	**83.33**	0	10	**100.00**	1	13	**92.86**	2	6	**75.00**	6	49	**89.09**
Mis1	4	11	**73.33**	9	6	**40.00**	16	8	**33.33**	12	4	**25.00**	9	3	**25.00**	50	32	**39.02**
Mis2	2	9	**81.82**	10	14	**58.33**	8	16	**66.67**	5	15	**75.00**	7	14	**66.67**	32	68	**68.00**
Mis3	18	10	**35.71**	10	5	**33.33**	14	7	**33.33**	6	9	**60.00**	13	4	**23.53**	61	35	**36.46**
Mis4	5	9	**64.29**	7	10	**58.82**	6	7	**53.85**	9	6	**40.00**	7	10	**58.82**	34	42	**55.26**
Mis5	3	20	**86.96**	6	15	**71.43**	17	9	**34.62**	8	6	**42.86**	10	5	**33.33**	44	55	**55.56**
Pen1	12	18	**60.00**	16	10	**38.46**	8	12	**60.00**	13	15	**53.57**	6	19	**76.00**	55	74	**57.36**
Pen2	14	14	**50.00**	7	14	**66.67**	0	13	**100.00**	9	16	**64.00**	2	21	**91.30**	32	78	**70.91**
Pen3	3	26	**89.66**	13	8	**38.10**	15	8	**34.78**	15	7	**31.82**	1	19	**95.00**	47	68	**59.13**

	N			S			E			W			C			Tot. M	Tot. C	%Comp.
	M	Comp.	% Comp.	M	Comp.	% Comp.	M	Comp.	% Comp.	M	Comp.	% Comp.	M	Comp.	% Comp.			(Mean)
a-Pen	4	9	**69.23**	0	12	**100.00**	0	10	**100.00**	3	16	**84.21**	2	10	**83.33**	9	57	**86.36**
Sev1	4	15	**78.95**	4	6	**60.00**	10	5	**33.33**	12	5	**29.41**	8	5	**38.46**	38	36	**48.65**
UC1	7	4	**36.36**	7	3	**30.00**	7	7	**50.00**	4	7	**63.64**	4	8	**66.67**	29	29	**50.00**
UC2	6	5	**45.45**	3	9	**75.00**	6	6	**50.00**	3	10	**76.92**	2	10	**83.33**	20	40	**66.67**
UC3	0	13	**100.00**	7	9	**56.25**	2	14	**87.50**	1	7	**87.50**	4	4	**50.00**	14	47	**77.05**
UC4	3	13	**81.25**	3	8	**72.73**	1	14	**93.33**	4	9	**69.23**	7	15	**68.18**	18	59	**76.62**
UC5	9	10	**52.63**	3	13	**81.25**	6	11	**64.71**	5	13	**72.22**	4	13	**76.47**	27	60	**68.97**
Ver1	1	9	**90.00**	0	11	**100.00**	2	11	**84.62**	6	13	**68.42**	2	9	**81.82**	11	53	**82.81**
Ver2	1	7	**87.50**	3	15	**83.33**	1	10	**90.91**	2	11	**84.62**	2	15	**88.24**	9	58	**86.57**
Ver3	3	18	**85.71**	6	7	**53.85**	4	15	**78.95**	0	11	**100.00**	3	16	**84.21**	16	67	**80.72**
Ver4	0	14	**100.00**	3	7	**70.00**	4	22	**84.62**	0	12	**100.00**	2	9	**81.82**	9	64	**87.67**

M = Male or staminate flower; Comp = Complete flower; % Comp. = Percentage of complete flowers; Tot. M = Total male flowers;
Tot. C. = Total Complete flowers
Bar: Barnea; Cor: Corregiola; a-Cor: Corregiola; Kal: Kalamata; Kor: Koroneiki; Kat: Katsourella; KKal: King Kalamata; Lec: Leccino;
a-Mk: atypical Manaki; Man: Manzanillo; Mis: Mission; Pen: Pendolino; a-Pen: atypical Pendolino; Sev: Sevillano; UC: UC13A6; Ver: Verdale.

Appendix 2

2a: Pollen vitality in olive cultivars at Milano Olives, Gumeracha, 2002-2003.

2b: Pollen vitality in olive cultivars at Milano Olives, Gumeracha, 2003-2004.

Appendix 2a

Pollen Vitality in olive cultivars at Milano Olives,Gumeracha (2002-2003)

Code	Pollen Vitality (Replicates)																		
	1			2			3			4			5						
	V	NV	%	V	NV	%	V	NV	%	V	NV	%	V	NV	%	Mean	SD	C.I. (1)	C.I.(2)
Bar1	22	18	55.00	25	15	62.50	62	20	75.61	38	19	66.67	52	24	68.42	65.60	7.60	65.6+/-9.44	62.4+/-4.79
Bar2	41	24	63.08	23	20	53.49	39	22	63.93	42	29	59.15	20	15	57.14	59.40	4.30	59.4+/-5.34	
Cor1	40	32	55.56	41	36	53.25	47	24	66.20	30	19	61.22	29	34	46.03	56.40	7.70	56.4+/-9.58	
Cor2	87	36	70.73	53	35	60.23	36	29	55.38	20	13	60.61	35	23	60.34	61.40	5.60	61.4+/-6.96	58.32+/-3.20
Cor3	28	22	56.00	36	29	55.38	42	26	61.76	25	19	56.82	31	25	55.36	57.10	2.70	57.1+/-3.35	
a-Cor	51	25	67.11	43	24	64.18	20	13	60.61	33	16	67.35	35	15	70.00	65.80	3.60	65.8+/-4.44	65.8+/-4.44
Kal1	23	20	53.49	33	22	60.00	37	35	51.39	23	32	41.82	31	34	47.69	50.90	6.80	50.9+/-8.40	
Kal2	25	26	49.02	23	22	51.11	38	30	55.88	15	25	37.50	39	48	44.83	47.70	6.90	47.7+/-8.62	
Kal3	23	43	34.85	19	27	41.30	17	33	34.00	32	46	41.03	45	51	46.88	39.60	5.30	39.6+/-6.59	47.39+/-3.21
Kal4	26	20	56.52	16	15	51.61	26	21	55.32	27	36	42.86	26	16	61.90	53.60	7.00	53.6+/-8.75	
Kal5	15	23	39.47	23	21	52.27	27	25	51.92	36	45	44.44	17	28	37.78	45.20	6.80	45.2+/-8.40	
Kor1	19	12	61.29	31	12	72.09	26	10	72.22	33	6	84.62	36	10	78.26	73.70	8.60	73.7+/-10.73	
Kor2	29	11	72.50	52	13	80.00	21	9	70.00	31	15	67.39	32	14	69.57	71.90	4.90	71.9+/-6.06	72.32+/-3.24
Kor3	46	27	63.01	54	16	77.14	45	18	71.43	41	9	82.00	22	5	81.48	75.00	7.90	75.9+/-9.87	
Kor4	37	20	64.91	25	11	69.44	27	17	61.36	29	9	76.32	52	24	68.42	68.10	5.60	68.1+/-6.92	
Kat1	36	23	61.02	41	20	67.21	41	29	58.57	42	10	80.77	26	13	66.67	66.90	8.60	66.9+/-10.70	59.06+/-10.51
Kat2	22	39	36.07	18	26	40.91	20	27	42.55	26	16	61.90	13	17	43.33	51.30	16.10	51.3+/-19.98	

Code	Pollen Vitality (Replicates)																		
	1			2			3			4			5						
	V	NV	%	V	NV	%	V	NV	%	V	NV	%	V	NV	%	Mean	SD	C.I. (1)	C.I. (2)
KKal1	10	37	**21.28**	13	32	**28.89**	11	36	**23.40**	8	49	**14.04**	10	44	**18.52**	21.20	5.60	21.2+/-6.89	
KKal2	19	31	**38.00**	14	26	**35.00**	7	21	**25.00**	8	22	**26.67**	9	34	**20.93**	29.10	7.10	29.1+/-8.86	**23.53+/-4.15**
KKal3	12	29	**29.27**	4	35	**10.26**	18	52	**25.71**	9	45	**16.67**	11	46	**19.30**	20.30	7.50	20.3+/-9.29	
Lec1	55	24	**69.62**	27	33	**45.00**	34	36	**48.57**	41	84	**32.80**	42	54	**43.75**	48.00	13.50	48+/-16.7	
Lec2	32	20	**61.54**	38	22	**63.33**	41	40	**50.62**	50	33	**60.24**	42	34	**55.26**	58.20	5.20	58.2+/-6.42	**57.91+/-5.92**
Lec3	43	33	**56.58**	27	36	**42.86**	23	16	**58.97**	38	20	**65.52**	41	44	**48.24**	54.40	8.90	54.4+/-11.1	
Lec4	30	9	**76.92**	37	21	**63.79**	42	6	**87.50**	33	19	**63.46**	40	23	**63.49**	71.00	10.90	71.0+/-13.5	
a-Mk1	No Pollen																		
a-Mk2	No Pollen																		
a-Mk3	No Pollen																		
a-Mk4	No Pollen																		
Man1	35	16	**68.63**	46	9	**83.64**	35	19	**64.81**	22	10	**68.75**	30	17	**63.83**	69.90	8.00	69.9+/-9.89	
Man2	23	15	**60.53**	19	9	**67.86**	24	11	**68.57**	18	16	**52.94**	23	27	**46.00**	59.20	9.70	59.2+/-12.1	**65.90+/-5.25**
Man3	40	17	**70.18**	43	13	**76.79**	27	10	**72.97**	56	26	**68.29**	35	29	**54.69**	68.60	8.40	68.6+/-10.43	
Mis1	24	22	**52.17**	31	22	**58.49**	46	28	**62.16**	42	17	**71.19**	20	18	**52.63**	59.30	7.80	59.3+/-9.74	
Mis2	39	36	**52.00**	21	43	**32.81**	35	31	**53.03**	34	18	**65.38**	27	16	**62.79**	53.20	12.80	53.2+/-15.93	
Mis3	31	16	**65.96**	32	23	**58.18**	14	17	**45.16**	14	15	**48.28**	26	22	**54.17**	54.40	8.20	54.4+/-10.22	**58.87+/-4.75**
Mis4	34	13	**72.34**	20	12	**62.50**	19	20	**48.72**	21	12	**63.64**	13	20	**39.39**	57.30	13.10	57.3+/-16.26	
Mis5	29	19	**60.42**	21	6	**77.78**	30	8	**78.95**	52	17	**75.36**	25	18	**58.14**	70.10	10.00	70.1+/-12.46	
Pen1	18	14	**56.25**	19	17	**52.78**	22	15	**59.46**	20	12	**62.50**	19	13	**59.38**	58.10	3.70	58.1+/-4.58	
Pen2	29	21	**58.00**	35	28	**55.56**	19	13	**59.38**	48	43	**52.75**	14	16	**46.67**	54.50	5.00	54.5+/-6.26	**50.18+/-5.6**
Pen3	18	35	**33.96**	25	56	**30.86**	54	66	**45.00**	27	41	**39.71**	51	76	**40.16**	38.00	5.60	38+/-6.89	

Code	Pollen Vitality (Replicates)																	
	1			2			3			4			5					
	V	NV	%	V	NV	%	V	NV	%	V	NV	%	V	NV	%	Mean	SD	C.I. (1)	C.I. (2)
a-Pen	38	13	**74.51**	41	19	**68.33**	26	8	**76.47**	27	10	**72.97**	30	33	**47.62**	68.00	11.80	68+/-14.63	**68+/-14.63**
Sev1	26	25	**50.98**	31	24	**56.36**	23	30	**43.40**	23	40	**36.51**	20	41	**32.79**	44.00	9.80	44+/-12.18	**44+/-12.18**
UC1	27	8	**77.14**	17	13	**56.67**	13	15	**46.43**	31	11	**73.81**	34	11	**75.56**	57.30	18.70	57.3+/-23.26	
UC2	43	30	**58.90**	75	57	**56.82**	68	46	**59.65**	71	36	**66.36**	38	21	**64.41**	55.30	12.00	55.3+/-14.9	
UC3	31	21	**59.62**	23	25	**47.92**	30	18	**62.50**	18	15	**54.55**	30	23	**56.60**	50.80	13.10	50.8+/-16.21	**51.19+/-5.64**
UC4	27	22	**55.10**	29	18	**61.70**	17	16	**51.52**	23	35	**39.66**	25	42	**37.31**	46.80	14.10	46.8+/-17.5	
UC5	40	43	**48.19**	25	46	**35.21**	45	29	**60.81**	34	33	**50.75**	36	28	**56.25**	45.70	11.40	45.7+/-14.1	
Ver1	No Pollen																	
Ver2	No Pollen																	
Ver3	No Pollen																	
Ver4	No Pollen																	

V = Viable; NV = Non viable; % = Percentage Vitality; C.I. (1) = Confidence Interval for Mean (Replicate Trees);
C.I. (2) = Confidence Interval for Mean (Cultivars).
Bar: Barnea; Cor: Corregiola; a-Cor: Corregiola; Kal: Kalamata; Kor: Koroneiki; Kat: Katsourella; KKal: King Kalamata; Lec: Leccino;
a-Mk: atypical Manaki; Man: Manzanillo; Mis: Mission; Pen: Pendolino; a-Pen: atypical Pendolino; Sev: Sevillano; UC: UC13A6; Ver: Verdale.

Appendix 2b																		
Pollen Vitality in Olive Cultivars at Milano Olives,Gumeracha, 2003-2004																		
Code	**Pollen Vitality (Replicates)**																	
	1			**2**			**3**			**4**			**5**			**Total V**	**Total NV**	**%Vitality (Mean)**
	V	**NV**	**%**	**V**	**NV**	**%**	**V**	**NV**	**%**	**V**	**NV**	**%**	**V**	**NV**	**%**			
Bar1	22	18	55.00	25	15	62.50	62	20	75.61	38	19	66.67	52	24	68.42	199	96	67.46
Bar2	41	24	63.08	23	20	53.49	39	22	63.93	42	29	59.15	20	15	57.14	165	110	60.00
Cor1	40	32	55.56	41	36	53.25	47	24	66.20	30	19	61.22	29	34	46.03	187	145	56.33
Cor2	87	36	70.73	53	35	60.23	36	29	55.38	20	13	60.61	35	23	60.34	231	136	62.94
a-Cor	51	25	67.11	43	24	64.18	20	13	60.61	33	16	67.35	35	15	70.00	182	93	66.18
Kal1	23	20	53.49	33	22	60.00	37	35	51.39	23	32	41.82	31	34	47.69	147	143	50.69
Kal2	25	26	49.02	23	22	51.11	38	30	55.88	15	25	37.50	39	48	44.83	140	151	48.11
Kal3	23	43	34.85	19	27	41.30	17	33	34.00	32	46	41.03	45	51	46.88	136	200	40.48
Kal4	26	20	56.52	16	15	51.61	26	21	55.32	27	36	42.86	26	16	61.90	121	108	52.84
Kal5	15	23	39.47	23	21	52.27	27	25	51.92	36	45	44.44	17	28	37.78	118	142	45.38
Kor1	19	12	61.29	31	12	72.09	26	10	72.22	33	6	84.62	36	10	78.26	145	50	74.36
Kor2	29	11	72.50	52	13	80.00	21	9	70.00	31	15	67.39	32	14	69.57	165	62	72.69
Kor3	46	27	63.01	54	16	77.14	45	18	71.43	41	9	82.00	22	5	81.48	208	75	73.50
Kor4	37	20	64.91	25	11	69.44	27	17	61.36	29	9	76.32	52	24	68.42	170	81	67.73
Kat1	36	23	61.02	41	20	67.21	41	29	58.57	42	10	80.77	26	13	66.67	186	95	66.19
Kat2	22	39	36.07	18	26	40.91	20	27	42.55	26	16	61.90	13	17	43.33	99	125	44.20
KKal1	10	37	21.28	13	32	28.89	11	36	23.40	8	49	14.04	10	44	18.52	52	198	20.80
KKal2	19	31	38.00	14	26	35.00	7	21	25.00	8	22	26.67	9	34	20.93	57	134	29.84
KKal3	12	29	29.27	4	35	10.26	18	52	25.71	9	45	16.67	11	46	19.30	54	207	20.69

	1			2			3			4			5			Total V	Total NV	%Viable
	V	NV	%	V	NV	%	V	NV	%	V	NV	%	V	NV	%			(Mean)
Lec1	55	24	**69.62**	27	33	**45.00**	34	36	**48.57**	41	84	32.80	**42**	54	**43.75**	199	231	46.28
Lec2	32	20	**61.54**	38	22	**63.33**	41	40	**50.62**	50	33	60.24	**42**	34	**55.26**	203	149	57.67
Lec3	43	33	**56.58**	27	36	**42.86**	23	16	**58.97**	38	20	65.52	**41**	44	**48.24**	172	149	53.58
Lec4	30	9	**76.92**	37	21	**63.79**	42	6	**87.50**	33	19	63.46	**40**	23	**63.49**	182	78	70.00
a-Mk1		No Pollen																
a-Mk2		No Pollen																
a-Mk3		No Pollen																
a-Mk4		No Pollen																
Man1	35	16	**68.63**	46	9	**83.64**	35	19	**64.81**	22	10	68.75	**30**	17	**63.83**	168	71	70.29
Man2	23	15	**60.53**	19	9	**67.86**	24	11	**68.57**	18	16	52.94	**23**	27	**46.00**	107	78	57.84
Man3	40	17	**70.18**	43	13	**76.79**	27	10	**72.97**	56	26	68.29	**35**	29	**54.69**	201	95	67.91
Mis1	24	22	**52.17**	31	22	**58.49**	46	28	**62.16**	42	17	71.19	**20**	18	**52.63**	163	107	60.37
Mis2	39	36	**52.00**	21	43	**32.81**	35	31	**53.03**	34	18	65.38	**27**	16	**62.79**	156	144	52.00
Mis3	31	16	**65.96**	32	23	**58.18**	14	17	**45.16**	14	15	48.28	**26**	22	**54.17**	117	93	55.71
Mis4	34	13	**72.34**	20	12	**62.50**	19	20	**48.72**	21	12	63.64	**13**	20	**39.39**	107	77	58.15
Mis5	29	19	**60.42**	21	6	**77.78**	30	8	**78.95**	52	17	75.36	**25**	18	**58.14**	157	68	69.78
Pen1	18	14	**56.25**	19	17	**52.78**	22	15	**59.46**	20	12	62.50	**19**	13	**59.38**	98	71	57.99
Pen2	29	21	**58.00**	35	28	**55.56**	19	13	**59.38**	48	43	52.75	**14**	16	**46.67**	145	121	54.51
Pen3	18	35	**33.96**	25	56	**30.86**	54	66	**45.00**	27	41	39.71	**51**	76	**40.16**	175	274	38.98
a-Pen	38	13	**74.51**	41	19	**68.33**	26	8	**76.47**	27	10	72.97	**30**	33	**47.62**	162	83	66.12
Sev1	26	25	**50.98**	31	24	**56.36**	23	30	**43.40**	23	40	36.51	**20**	41	**32.79**	123	160	43.46

	1			2			3			4			5			Total V	Total NV	%Viable
	V	NV	%	V	NV	%	V	NV	%	V	NV	%	V	NV	%			(Mean)
UC1	27	8	**77.14**	17	13	**56.67**	13	15	**46.43**	31	11	73.81	**34**	11	**75.56**	122	58	67.78
UC2	43	30	**58.90**	75	57	**56.82**	68	46	**59.65**	71	36	66.36	**38**	21	**64.41**	295	190	60.82
UC3	31	21	**59.62**	23	25	**47.92**	30	18	**62.50**	18	15	54.55	**30**	23	**56.60**	132	102	56.41
UC4	27	22	**55.10**	29	18	**61.70**	17	16	**51.52**	23	35	39.66	**25**	42	**37.31**	121	133	47.64
UC5	40	43	**48.19**	25	46	**35.21**	45	29	**60.81**	34	33	50.75	**36**	28	**56.25**	180	179	50.14
Ver1	No Pollen																	
Ver2	No Pollen																	
Ver3	No Pollen																	
Ver4	No Pollen																	

V = Viable; NV = Non viable; % = Percentage Vitality; Total V= Total viable pollen; Total NV = Total non viable pollen; % Vitality= Pollen vitality (%).

Bar: Barnea; Cor: Corregiola; a-Cor: Corregiola; Kal: Kalamata; Kor: Koroneiki; Kat: Katsourella; KKal: King Kalamata; Lec: Leccino;

a-Mk: atypical Manaki; Man: Manzanillo; Mis: Mission; Pen: Pendolino; a-Pen: atypical Pendolino; Sev: Sevillano; UC: UC13A6; Ver: Verdale.