Sexual Compatibility and Construction of Molecular Linkage Maps in Olives (*Olea europaea* L.)

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B.Sc., M.Sc.

submitted in fulfilment of the requirements for the degree of

Doctor of Philosophy

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The University of Adelaide

September 2002

Olive, *Olea europaea* L.

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Abstract

The olive, *Olea europaea* L., is one of the oldest agricultural tree crops. The genus *Olea* consists of more than 30 different species, with only *Olea europaea* L. being cultivated. The olive tree is a perennial evergreen and its flowers show varying levels of self-incompatibility. It is a widely planted in regions with Mediterranean climate and is economically important both for oil and table use.

Olive trees were introduced into Australia in the early 1800s, but in spite of many efforts made by the government and the new colonists, a viable commercial industry did not develop for a variety of reasons. In recent decades, however, the potential profits to be made from olives has stimulated a current investment boom. To be competitive, the Australian industry will need to concentrate on yield and quality by planting cultivar combinations with appropriate pollinations, and by developing cultivars that are well adapted to local environmental conditions. This objective will be achieved in part by determining the compatibility relationships between and among the common commercial cultivars, and by the use of molecular markers for quality traits that can be used in plant improvement. The construction of a genetic linkage map would be an important approach to aid marker-assisted selection. These topics were the focus of the research described in this thesis.

Self- and cross-compatibility was investigated in 1999 and 2000 using five common commercial cultivars. The results showed that Frantoio was cross-compatible, as either a male or female parent, with each of the other cultivars, but showed a high degree of self-incompatibility. Manzanillo, Kalamata, Pendolino, and Picual were cross-incompatible, and except for Manzanillo, were self-incompatible. It is concluded that Frantoio is a good general polleniser for the other cultivars investigated. Pollen tube growth decreased in discrete steps from stigma to upper style, and from upper style to lower style, with the result that only one, or rarely two, pollen tubes penetrated ovules. Self-incompatibility in olive is probably gametophytic, but more research is needed to clarify this. The sex ratio of flowers, pollen viability, and male sterility were also examined in the study. The results showed that complete flowers were predominant in Frantoio, Manzanillo, and Pendolino, but Kalamata and Picual had mainly male flowers. Frantoio had the highest pollen viability, Kalamata and Picual were intermediate, and Manzanillo and Pendolino the lowest. Male sterility was found in the cultivar SA Verdale during most of the flowering period of 2000 but it was male fertile in 1999.

Fruit development continued about 5 months after pollination. The initial percent fruit set varied from 79.3% for SA Verdale x Manzanillo to 3.4% for Kalamata x Frantoio. Fruit abscission was evident as early as one month after pollination, and by two months, the highest fruit set of any cross was only 10.1%. Between two and five months after pollination, fruits retained declined to between 1.8% and 5.3%.

While fruit number was an important outcome from controlled pollinations, seed germination was needed for mapping studies. However, olive seed germination is slow and germination percentage is low. Therefore, germination conditions were studied and a germination percentage of 94% was obtained through optimised treatment of the seeds. The results showed that the best combination is a storage period of ten months at room temperature followed by four weeks at 4°C on ½ MS solidified medium; and then growth on the same medium at 20°C.

Molecular linkage maps are a powerful approach for the selection of quality traits in plant improvement programs. However, limited information is available for the linkage groups of olive. Therefore, the construction of a molecular linkage map was an objective of this project. A mapping population of 104 progeny was generated from a cross between the cultivars Frantoio and Kalamata, and molecular linkage maps were constructed based on a combination of RAPD, SCAR, and microsatellite markers using the pseudo-testcross strategy. The hybridity of the mapping population was confirmed by genetic similarity using 300 RAPD markers, nonmetric multidimensional scaling using 300 RAPD markers, and genotype matching based on 9 SSR markers. 194 molecular markers were used for map construction and 152 were mapped in the linkage groups.

Separate maps were produced using markers that were heterozygous in each of the parents, and these were integrated to produce a map for the species using Joinmap v. 2.0. Twenty-three linkage groups were obtained for Kalamata, 27 for Frantoio, and 15 for the integrated map. The sizes of the genomic DNAs were estimated to be 2614 cM and 3427 cM for Frantoio and Kalamata respectively, and therefore the genomic size of the species was estimated around 3000 cM. The linkage groups for Frantoio cover 798 cM of the genome with 92 loci, and the average distance between loci is 12.3 cM. The linkage groups for Kalamata cover 759 cM of the genome with 89 loci, and the average distance between loci is 11.5 cM. The linkage groups for the integrated map cover 879 cM of the genome with 101 loci, and the average distance is 10.2 cM. It is estimated that 1333 cM of the olive genome was mapped with 152 markers in the present study, and this would account for about 45% of the genome.

One SCAR marker, G07-700, which is linked to olive peacock disease resistance, was mapped in linkage group 1 of the Frantoio map, and linkage group 2 of the integrated map. When the mapping population pass through their juvenile phase and adopt their adult characters, the morphological markers will be added.

This study has contributed significantly to the information available for olive improvement in Australia, and the renaissance of the olive industry. However, further studies are needed to determine the sexual compatibility of other cultivars under Australian conditions, and to increase the number of molecular markers on the genetic maps.

Declaration and authorisation of access to coping

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Acknowledgements

This work was conducted with support from an International Postgraduate Research Scholarship, and a University of Adelaide Scholarship. I would like to express my gratitude to my supervisors, Professor Margaret Sedgley and Dr Graham Collins for their valuable supervision, encouragement, and dedication throughout my studies. I am deeply impressed by their rigorous scholarship, noble personality, and helpfulness.

I am grateful to Dr Michelle Wirthensohn, for her help on pollination and supportive advice; Drs Jenny Guerin and Meredith Wallwork for their help with ordinary lab techniques and valuable discussions. I also wish to thank my fellow postgraduates in the department, especially Genet Mekuria, Chockpisit Channuntapipat (Nid), Leanne Pound, and Toby Knight for their friendship and kind help. Thanks also to Steven Choimes for his help on seed germination, Angelo Karakousis for his technical help, and Michelle Lorimer of Biometrics SA for her help on statistical analysis.

I would also gratefully acknowledge the generous help of the olive growers who allowed me access to their properties and olive trees, especially Chaz Chamberlain, Peter Grilli, Sam Hristofis, Nick Kyriakou, and Milan Palvic, and Susan Sweeny, who allowed me to use some of NOVA trees.

Finally, I wish to pay my special thanks to my wife, Xuemei Han, and son Chengzhe, for their understanding, support, patience, and love throughout my studies, which gave me the strength and motivation to fulfil my study. This thesis is also dedicated to my parents Wu Ji-Qi, Ma Bu-Xiang, and parents-in-law Han Sha-Ge-La and Hu Yu-Zhen for their great love and encouragement.

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CHAPTER ONE

General Introduction

1.1 Introduction

The olive, *Olea europaea* L., is one of the oldest agricultural tree crops. It belongs to the family Oleaceae, which includes about 30 genera and 600 species (Cronquist, 1981). The genus *Olea* consists of more than 30 different species, with only *Olea europaea* L. being cultivated. The olive tree is a perennial evergreen and its flowers show varying levels of self-incompatibility. It is widely planted in regions with Mediterranean climates and is economically important both for oil and table use.

The olive was introduced to Australia in the first half of the 19th century and, although there was an early industry (Booth and Davies, 1996), it subsequently went into decline and almost all olive products consumed are now imported. The olive is ideally suited to the southern Australian environment and superior wild material can be identified and propagated, which has the potential to increase Australian olive production (Sedgley and Wirthensohn, 1999, 2000). Therefore, selection and breeding of superior olive cultivars is important to the renaissance of the Australian olive industry.

The olive is thought to have been cultivated and traded for its oil in Syria as early as 3000 BC (Zohary, 1994). Many varieties are of ancient origin, and breeding and genetic improvement of olive was rather limited until recently, owing to the long juvenile phase, which may be up to 10 years (Rugini and Fedli, 1990). Breeding approaches have focused on clonal selection, induced mutagenesis, cross-breeding, and biotechnology (Fontanazza and Baldoni, 1990; Lavee, 1990; Rugini and Caricato, 1995; Rugini *et al.*, 1996). Clonal selection and induced mutagenesis are currently the main methods for plant improvement, although crossbreeding has been considered as a potential and important protocol to meet the needs of olive improvement for the current international industry. Clonal selection is limited to the improvement of an existing cultivar (Lavee, 1990), and biotechnological breeding faces many difficulties, such as plantlet regeneration and suitable genetic transformation systems (Rugini and Lavee, 1992).

Cross-breeding programs have been set up in some countries (Fontanazza and Baldoni, 1990) and some cultivars have been released. The objectives of genetic improvement are increased yield, reduced alternate bearing, resistance to pests and diseases, adaptation to intensive

cultivation systems, and improved quality of both oil and table olives (Fontanazza and Baldoni, 1990; Rugini and Lavee, 1992). However, cross-breeding is difficult in the olive due to its low breeding efficiency. One problem is the slow morphological discrimination of promising traits in the progenies owing to the long juvenile phase. Another is low fruit set because of sexual incompatibility and the physiological competition between flowers, fruits and vegetative shoots during development.

To attempt to address some of these problems, this project will comprise two parts, (i) to investigate the mechanism of self-incompatibility, to identify cross compatible cultivars and to explore the mechanism of fruit fall after compatible pollination. This will optimise pollination for fruit productivity and for the University of Adelaide olive breeding program (Collins, 1998; Sedgley and Wirthensohn, 1999, 2000); (ii) to construct a linkage map for olive based on various genetic markers. Eventually, this will enable desirable traits to be identified through the analysis of DNA samples from leaves of seedlings rather than by phenological investigation of the adult stage of the trees.

1.2 Literature Review

1.2.1 Breeding systems in the olive

1.2.1.1 Floral morphology

The flower of the olive is produced in a short axillary raceme, often compound towards the base. The calyx is about 2 mm long with four short teeth. The corolla is small, white, with a very short tube and four spreading valvate lobes. Two stamens are exserted. The ovary is two-celled with two ovules in each cell (Fig. 1.1) (McGregor, 1976; Black *et al.*, 1980). Individual flowers may be either perfect or imperfect. Perfect flowers contain both pistils and stamens but imperfect ones are usually staminate, with the pistil either lacking or rudimentary. The percentage of perfect flowers is generally small but varies widely (Griggs *et al.*, 1975).

1.2.1.2 Stigma and style

The stigma of the olive is of the wet type, about 6 mm in height and saddle-shaped. At the time of pollination, there is the potential for a large number of pollen grains to alight on the stigma, to come in contact with the stigmatic exudate, and to germinate. The stigma comprises two parts: the terminal, papillar outer surface and the subterminal, inner stigmatic zone (Ciampolini *et al.*, 1983).

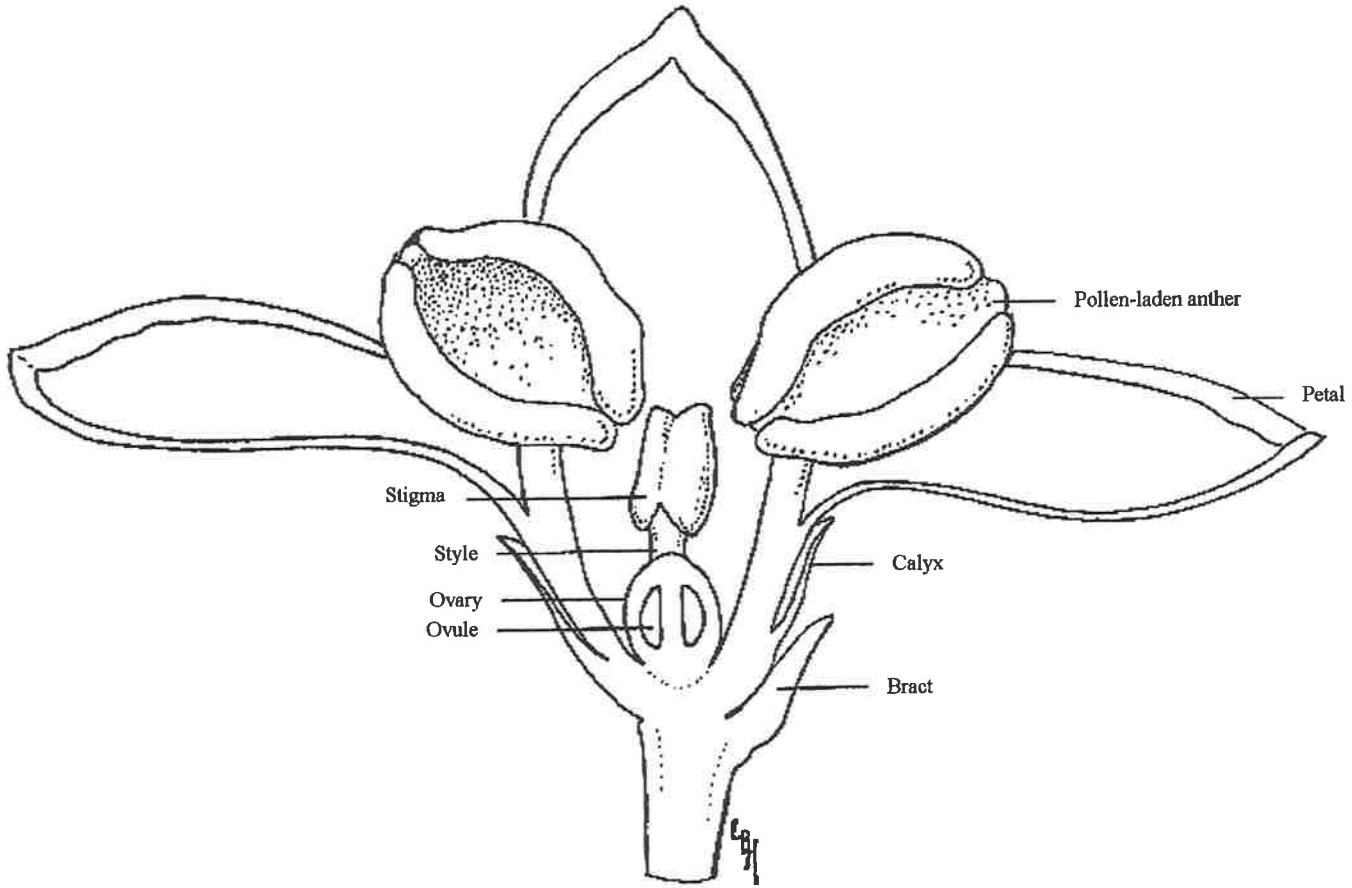

Fig. 1.1 A diagram showing longitudinal section of an olive flower (from S.E. McGregor, 1976)

The adult style is about 4 mm long and yellow in colour. The central transmitting tissue is bordered by two to three layers of cells and the cortical region is covered by an undulating epidermis with a thick cuticle. Two vascular bundles with xylem and phloem are symmetrically located in the parenchymatous cortex and extend up to the tip of the stigma (Ciampolini *et al.*, 1983).

1.2.1.3 Anthesis and pollination

Usually, more perfect flowers are borne in the apical portion of an inflorescence than at the base, and perfect flowers reach anthesis before staminate ones on the same inflorescence (Brooks, 1948). Pollen is produced in great abundance. Self-pollination can be accomplished by pollen falling, after anther dehiscence, to the stigma in the same flower, and flowers are also wind-pollinated by air currents. Honeybees often visit olive flowers to gather pollen but are not considered to be essential for olive pollination (Griggs *et al.*, 1975).

1.2.1.4 Embryo and endosperm development

Fertilization occurs in four to eight days (Villemur *et al.*, 1984), and embryo development lasts approximately 10 wk. The developmental stages include a young filamentous embryo with a single terminal cell, globular embryo, heart-shaped embryo, and mature embryo (Rapoport *et al.*, 1994). The endosperm persists in the mature seed.

1.2.1.5 Fruit development

The fruit is an oblong fleshy drupe, black when ripe, about 25 mm long, with a bony endocarp containing one to two seeds (Black *et al.*, 1980). The fruit results from the development of the ovary in which generally a single ovule develops and grows. Both fruit fresh weight and fruit diameter exhibit a sigmoid curve with a rapid increase until about 45 days after full bloom, then they show moderate growth until 90 days after full bloom. Finally the fruit weight remains constant or undergoes a small reduction (Proietti, 1990; Tombesi *et al.*, 1994). The fruits get mature about 4-5 months after pollination.

Fruit set rate in the olive is as low as 1-2% (Griggs *et al.*, 1975; Lavee *et al.*, 1996). This may be due to water deficiency in the soil when the floral parts are forming in the bud (Hartmann and Hoffman, 1953; Hartmann and Panetsos, 1962), competition among flowers (Rallo and Fernandez, 1985), and pollen-pistil incompatibility (Griggs *et al.*, 1975; Voyiatzis, 1993).

1.2.1.6 Seed germination

Olive seed germination is slow and the germination rate is low. Seed germination might continue for three years and even longer (Sotomayor-Leon and Caballero, 1990). The underlying factors for the slow germination and low germination rate may be the mechanical resistance of the endocarp (Crisosto and Sutter, 1985b), dormancy imposed by the endosperm (Lagarda and Martin, 1983; Mitrakos and Diamantoglou, 1984), and dormancy in the embryo itself (Lagarda and Martin, 1983; Voyiatzis, 1995). Removal of the endocarp using various methods may increase the germination rate (Crisosto and Sutter, 1985a; Sotomayor-Leon and Caballero, 1990). Pre-treatments of naked seeds may further improve germination, including chilling at 10°C (Voyiatzis, 1995), rinsing with running tap water (Sotomayor-Leon *et al.*, 1994), and applying chemicals in the soaking solution such as norflurazon (4-chloro-5-(methylamine)-2-(α,α,α,-trifluoro-m-tolyl)-3-(2H)-pyridazinone) (Sotomayor-Leon *et al.*, 1994) and Na_2CO_3 (Vachkoo *et al.*, 1993). Culturing the excised embryo may result in germination up to 100% (Acebedo *et al.*, 1997; Voyiatzis, 1995).

1.2.2 Self-incompatibility

The widely accepted definition of self-incompatibility (SI) is 'the inability of a fertile hermaphrodite seed plant to produce zygotes after self-pollination' (de Nettancourt, 1977), which emphasises presyngamic phenomena. In addition, postzygotic recognition occurs in many plants which express late-acting ovarian self-incompatibility (Sage *et al.*, 1994; Seavey and Bawa, 1986). Pre-zygotic SI is one of the most important mechanisms responsible for outbreeding, and hence heterozygosity, in flowering plants. In many cases it is controlled by a multiallelic single locus, the S-locus. Self-incompatibility may be of three types, gametophytic, sporophytic, and late-acting.

In woody plants, outbreeding is quite common (Sutherland, 1986) and self-incompatibility may be one of the mechanisms responsible for cross-pollination (Sedgley, 1994). Woody plants make up three quarters of angiosperm species exhibiting self-incompatibility (Sutherland, 1986). However, the mechanism is complicated. The incompatibility may occur at the pre- or post-zygotic stages, and the degree of self-incompatibility may vary in woody species subject to different taxa and environmental conditions (Sedgley, 1994).

The olive is a partly self-incompatible species with some cultivars considered to be self-compatible and others self-incompatible. The SI mechanism may be gametophytic (Bini and

Lensi, 1981) and late-acting (Cuevas and Polito, 1997). Further studies need to be conducted for complete understanding of the self-incompatibility system in this species. Since there is self-incompatibility in many cultivars, interplanting with several other cultivars is recommended in the olive orchard. Therefore, cross compatible cultivars should be selected to improve the fruit set of olives.

1.2.2.1 Gametophytic self-incompatibility (GSI)

In GSI, haploid genotypes of pollen are responsible for the recognition between pollen and pistil. GSI exists in Gramineae, Liliaceae, Rosaceae, Solanaceae, and Leguminosae. In this system the products involved in the compatibility reaction, which may be protein-containing substances, are formed in the pollen grains during development. These products are stored in the intine, released during pollination on the stigma, and act during or shortly after pollination. In many families with gametophytic incompatibility the wet stigma condition dominates over the dry stigma, and pollens are mostly binucleate (Heslop-Harrison and Shivanna, 1977).

In most GSI systems, the incompatible pollen germinates successfully on the stigma surface, penetrates the stigma, and grows into the style. The tube grows between the longitudinal files of cells of the central transmitting tract and at some point, pollen tube growth through the transmitting tract toward the ovary is arrested (Newbigin *et al.*, 1993).

The structure of incompatible pollen tubes is different from that of compatible tubes. In compatible tubes, numerous callose plugs form within each pollen tube along their entire length (Sarker *et al.*, 1997). The presence of callosic cross walls gives the tube a ladder-like appearance and the walls cut off the growing tip of the tube from the spent pollen grain (Newbigin *et al.*, 1993). In incompatible tubes, the pattern of growth is similar to that initially seen during compatible pollination, but at some stage, the growth rate of the pollen tube begins to diminish and eventually ceases. The pollen tube walls become thicker, the tips may burst, and lysis of the contents follows (Bell, 1995).

1.2.2.2 Sporophytic self-incompatibility (SSI)

In SSI, diploid genotypes of a plant determine the compatibility between pollen and pistil. SSI may be divided into two groups according to the morphology of the flowers, homomorphic and heteromorphic.

All the mating types within a species with homomorphic self-incompatibility are morphologically identical and require a breeding test for their recognition. In this system, dry and papillate stigmas are common compared to GSI, except in the Gramineae (Heslop-Harrison and Shivanna, 1977).

In contrast to homomorphic SSI, heteromorphic SSI is characterised by the occurrence of morphologically distinct mating types within a species, which can be easily recognised without a breeding test. The differences in the mating types are generally with respect to the position of their stigma and anthers (heterostyly).

1.2.2.3 Late-acting self-incompatibility

Late-acting self-incompatibility implies that the SI mechanism does not act in the stigma or in the style (Sedgley, 1994). In many previous reviews, late-acting self-incompatibility (LSI) was neglected until Seavey and Bawa (1986) drew attention to about 20 species with this type of self-incompatibility. Obligate or partial late-acting SI systems have since been found in many species.

Two broad categories of late-acting self-incompatibility can be distinguished (Gibbs and Bianchi, 1993). In the first, the pollen tubes reach the ovary after selfing, and may even penetrate the ovules, but fertilization does not occur. In the second, self-fertilization occurs after self-pollination, but either the zygote does not divide further, or the embryo aborts at a later stage.

1.2.2.4 Self-incompatibility in woody plants

Self-incompatibility is widespread in woody plants, as reviewed by Sedgley (1994), where the author listed more than seventy species that show reduced fruit or seed set following selfing. All SI systems are involved in woody plant self-incompatibility, and the occurrence of late-acting SI is more common in woody plants than in herbaceous ones. Therefore, investigations of the growth of the pollen tube in the ovary, the fertilization process, and embryo development are important for self-incompatibility studies in tree crops.

1.2.2.5 Self-incompatibility in olive

Both self-incompatibility and self-compatibility have been reported in olive. Table 1.1 shows the cultivars with self-compatibility (SC) or self-incompatibility (SI). Cross compatibility varies between cultivars as shown in Table1.2.

Leccino, Manzanilla (Monzanillo), Moraiolo, and Pendolino (Pendulino) are reported as self-incompatible by some researchers but self-compatible by others. Environmental factors may play an important role in the reproductive behaviour (Androulakis and Loupassaki, 1990), or else the genotypes tested may be different (Mekuria *et al.*, 1999).

There are few reports on the detailed cytological mechanism of SI in olives. A report on the cultivar Moraiolo showed that self pollen tubes did not grow beyond the stylar region, and self incompatibility of the gametophytic type seems possible (Bini and Lensi, 1981; Bartolini and Guerriero, 1995). In Leccino, self pollen tubes penetrated only a short distance into the style (Bartolini and Guerriero, 1995).

1.2.3 Genetic research in olives

1.2.3.1 Conventional genetics

There is little information on the genetic control of characters in olive. A cytological study showed that the chromosome number of the olive is 2n = 2x = 46 (Taylor, 1945). The genetic background is highly heterozygous as shown by morphological investigation and isoenzymatic analysis (Dionigi, 1973; Trujillo *et al.*, 1990). Allozyme variability is higher in wild trees (66%) than in cultivated (19%) (Ouazzani *et al.*, 1993). However, the difficulty of measuring heterozygosity is that olive morphology is known to be affected by environmental factors (Loukas and Pontikis, 1981; Pontikes *et al.*, 1981).

1.2.3.2 Molecular genetics

Molecular marker studies started later in olives than in some other crops, and studies in olives have been reported by several authors since the first research was published in 1994. The differentiation of olive cultivars by DNA fingerprinting using the randomly amplified polymorphic DNA (RAPD)-PCR technique, amplified fragment length polymorphism (AFLP), restriction fragment length polymorphism (RFLP) and simple sequence repeat (SSR) or microsatellite has been successfully studied by Bogani *et al.* (1994), Fabbri *et al.* (1995), Cresti *et al.* (1997), Wiesman *et al.* (1998), Beloj *et al.* (1999), Mekuria *et al.* (1999), Angiolillo *et al* (1999), Besnard and Berville (2002), de Caraffa *et al*, (2002), Sefc *et al.* (2000), Rallo *et al.*(2000), Carriero *et al.* (2002), and Cipriani *et al.*(2002). The results of these studies showed

Table 1.1 Self-compatibility and self-incompatibility in olive cultivars

Cultivars	Self-compatibility	References
Aglandaou	SI*	Morettini *et al.* (1972)
Ascoiterana	SC*	Singh and Kar (1980)
Bouteillan	SC	Morettini *et al.* (1972)
Canino	SC	Singh and Kar (1980)
Carolea	SI	Iannotta *et al.* (1996)
Conservolia	SI	Caruso *et al.* (1993)
Coratina	SC	Sharma *et al.* (1976)
Dritta di Moscufo	SI	Antognozzi and Standardi (1978)
Frantoio	SC	Sharma *et al.* (1976); Bini (1984)
Gentile di Chieti	SI	Antognozzi and Standardi (1978)
Giarraffa	SI	Baratta *et al.*(1985); Caruso *et al.*(1993)
Grossanne	SC	Morettini *et al.* (1972)
Gordales	SC	Morettini *et al.* (1972)
Kalokairida	SC	Androulakis and Loupassaki (1990)
Koutsourelia	SC	Androulakis and Loupassaki (1990)
Leccino	SC	Bartolini and Guerriero (1995)
	SI	Antognozzi and Standardi (1978); Sharma *et al.* (1976); Ugrinovic and Stampar (1996)
Manzanilla	SC	Androulakis and Loupassaki (1990)
(Manzanollo)	SI	Caruso *et al.* (1993); Cuevas and Polito (1997); Morettini *et al.* (1972)
Megaritiki	SC	Androulakis and Loupassaki (1990),
Moraiolo	SC	Singh and Kar (1980)
	SI	Antognozzi and Standardi (1978); Bini and Lensi (1981)
Nocellara del Belice Picholine	SI	Caruso *et al.* (1993)
Olivo da Olio	SI	Rotundo and Rotundo (1988)
Pendolino	SC	Singh and Kar (1980)
(Pendulino)	SI	Ugrinovic and Stampar (1996)
Picholine	SI	Morettini *et al.* (1972)
Pidicuddara	SI	Baratta *et al.* (1985)
Salonenque	SI	Morettini *et al.* (1972)
Tanche	SI	Morettini *et al.* (1972)
Thiaki	SC	Androulakis and Loupassaki (1990)
Verdale	SI	Morettini *et al.* (1972)

* SC: self-compatible; SI: self-incompatible

Table 1.2 Cross compatibility among cultivars

Female parent	Pollenizer	Compatibility	Reference
Ascoiterana	Ascolano	CC*	Singh and Kar (1980)
----------	Coratina	CC	Singh and Kar (1980)
----------	Cornicobra	CC	Singh and Kar (1980)
----------	Frantoio	CC	Singh and Kar (1980)
----------	Pendolino	CC	Singh and Kar (1980)
Cailletier	Aglandau	CC	Carles (1983)
Canino	Ascolano	CC	Singh and Kar (1980)
--------	Coratina	CC	Singh and Kar (1980)
--------	Cornicobra	CC	Singh and Kar (1980)
--------	Frantoio	CC (partially)	Singh and Kar (1980)
--------	Pendolino	CC	Singh and Kar (1980)
Coratina	Cornicobra	CC	Kar and Singh (1984)
----------	Frantoio	CC	Sharma *et al.* (1976)
----------	Leccino	CI**	Sharma *et al.* (1976)
----------	Moraiolo	CC	Singh and Kar (1980)
Corniale	Verdale	CC	Carles (1983)
Frantoio	Coratina	CC	Sharma *et al.* (1976)
---------	Leccino	CI	Sharma *et al.* (1976)
Gordal Sevillana	Lechin de Sevilla	CC	Fernandez and Gomez (1985)
---------	Manzanillo	CC	Fernandez and Gomez (1985)
---------	Picudo	CC	Fernandez and Gomez (1985)
Istrska Belica	Leccino	CC	Ugrinovic and Stampar (1996)
Itrana	Leccino	CC	Rotundo and Fiume(1978)
Leccino	Coratina	CC	Sharma *et al.* (1976)
---------	Frantoio	CC	Sharma *et al.* (1976)
---------	Istrska Belica	CC	Ugrinovic and Stampar (1996)
---------	Pendolino	CC	Ugrinovic and Stampar (1996)
Manzanillo	Ascolano	CI	Cuevas and Polito (1997)
----------	Merhavia	CI	Lavee and Datt (1978)
----------	Mission	CI	Cuevas and Polito (1997)
----------	Sevillano	CC	Cuevas and Polito(1997); Sibbett *et al.* (1992)
----------	Suri	CI	Lavee and Datt (1978)
----------	Uovo de Piccione	CC	Lavee and Datt (1978)
Moraiolo	Ascolano	CC	Singh and Kar (1980)
----------	Cornicobra	CI	Singh and Kar (1980)
----------	Coratina	CC	Singh and Kar (1980)
----------	Frontoio	CC	Singh and Kar (1980)
----------	Pendolino	CC	Singh and Kar (1980)
Pendolino	Ascolano	CC	Singh and Kar (1980)
(Pendulino)	Canino	CC (partially)	Singh and Kar (1980)
----------	Coratina	CC (partially)	Singh and Kar (1980)
----------	Cornicobra	CC	Singh and Kar (1980)
----------	Frontoio	CC	Singh and Kar (1980)
----------	Leccino	CC	Ugrinovic and Stampar (1996)
----------	Moraiolo	CC (partially)	Singh and Kar (1980)

Table 1.2 continued

Female parent	Pollenizer	Compatibility	Reference
Sevillano	Manzanillo	CI	Lavee and Datt (1978)
----------	Merhavia	CI	Lavee and Datt (1978)
----------	Suri	CI	Lavee and Datt (1978)
----------	Uovo de Piccione	CI	Lavee and Datt (1978)

*CC: cross compatible; ** CI: cross incompatible

that olive cultivars have a high degree of diversity and the markers employed are efficient for detecting polymorphisms in olives. However, little information is available on molecular genetic mapping in olives, which is important for genetic study and breeding.

1.2.3.3 Molecular mapping in olives

One preliminary molecular map has been constructed using a population from a cross between cvs. Dolce Agogia and Leccino (Baldoni *et al.*, 1999). The linkage map of Dolce Agogia contains 9 linkage groups with 22 markers covering 178 cM of the genome, and the linkage map of Leccino contains 2 linkage groups with 4 markers covering 22 cM of the genome.

1.2.4 Genetic linkage mapping

Genetic linkage maps can provide information about the location of agronomically important traits in a genome and help to track them without the necessity of observing the phenotypes of the offspring. Therefore, genetic dominance and the developmental stage are no longer problems for selecting promising individuals. Molecular genetic maps are also useful for breeding programs involving quantitative trait loci (QTL) (Beckmann and Soller, 1983). Some regions of the genome may contain genes involved in the expression of the quantitative trait (McClean, 1998).

Genetic mapping, based on establishing linkage groups, has been carried out in a wide range of plants and has provided sound information on genomic linkage relationships and chromosome structure. The study of genetic linkage and the construction of genetic maps using molecular markers has developed rapidly in recent years. The most frequently used molecular marker techniques for this purpose are RFLP, AFLP, SSR, and RAPD. Among the above-mentioned molecular marker techniques, RAPD requires only small amounts of DNA and no prior knowledge of the structure of the genome and produced dominant marker, while SSR require

sequence information and produces codominant. Although markers produced by RFLP, AFLP and SSR are regarded as more reproducible markers, the techniques generally are more time-consuming, and expensive, and may require the use of radioisotopes. However, to obtain more specific and reliable markers, RAPDs can also be converted into sequence-characterised amplified region (SCAR) markers.

1.2.4.1 Molecular marker techniques

RFLP

RFLP analysis was the first and most widely used method for detecting DNA polymorphisms and has been used for genetic mapping and the investigation of genetic diversity in eukaryotic organisms (Beckman and Soller, 1986; Bostein *et al.*, 1980; Tansley *et al.*, 1989). RFLP involves the digestion of genomic DNA with one or more restriction endonucleases and then the detection of length polymorphisms by the hybridization of the fragments with labelled DNA probes (Helentjaris *et al.*, 1985). Genetic mapping studies based on RFLP markers have been reported in many horticulturally important crops, for example, apple (Conner *et al.*, 1997; Gardiner *et al.*, 1994; Maliepaard *et al.*, 1998; Roche *et al.*, 1997) and citrus (Cai *et al.*, 1994).

AFLP

This is a multilocus polymorphic marker technique, which relies on the selective PCR amplification of restriction fragments from a total digestion of genomic DNA (Vos *et al.*, 1995). AFLP markers have already been used successfully in genetic linkage maps in fruit tree crops such as apple (Maliepaard *et al.*, 1998) and peach (Dirlewanger *et al.*, 1998; Shimada *et al.*, 1999).

SSR

SSR, also known as microsatellites or variable tandom repeats (VTRs), is a powerful approach (Litt and Luty, 1989; Zietkiewicz *et al.*, 1994). This kind of repeat consists of tandemly repeated motifs of di-, tri- and tetra-nucleotides. According to Morgante and Vogel (1999), the repeat motif can be classified as perfect, imperfect, and compound. Perfect repeat motifs contain a single type of nucleotide repeat unit without interruption among the sequence, whereas imperfect repeat motifs contain short arbitrary nucleotide sequences among the repeat sequences. A compound motif is defined as one where more than one type of nucleotide unit occurs in the repeat sequences. SSR studies were established firstly in animals, and extensive

analysis have also been carried out in a wide range of plant species (Lagercrantz *et al.*, 1993; Morgante and Olivieri, 1993; Wang *et al.*, 1994; Zietkiewicz *et al.*, 1994). In recent years, SSR markers have been identified in olive, and polymorphism has been assessed in some cultivars and progeny obtained from a cross between Dolce Agogia and Leccino (Rallo *et al.*, 2000; Sefc *et al.*, 2000; Cipriani *et al.*, 2002; Carriero *et al.*, 2002). SSRs have been used for linkage mapping in tree crops such as apple (Gianfranceschi *et al.*, 1998; Hemmat *et al.*, 1997) and peach (Cipriani *et al.*, 1999).

RAPD

Williams *et al.* (1990) and Welsh and McClelland (1990) independently developed this method in different laboratories. Arbitrarily chosen short oligodeoxyribo nucleotide primers of known sequence are used to amplify regions of the genome by the polymerase chain reaction (PCR). If the primers bind sufficiently close on opposite strands, amplification of the DNA bounded by the primers occurs (Newbury and Ford-Lloyd, 1993; Tingy and Tufo, 1993). With appropriate primers the procedure can produce polymorphic DNA fragments among and within species. The differences between genotypes are revealed by the differences in the number and size of fragments amplified. RAPD has been regarded as a rapid, inexpensive and convenient molecular method, which requires no prior knowledge of the genomes investigated and can therefore be applied to many different species. RAPD has been successfully used for genetic mapping in apple (Conner *et al.*, 1997; Maliepaard *et al.*, 1998), citrus (Cai *et al.*, 1994; Cristofani *et al.*, 1999; Fang *et al.*, 1997), and peach (Dirlewanger and Bodo, 1994; Dirlewanger *et al.*, 1998; Rajapakse *et al.*, 1995; Warburton *et al.*, 1996). An important consideration should be the reproducibility of the RAPD markers. Stable bands should be chosen through several repeated experiments. In addition, to achieve DNA polymorphisms among a population or taxa, a number of random primers are usually screened.

SCAR

To improve the reliability of RAPDs and to convert them to more specific markers, Paran and Michelmore (1993) developed sequence-characterised amplified regions (SCARs). RAPD fragments can be cloned and sequenced, and primers synthesised used to amplify the specific regions of genomic DNA. SCAR markers are advantageous over RAPD markers because they usually detect only a single locus, their PCR amplification is less sensitive to reaction conditions, and they can be codominant markers. SCARs have been used for mapping genes of interest, map based cloning, and marker-assisted selection (MAS) on several plants including

lettuce (Paran and Michelmore, 1993), common bean (Adamblondon *et al.*, 1994; Fall *et al.*, 2001), oak (Barreneche *et al.*, 1998), and citrus (Deng *et al.*, 1997; Fang *et al.*, 1997).

1.2.4.2 Genetic mapping

Genetic mapping aims to analyse the position of genetic loci on the chromosome and their corresponding order. A genetic linkage map shows the arrangement of genes and markers along the chromosomes as calculated by the frequency with which they are co-inherited. A map unit of linkage maps is a centimorgan (cM) which corresponds to an observation of recombination in 1% of the gametes in the samples. Conventional linkage maps have been based on polymorphisms in morphological, anatomical, and physiological traits segregating in the progeny, whereas molecular linkage maps are constructed using molecular markers that are polymorphic in the progeny (Young, 1994). Therefore, the segregation and recombination of DNA fragments among the progeny of a cross (F1 for outbred lines as parents and F2 and/or BC1 for inbred lines as parents) may be monitored and as a result a linkage map can be constructed. Genetic maps using molecular markers have been established in many cereal crops such as wheat (Dubcovsky *et al.*1996) and rice (Nagamura *et al.*, 1997) as well as fruit crops such as apple (Hemmat *et al.*, 1994; Conner *et al.*, 1997; Maliepaard *et al.*, 1998), citrus (Cai *et al.*, 1994; Cristofani *et al.*, 1999; Fang *et al.*, 1997), and peach (Dirlewanger and Bodo, 1994; Dirlewanger *et al.*, 1998; Rajapakse *et al.*, 1995; Warburton *et al.*, 1996).

Molecular mapping in Oryza sativa *(rice)*

In the past decade, notable progress has been made in genetic mapping using genomic or cDNA clones. The first molecular map was reported in rice in 1988. Since then, several genetic maps have been independently constructed using either segregating F2 populations or backcross populations. A total of over 3000 RFLP and RAPD markers were involved in the map construction for the rice genome. The population used in the screening contained from 113 to 186 individuals in the progenies (Nagamura *et al.*, 1997).

Molecular mapping in Triticum monococcum *(diploid wheat)*

Since cultivated hexaploid wheat (*Triticum aestivum*) cultivars have a low level of polymorphism (Dubcovsky *et al.*, 1996), it is not easy to map the genome. However, cultivated and wild genotypes of *T. monococcum* show high levels of restriction fragment length polymorphism. A genetic map was constructed in the species, which included RFLP DNA markers, isoenzymes, and morphological loci (Dubcovsky *et al.*, 1996). Three-hundred-

and-thirty-five markers were screened in the map. When the genome of *T. monococcum* is compared with that of barley, the linkage groups are fairly similar in these two species indicating a close phylogenic relationship.

Molecular mapping in apple

Genetic linkage maps based on RAPD markers, as well as isozyme loci and morphological markers, were created for three apple cultivars by Conners (1997). Maps were constructed using a double pseudo testcross mapping format, in which two combinations of these three cultivars were involved. The cultivars were heterozygous, so markers in the resulting F1 progenies segregated. An integrated map was produced by combining marker data from both progenies into a single linkage map. The map consist of 238 markers in 19 linkage groups and the average distance between markers in the maps was approximately 5.0 cM.

To sum up, the generation of genetic linkage maps in plants is a time-consuming but important objective. RFLP, RAPD AFLP and SSR have all been used in plant genetic mapping.

1.3 Conclusion

The olive is an agronomically important tree crop which can be used for both oil and table olives. The olive industry in Australia can become more competitive on the world market if olive cultivars, with high yield and oil quality, can be obtained by planting combinations of cultivars that are cross-compatible, and by developing cultivars that are well adapted to Australian environmental conditions. This will be achieved in part by determining the compatibility relationships between and among the common commercial cultivars, and by the use of molecular markers for quality traits that can be used in plant improvement. The construction of a linkage map would be an important approach to aid marker assisted selection.

The floral biology of olive has been studied by different authors. However, in spite of frequent reports published, information on olive sexual compatibility is still fragmented in terms of more than 2000 cultivars planted around the world, and there is no report on sexual compatibility of olives under Australian environmental conditions, so far. Diallel matrix design has been frequently used for the investigation of self- and cross-compatibility in plant species (Sedgley, 1994). This design involves the examination of pollen tube growth in different levels of the pistils. A 5 x 5 diallel matrix will thus be applied to examine self- and cross-compatibility

between and within olive cultivars commonly cultivated in Australia. The fluorescence microscopic techniques will be employed in this study.

There has been little genetic linkage reported in the olive (Baldoni *et al.*, 1999), and little genetic information, either molecular or classical, is available in olive to date. Therefore, specific DNA fragment detection, using restriction endonucleases and/or labelled probes as in RFLP, is difficult, although RFLP is regarded as reliable and has been used for long time. In addition, RFLP and AFLP are time-consuming and expensive techniques. In contrast, RAPD is an easy, time-saving and cheap fingerprinting approach, which requires no prior knowledge of the genome studied, and RAPD markers have been used in a range of areas in the olives. Microsatellite markers have also been reported in the olives, so it is possible to generate new microsatellite primers and to use published primers in olive molecular mapping. This study will employ RAPD and microsatellite techniques to detect the segregation of molecular markers in the offspring of olive cultivars and construct molecular genetic maps.

This project includes two main parts, 1. a study of self- and cross-compatibility in selected olive cultivars, and 2. the development of a molecular map of the olive. The first part of this research is to observe the reproductive behaviour of some olive cultivars and to define the mechanism of self-incompatibility. The research includes: i) self and cross pollen germination on stigma in various combinations; ii) pollen tube growth and the location where pollen tubes cease to grow in style; iii) pollen tube growth into ovary; iv) comparison of morphology and cytology of pollen and pistil reaction among self-compatible, self-incompatible, cross-compatible and cross-incompatible combinations; v) investigate the compatibility between the cultivars being studied; vi) possible cytological mechanism of low fruit set in the olive. The study will determine the self-incompatible and compatible status of the cultivars used in the study under the environment of South Australia, and the cytological mechanisms of SI. The second part is to construct molecular linkage maps for olives based on RAPD, SSR and SCAR markers and to tag mapped markers to traits that have agronomical or biological importance.

CHAPTER TWO

General Materials and Methods

This Chapter includes the materials and methods which are used in more than one chapters.

2.1Materials

2.1.1 Leaves

Young, healthy leaves of the olive cultivars studied (Chapters 3 and 6) were collected and stored either at 4°C (for use within one week), or at –80°C (for storage up to 1 year). For macro extraction, 6-8 leaves were wrapped in thin polythene film, whereas for micro extraction, about 200 mg leaf material was placed in a 2 mL Eppendorf tube.

2.1.2 Trees for pollination

Trees having had at least one flowering season and with over 6 bud-bearing shoots were chosen for pollination. The identity of all cultivars was determined by comparing their DNA fingerprints against those of recognised cultivars.

2.2 Methodology

2.2.1 Controlled pollination

2.2.1.1 Emasculation

An inflorescence was chosen for emasculation when most of the flowers on the inflorescence were about to open. A nearly opened flower had white petals whereas the petals of young buds were green. Open flowers and young buds were removed from the inflorescences, and the anthers of the remaining buds were removed using forceps. Prior to emasculation of every flower, the forceps were dipped in 70% ethanol for 15 seconds to kill pollen grains from the previous emasculation.

2.2.1.2 Pollination

Emasculated flowers were pollinated by brushing more than 100 pollen grains of male parents on their stigmas on the day of flower opening. The inflorescence was bagged using an 80 x 69 mm white paper bag secured with a twist tie to guard against contamination.

2.2.2 Pollen tube microscopy

2.2.2.1 Pistil collection and fixation

Prior to fixation, the paper bags on pollinated inflorescences were opened. Pistils were removed and fixed in Carnoy's fluid (absolute ethanol : chloroform : acetic acid, 6:3:1) in a glass specimen tube (50 x 13 mm) for 24 hr. Then they were transferred in 70% ethanol through 95% ethanol and stored at 4°C until required.

2.2.2.2 Specimen preparation

Fixed pistils were hydrated through 50% and 30% ethanol to distilled water, 30 min for each. They were softened with 0.8 N NaOH for 6 hours at room temperature and washed in running water overnight. Prior to the preparation of slides, the style was separated from the ovary and the four ovules were dissected out in 70% ethanol in a Petri dish. The style and four ovules were placed on two slides (0.8 mm) separately and the slides were labelled accordingly.

The style and ovules were stained with a drop of 0.1% aniline blue in alkaline phosphate buffer (pH 11.5) for 1 hour (Martin, 1959). The style was gently squashed in a drop of 80% glycerol and mounted with a coverslip (22 x 22 mm). The ovules, however, were mounted directly in 80% glycerol with a coverslip without squashing. The style and ovules were observed under UV using a Zeiss photomicroscope (Axiophot) equipped with a filter set of exciter filter 395-440, interference beam splitter FT 460, and barrier filter LP 470. The numbers of pollen grains and pollen tubes on stigma, pollen tubes in upper and lower style, and pollen tubes in ovules were counted.

2.2.3 RAPD fingerprinting

2.2.3.1 DNA extraction

Macro DNA extraction Macro DNA extraction used the method of Mekuria *et al* (1999). Approximately 2.0 g of frozen leaf lamina was ground to a fine powder in liquid nitrogen in a chilled mortar using a pestle. The ground leaf tissue was mixed with 7.5 mL of extraction buffer (3% (w/v) CTAB (SIGMA), 2 M NaCl (UNIVAR), 0.02 M EDTA (AnalaR), 1 M Trizma base) (Steenkamp *et al.*, 1994) containing 15 μL 2-mercaptoethanol and 15 mg PVP-40T. The mixture was incubated at 60°C for 30 minutes, with inversion every ten minutes. After incubation, the tubes were placed on ice and extracted with 7.5 mL of

chloroform/isoamyl alcohol (24:1), and mixed gently on a spinning wheel for 10 minutes at room temperature, followed by centrifugation at 3000 rpm for 20 minutes at room temperature. The upper aqueous phase was transferred to a clean tube using a wide bore pipette, 2/3 volume of cold isopropanol (-20°C) was added with gentle mixing of the solution, and the tube was placed on ice for 20 minutes to precipitate the DNA. The DNA was spooled onto a glass rod, transferred to a clean, sterile tube containing 20 mL of wash buffer, and washed gently until it was white.

Excess wash buffer was removed by gently squeezing the DNA, and the end of the glass rod with the DNA was placed into an Eppendorf tube containing 1 mL of TE buffer (10mM Tris HCl, 0.1mM EDTA) to dissolve the DNA. When the DNA was completely dissolved, 2 µL of RNase (10mg/mL, DNase free) was added and the tube was incubated at 37°C for 30 minutes. The solution was transferred to a clean, sterile 15 mL Corex tube, 2mL of TE buffer and 1mL of 7.5 M NH_4Ac were added, and protein precipitated for 20 minutes after the tube was placed on ice, then centrifuged at 10,000 rpm for 20 minutes at 4°C. The upper aqueous layer was transferred to a clean, sterile 30 mL Corex tube, two volumes of cold ethanol (-20°C) were added with gentle mixing, and placed on ice for 20 minutes, then centrifuged at 8,000 rpm for ten minutes at 4°C. The ethanol was discarded and the tube was drained on a tissue to air dry. The DNA was dissolved in 1 mL TE buffer and placed in a 1.5 mL Eppendorf tube for storage at –20°C until required. DNA concentration and quality were calculated following measurement of the spectrum of the DNA solution using a UV spectrophotometer.

$$\text{DNA concentration (ng/µL)} = \frac{\text{O.D.}_{260} \times 1000 \times 50}{\text{Volume of DNA added in } H_2O \text{ to make up 1 mL solution for spectrum}}$$

DNA was regarded as of good quality when the ratios of $\text{O.D.}_{260/280}$ and $\text{O.D.}_{260/230}$ were greater than 1.8 (Sambrook *et al.*, 1989).

Micro DNA extraction A micro DNA extraction method was developed by modifying the macro extraction. Approximately 200 mg of frozen leaf lamina was ground with a steel rod in a 2 mL Eppendorf tube on a rack cooled by liquid nitrogen. 700 µL of extraction buffer containing 1.5 µL 2-mercaptoethanol and 1.5 mg PVP-40T was added to the tube and mixed with the leaf powder. After incubation, 700 µL of chloroform/isoamyl alcohol (24:1) was added and mixed gently on a spinning wheel for 10 minutes at room temperature, followed by centrifugation at 8,000 rpm for 20 minutes at room temperature. The upper aqueous phase was

transferred to a clean 2 mL Eppendorf tube, 2/3 volume (370-420 mL) of cold isopropanol (-20°C) was added, and the tube was placed on ice for 20 minutes to precipitate the DNA. The DNA was spooled onto a glass rod and transferred to a clean, sterile 2 mL Eppendorf tube containing 1.2 mL of wash buffer. Following the DNA wash in buffer, the DNA was placed into 100 µL of TE buffer in a 1.5 mL Eppendorf tube. When the DNA was completely dissolved, 0.5 µL of 10 mg/mL RNase was added. After 30 minutes of incubation at 37°C 200 µL of TE buffer and 100 µL of 7.5 M NH_4Ac were added, and protein was allowed to precipitate for 20 min at 4°C. The solution was then centrifuged at 14,000 rpm for 20 minutes at 4°C. The upper aqueous layer was transferred to a clean, sterile 1.5 mL Eppendorf tube, 1 mL of cold ethanol (-20°C) added, allowed to stand on ice for 10 minutes and centrifuged at 8,000 rpm for 10 minutes at 4°C. The DNA was dissolved in 100 µL TE buffer and placed in 1.5 mL Eppendorf tubes for storage at -20°C until required.

2.2.3.2 PCR program

PCR amplifications were performed in a 20 µL volume containing 40 ng of genomic DNA, 1.5 mM $MgCl_2$, 0.25 µM 10-mer oligodeoxy ribonucleotide primer 200 µM of each dNTP, 1 x PCR buffer (10 mM Tris HCl, pH 8.3, 50 mM KCl, 0.001% (w/v) gelatine) and 1 unit *Taq* DNA polymerase (Invitrogen Australia Pty. Ltd., Mt. Waverly, Victoria), and covered with a drop of paraffin oil. A negative control tube containing all PCR reagents except for DNA was added in each run to check DNA contamination.

All DNA amplifications were performed in a thermocycler (Programmable Thermal Controller, MJ Research Inc., USA) under the following conditions: an initial denaturation at 95°C for 2 min followed by 39 cycles of 1 min at 95°C, 10 s at 50°C, 15 s at 45°C, 20 s at 40°C, 1 min at 35°C, 30 s at 45°C, 1 min 45 s at 72°C, with final extension step of 5 min at 72°C (Collins and Symons, 1993).

2.2.3.3 Gel electrophoresis

DNA fragments from PCR amplification were separated on 1.5% agarose (Seakem®) gels in 1 x TBE buffer (89 mM Trizma base, 89mM Boric acid, 5mM EDTA) at a constant current of 80 mA. 8 µL of PCR products was added in a well on the gel together with 2 µL of loading buffer (50% (v/v) glycerol, 0.075M EDTA, 0.2% (w/v) SDS, 0.1% (w/v) bromophenol blue, 0.1% (w/v) xylene cyanol FF) (Watson and Thompson, 1986). One lane on each gel contained

2 μL of a 100 bp DNA ladder (DMW-100M, GeneWorks Pty. Ltd, Adelaide, Australia) to aid in the interpretation of band homology between gels.

After electrophoresis, the gel was stained with ethidium bromide (0.5 μg/mL) for 10 minutes and destained with milliQ water for 5 minutes with mild shaking. The band data were collected either by photography under UV light using Polaroid 667 film or by direct image capture using the program Tekcap (version 1.0. Tekram Corporation, 1998). The digital image was converted to bitmap (BMP) format using PaintShop Pro (version 5.00, Jasc Software Inc, 1998).

2.2.3.4 Data analysis

The presence and absence of RAPD fragments was recorded as a binary matrix using Gel-Pro Analyzer (Version 3.1, Media Cybernetics, Silver Spring, Maryland, USA). Genetic similarities among all pairs of individuals were estimated using the hierarchical distance method of simple matching coefficient with NTSYS-pc (version 2.02) (Exeter software, Setanket, NY, USA). Cluster analysis was performed on the estimated similarity using the unweighted pair group method with arithmetic average (UPGMA).

CHAPTER THREE

Sexual Compatibility of Olives-1999 Experiment

3.1 Introduction

A knowledge of the self- and cross-compatibility of olive cultivars is important for the industry so that orchards can be planted to maximise yields. Reports on this character for cultivars grown around the world show that there are both genetic and environmental effects. Up to now, there are no reports on sexual compatibility of olives in the Australian environment. This chapter presents a preliminary investigation of compatibility between olive cultivars conducted during the 1999 season in South Australia. The aim of the study was to test the best time to fix styles after pollination to observe pollen tube growth, and to investigate the compatibility status of three commercial cultivars.

3.2 Materials and methods

3.2.1 Trees

The cultivars used were Frantoio, Kalamata, and SA Verdale, located at Virginia (34°40'S, 138°34'E), Two Wells (34°35'S, 138°30'E), and Angle Vale (34°38'S, 138°38'E), South Australia respectively. The identities of two trees of each were confirmed by comparing their DNA fingerprints with those of standard cultivars (Chapter 2).

3.2.2 Pollination

Controlled pollinations were applied on the day of flower opening using the methods described previously (sections 2.2.1 and 2.2.2). Combinations were made as shown in Table 3.1.

3.2.3 Pistil collection

Ten pistils were collected and fixed in Carnoy's fluid at each of 1, 3, 7, and 14 d after pollination, and stored at 4°C. The styles and ovules were stained and squashed on slides as described in sections 2.2.1 and 2.2.2.

Table 3.1 Combinations of cultivars for controlled pollinations. For each combination ten flowers were collected at 1, 3, 7, and 14 days after pollination.

Female		Male
Frantoio tree 1	x	SA Verdale tree 2
SA Verdale tree 1	x	Frantoio tree 2
Frantoio tree 1	x	Kalamata tree 1
Kalamata tree 1	x	Frantoio tree 1
Kalamata tree 2	x	SA Verdale tree 1
Kalamata tree 1	x	SA Verdale tree 2
SA Verdale tree 1	x	Kalamata tree 2
SA Verdale tree 2	x	Kalamata tree 1
SA Verdale tree 1	x	SA Verdale tree 1
Frantoio tree 1	x	Frantoio tree 1
Kalamata tree 2	x	Kalamata tree 2
Kalamata tree 1	x	Kalamata tree 1

3.2.4 Data collection and analysis

The numbers of pollen grains and pollen tubes on the stigmas, and the numbers of pollen tubes in the upper and lower styles and ovules were counted using a fluorescence microscope equipped with exciter filter 395-440, interference beam splitter FT 460, and barrier filter LP 470. For each controlled pollination, the counts were analysed by one-way ANOVA using MINITAB (Version 13.20, Minitab Inc). The compatibilities between cultivars, and the optimal time of pistil fixation were determined according to significant differences at the 0.05 probability level.

The numbers of pollen tubes at different positions within the pistil were subjected to cluster analysis using the unweighted pair group average as the linkage rule, and Euclidean distance as the distance measure using Statistica for Windows Release 5.1 (Statsoft Inc.).

3.3 Results

3.3.1 Identification of trees

The DNA fingerprints of all trees used were consistent with standards in the database at the Department of Horticulture, Viticulture, and Oenology, Adelaide University (Fig 3.1). The standard samples were: Frantoio from CO.RIPR.OL, Pescia, Italy; Kalamata from Olives Australia, Queensland, Australia; SA Verdale from University of California, Davis, California, USA.

3.3.2 Cytology of pollen germination and pollen tube growth

The germination of pollen grains on stigmas peaked after 1 d for crosses between SA Verdale tree 1 and Frantoio tree 2, Kalamata tree 2 and SA Verdale tree 1, SA Verdale tree 1 and Kalamata tree 2, Frantoio tree 1 and SA Verdale tree 2, and Kalamata tree 2 selfed (Table 3.2). Some abnormal growth of pollen tubes was observed on stigmas (Fig 3.2), but most tubes appeared normal (Fig 3.3). The abnormalities included branched tubes, thickened walls, bulbous swellings (Fig 3.2), and burst tips.

For crosses between Frantoio tree 1 and Kalamata tree 1 and the reciprocal, and SA Verdale selfed, the peak occurred 3 d after pollination, and for Kalamata tree 1 and SA Verdale tree 2, the peak was at 7 d. For Frantoio selfed, and Kalamata selfed, the maximum germination was at 14 d.

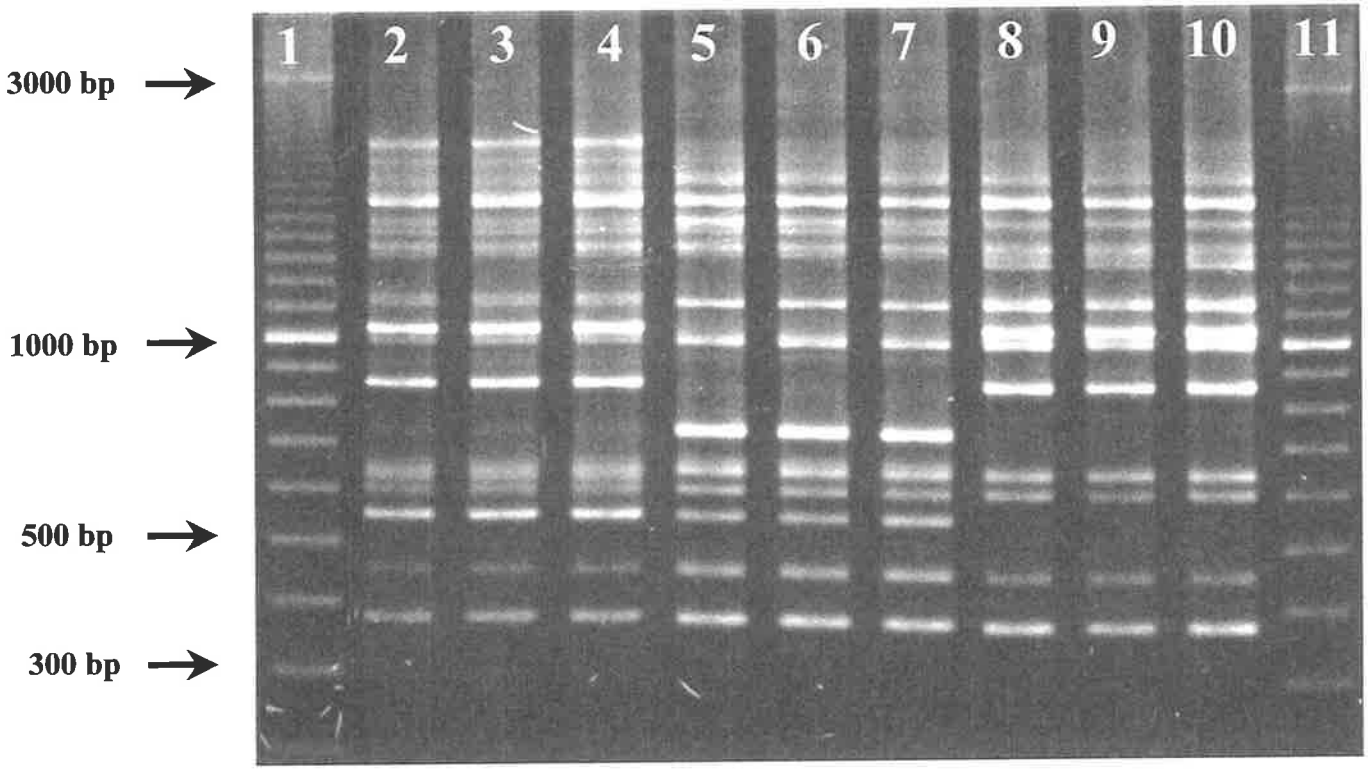

Fig 3.1 RAPD fingerprinting of olive cultivars, showing that all the trees of the same cultivars are identical to the standard samples. Lane 1: 100 bp molecular standards. Lane 2: Frantoio standard (Cordoba, Spain). Lane 3: Fra 1. Lane 4: Fra 2. Lane 5: Verdale standard (Davis, California). Lane 6: Ver 1. Lane 7: Ver 2. Lane 8: Kalamata standard (Perugia, Italy). Lane 9: Kal 1. Lane 10: Kal 2. Lane 11: 100 bp molecular standards. Fra: cv Franotio; Kal: cv Kalamata; Ver: cv SA Verdale.

Table 3.2 Germination of pollen grains on the stigma

Cross	Germination (%)			
	1d	3d	7d	14d
Fra1 x Ver2	1.77	0.28	0.00	0.43
Ver1 x Fra2	34.50	23.99	15.13	6.78
Fra1 x Kal1	20.72	26.36	20.00	19.38
Kal1 x Fra1	13.75	22.00	20.26	17.07
Kal2 x Ver1	39.36	32.66	19.39	4.44
Kal1 x Ver2	3.29	4.87	9.02	0.98
Ver1 x Kal2	32.55	21.66	12.41	4.10
Ver2 x Kal1	0.00	0.00	0.00	N/A*
Ver1 selfed	15.90	20.15	9.28	12.61
Fra1 selfed	0.87	0.15	0.68	4.08
Kal2 selfed	47.95	38.22	24.41	8.74
Kal1 selfed	0.87	0.46	0.74	1.67

* not available

Fra : Frantoio; Kal : Kalamata; Ver: SA Verdale.

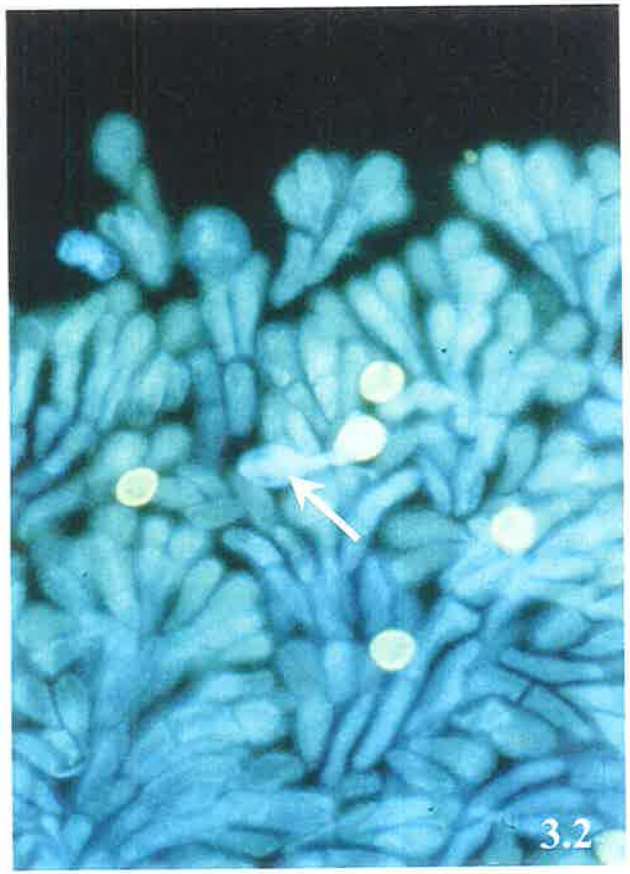

Fig 3.2 Abnormal pollen tube on stigma. Arrow shows swollen tip.

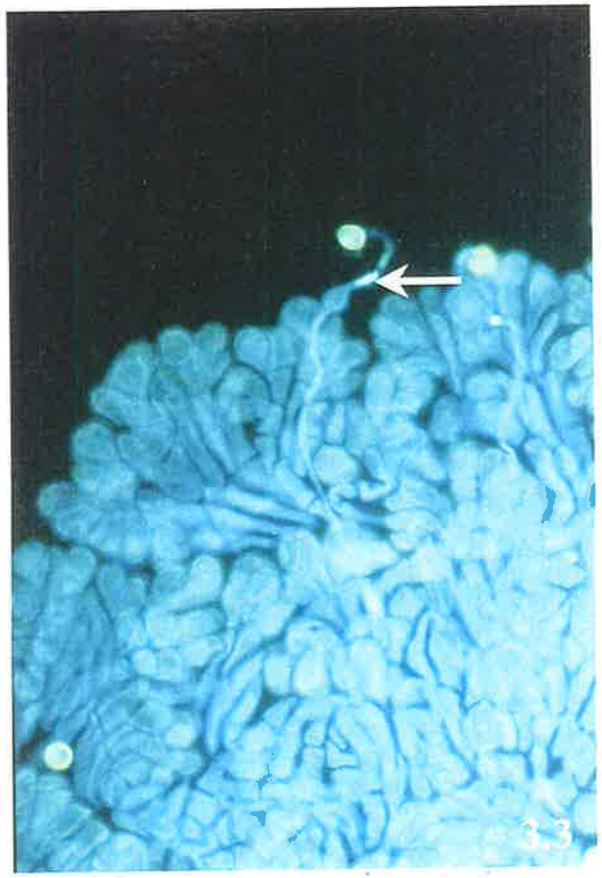

Fig 3.3 Pollen tube growth in the stigma. Arrow shows callose plug in the tube.

Regardless of the number of pollen grains that germinated on the stigma, and the number of pollen tubes in the stigma, only one, or rarely two, pollen tubes reached the lower style. The unsuccessful pollen tubes ceased growth in the upper style at the conjunction of the two stigmas (Fig 3.4). Usually, pollen tubes penetrated the ovules (Fig 3.5) 3 to 7 d after pollination, but for Kalamata tree 1 x SA Verdale tree 2 penetration took only one day.

3.3.3 Variation of pollen germination on stigmas

Pollen germination varied from 0 to 48.0% for different crosses (Table 3.2). The highest germination (48.0%) for self-pollination of Kalamata tree 2 was observed when the sample was collected 1d after pollination. Conversely, the pollen from Kalamata tree 1 on the stigma of SA Verdale tree 2 did not show any germination. Frantoio tree 1 pollen had maximum germination of 22% on Kalamata tree 1, but only 4.1% on its own stigma. Similarly, Kalamata tree 1 pollen germinated at 26.4% on Frantoio tree 1, but only 1.7% when selfed. 34.5% of Frantoio tree 2 pollen germinated on SA Verdale tree 1 stigmas, while SA Verdale tree 2 pollen produced only 1.8% pollen tubes on Frantoio tree 1 stigmas. The pollen of SA Verdale showed different germination rates on the stigmas of different trees of Kalamata, for example, 39.4% and 9.0% for Kalamata tree 2 x SA Verdale tree 1, and Kalamata tree 1 x SA Verdale tree 2 respectively. A similar effect occurred for the pollen of Kalamata on stigmas of SA Verdale where 32.6% germination resulted from the cross SA Verdale tree 1 x Kalamata tree 2, and 0% from SA Verdale tree 2 x Kalamata tree 1. Self pollen also showed variable germination, for example, Kalamata tree 2 had 48.0% pollen germination, whereas Kalamata tree 1 had 1.7%.

3.3.4 Pollen tube growth in upper style

Table 3.3 shows the number of pollen tubes in the upper style in all the crosses following different times of collection. The highest number was observed for the cross SA Verdale tree 1 x Frantoio tree 2 for pistils collected 3 d after pollination. There are several crosses for which pollen tubes failed ($P<0.05$) to reach the upper style. These included Frantoio tree 1 x SA Verdale tree 2, Kalamata tree 2 x SA Verdale tree 1, and selfed Frantoio tree 1, Kalamata tree 1, and Kalamata tree 2. SA Verdale tree 2 x Kalamata tree 1 also showed failure of pollen tube growth, but the value 0.00 was not significantly different from that obtained from the crosses showing significant number of pollen tubes in the upper style statistically, due to fewer samples being collected for the observation. Only SA Verdale tree 1 x Frantoio tree 2, and Frantoio

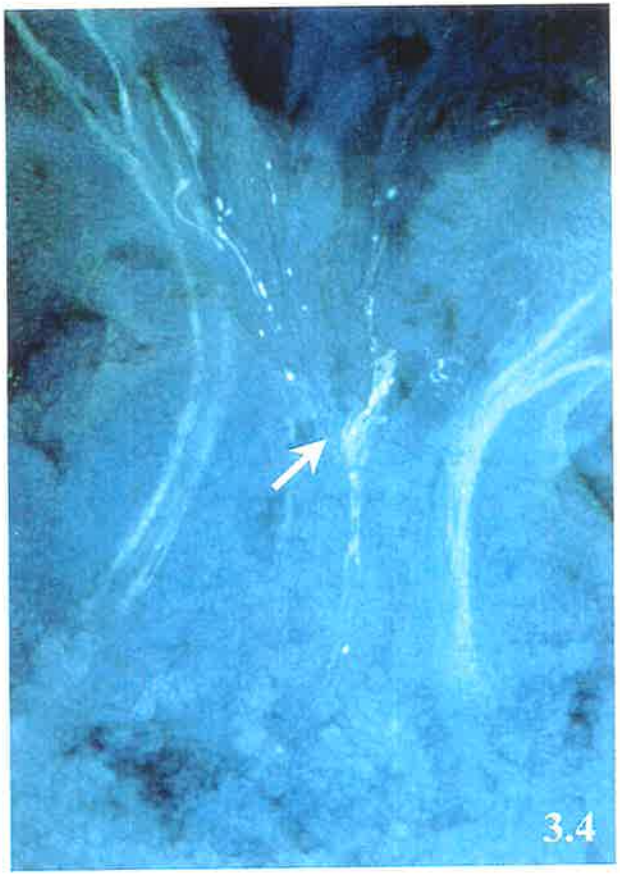

Fig 3.4 Pollen tube growth in the style. Arrow shows the inhibition site of unsuccessful pollen tubes.

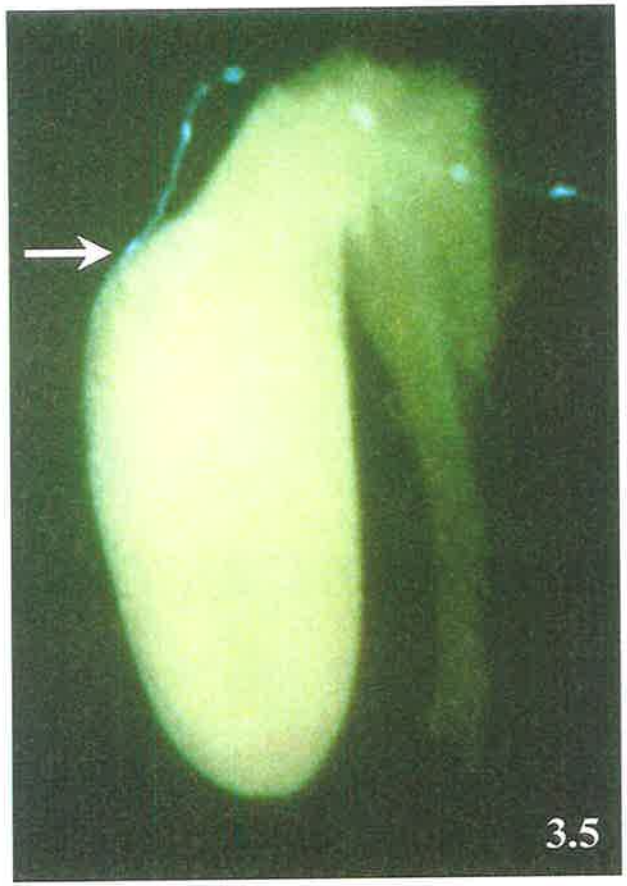

Fig 3.5 Pollen tube penetration of the ovule. Arrow shows a pollen tube penetrating the micropyle of the ovule.

Table 3.3 Pollen tubes in the upper style

	Day 1		Day 3		Day 7		Day 14	
Cross	Mean	Significance	Mean	Significance	Mean	Significance	Mean	Significance
Fra1 X Ver2	0.00	a*	0.00	a	0.00	a	0.00	a
Ver1 X Fra2	1.30	ab	12.70	c	10.90	c	4.33	b
Fra1 X Kal1	4.80	b	9.80	bc	4.11	b	3.70	b
Kal1 X Fra1	3.56	b	5.67	b	4.44	b	0.00	a
Kal2 X Ver1	0.00	a	0.00	a	0.00	a	0.00	a
Kal1 X Ver2	0.00	a	0.00	a	0.25	ab	0.00	a
Ver1 X Kal2	0.44	a	0.20	ab	0.40	ab	0.22	a
Ver2 X Kal1	0.00	a	0.00	ab†	0.00	ab†	N/A§	N/A
Ver1 selfed	0.60	a	0.56	ab	0.50	ab	0.20	a
Fra1 selfed	0.00	a	0.00	a	0.00	a	0.00	a
Kal2 selfed	0.00	a	0.00	a	0.00	a	0.00	a
Kal1 selfed	0.00	a	0.00	a	0.00	a	0.11	a

* Within a column, mean values followed by different letters are significantly different at the 0.(probability level

† The value 0.00 is not significantly different from the values with letter b, due to small sample size

§ not available

tree 1 x Kalamata tree 1, and the reciprocal crosses showed significant numbers of pollen tubes ($P<0.05$) in the upper style for at least one collection. The other crosses showed no significant difference in pollen tubes in the upper style, either from the higher group, such as SA Verdale tree 1 x Frantoio tree 2, Frantoio tree 1 x Kalamata tree 1, and Kalamata tree 1 x Frantoio tree 1, or the lower group such as Frantoio tree 1 x SA Verdale tree 2 and Kalamata tree 2 x SA Verdale tree 1.

3.3.5 Pollen tube growth in lower style

The number of pollen tubes in the lower style was lower than in the upper style (Table 3.4). On day 1 there were no significant differences between any of the crosses for the number of pollen tubes in the lower style. SA Verdale tree 1 x Frantoio tree 2 showed a significantly higher number of pollen tubes in the lower style for days 3, 7 and 14 compared to other crosses, as did Kalamata tree 1 x Frantoio tree 1 for day 3. Pollen tubes failed to arrive at the lower style in the following combinations, Frantoio tree 1 x SA Verdale 2, Kalamata tree 2 x SA Verdale tree 1 and the reciprocal crosses, Kalamata tree 1 x SA Verdale tree 2, and selfed Frantoio tree 1 and Kalamata trees 1 and 2.

3.3.6 Pollen tube penetration of ovule

The number of pollen tubes penetrating the ovule was reduced in comparison to those in the lower style (Table 3.5). Kalamata tree 1 x SA Verdale tree 2 was the only cross with pollen tubes penetrating the ovules on day 1, other crosses showed pollen tube penetration on day 3, and SA Verdale tree 1 x Frantoio tree 2 and SA Verdale tree 1 x Kalamata tree 2 showed significantly high pollen tube penetration on days 7 and 14 respectively.

3.3.7 Correlation between the numbers of pollen tubes in different levels of pistil

Cluster analysis of the number of pollen tubes at different levels within the pistil showed that the lower the pollen tubes grow down the pistil, the more likely they are to penetrate the ovules. The number of pollen tubes in the lower style was closely correlated to the number of pollen tubes penetrating the ovule (Fig 3.6).

3.4 Discussion

In this preliminary experiment on sexual compatibility of olives, it was found that the use of fluorescence microscopy to observe pollen tube growth in either style or ovary was best at 3

Table 3.4 Pollen tubes in lower style

	Day 1		Day 3		Day 7		Day 14	
Cross	Mean	Significance	Mean	Significance	Mean	Significance	Mean	Significance
Fra1 x Ver3	0.00	a*	0.00	a	0.00	a	0.00	a
Ver1 x Fra3	0.20	a	0.60	b	0.70	b	1.50	b
Fra1 x Kal2	0.10	a	0.40	ab	0.33	ab	0.20	a
Kal1 x Fra2	1.60	a	0.70	bc	0.50	ab	0.11	a
Kal2 x Ver2	0.00	a	0.00	a	0.00	a	0.00	a
Kal1 x Ver3	0.00	a	0.00	a	0.13	a	0.00	a
Ver1 x Kal3	0.11	a	0.20	ab	0.11	a	0.20	a
Ver2 x Kal2	0.00	a	0.00	a	0.00	a	N/A[§]	N/A
Ver1 selfed	0.40	a	0.22	ab	0.50	ab	0.20	a
Fra1 selfed	0.00	a	0.00	a	0.00	a	0.00	a
Kal2 selfed	0.00	a	0.00	a	0.00	a	0.00	a
Kal1 selfed	0.00	a	0.00	a	0.00	a	0.00	a

* Within a column, mean values followed by different letters are significantly different at the 0.05 probability level.

§ not available

Table 3.5 Pollen tubes in ovule

Cross	Day 1		Day 3		Day 7		Day 14	
	Mean	Significance	Mean	Significance	Mean	Significance	Mean	Significance
Fra1 x Ver2	0.00	a*	0.00	a	0.00	a	0.00	a
Ver1 x Fra2	0.00	a	0.20	a	0.30	b	0.00	a
Fra1 x Kal1	0.00	a	0.11	a	0.22	ab	0.00	a
Kal1 x Fra1	0.00	a	0.20	a	0.00	a	0.00	a
Kal2 x Ver1	0.00	a	0.00	a	0.00	a	0.00	a
Kal1 x Ver2	0.11	a	0.00	a	0.00	a	0.00	a
Ver1 x Kal2	0.00	a	0.00	a	0.10	ab	0.30	b
Ver2 x Kal1	0.00	a	0.00	a	0.00	a	N/A[§]	N/A
Ver1 selfed	0.00	a	0.11	a	0.10	ab	0.10	ab
Fra1 selfed	0.00	a	0.00	a	0.00	a	0.00	a
Kal2 selfed	0.00	a	0.00	a	0.00	a	0.00	a
Kal1 selfed	0.00	a	0.00	a	0.00	a	0.00	a

* Within a column, mean values followed by different letters are significantly different at the 0.05 probability level.

[§] not available

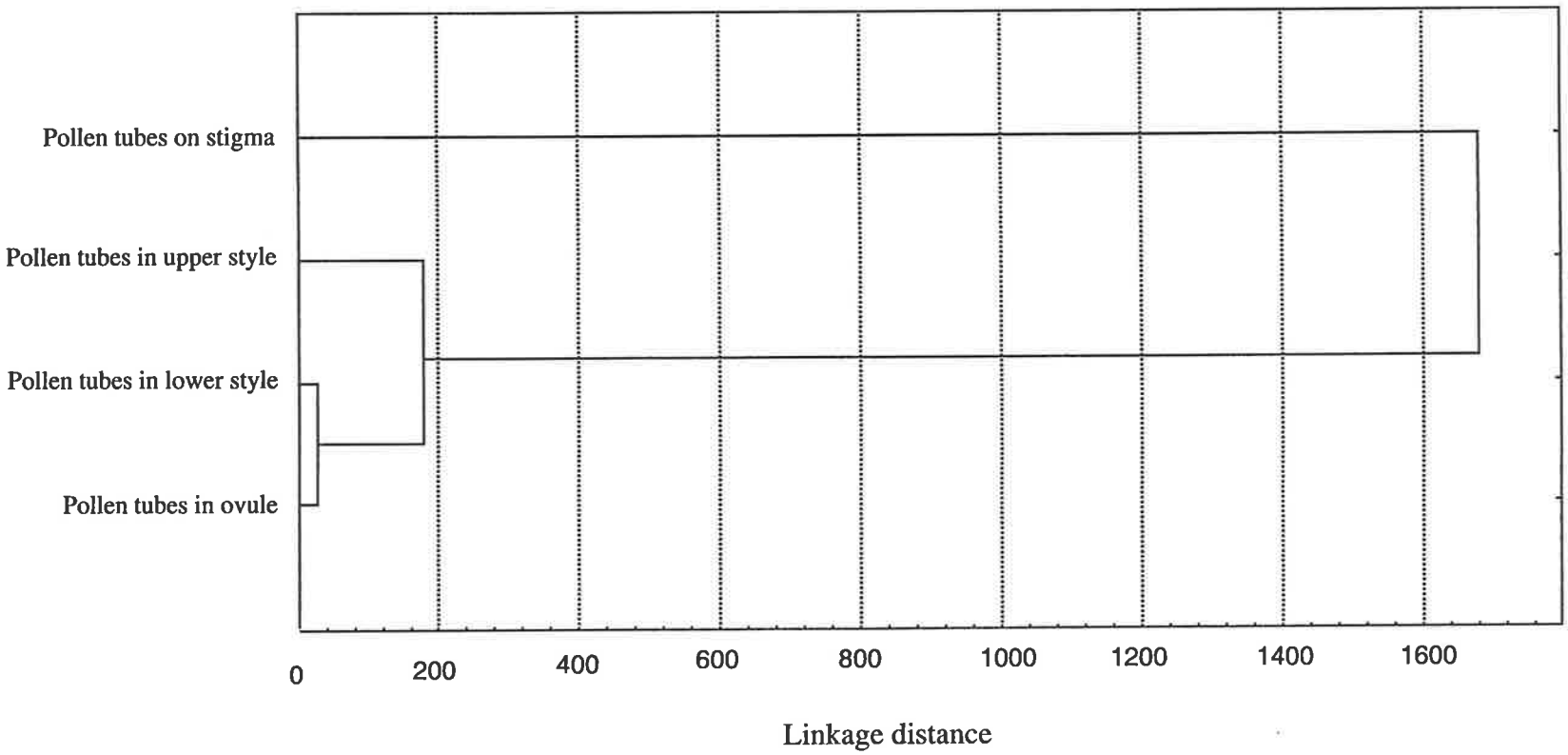

Fig.3.6 Linkage between locations of pollen tube presence in the olive pistil using unweighted pair group average a: the linkage rule, and Euclidean distance as the distance measure.

and 7 days after pollination. Based on the number of pollen tubes in the lower style, and those penetrating the ovule, day 7 after pollination was considered to be optimal for the collection of pistils for pollen tube observation.

Pollen germination on styles showed a wide range of variation. Possible reasons for this include differences in pollen viability, differences in stigma receptivity, differences in pollen-pistil interaction on the stigma surface, environmental effects, experimental variation, and small sample size. From these results, it is suggested that both pollen viability and stigma receptivity should be tested before pollination to ensure that pollen grains are viable and that stigma receptivity is adequate. The sample size should also be increased to allow for the variability in results.

The results in Table 3.2, show that pollen germination on the stigma decreases with an increase in the time of pistil collection after flowering. This result is unlikely, and possible explanations are as follows. 1. Sample size was too low to allow for the variability within the system. 2. With an increase in time of collection after pollination, fewer pollen tubes show fluorescence when stained by aniline blue (Cuevas *et al.*, 1994). 3. During the preparation of slides, pistils were softened using NaOH and then rinsed in running water, and it is possible that some pollen grains were washed off during the process. The stigma produces an exudate after anthesis, and if pistil collection is delayed, this secretion may result in an increase in the total number of pollen grains adhering to the stigma after washing. However, the number of germinated pollen grains may be the same. This would result in a reduction of the ratio of germinated pollen grains to total pollen grains. The fact that the total number of pollen grains increased in most of the crosses (data not shown) supports this assumption.

Significant differences between some of crosses were found between day 1 and day 14 for numbers of pollen tubes in the upper style, from day 3 to day 14 for the lower style, and on day 7 and day 14 for pollen tube penetration into the ovule (Table 3.3-3.5). However, the cross- and self-compatibility of the cultivars could not be clearly determined because of the limited number of cultivars used and the small sample size. A larger experiment was designed, and the results are the subject of Chapter 4.

CHAPTER FOUR

Sexual Compatibility of Olives-2000 Experiment

4.1. Introduction

Olive is a partially self-incompatible species, and cultivars may be totally self-incompatible or show some level of self-compatibility (Cuevas *et al.*, 2001; Griggs *et al.*, 1975). Sexual compatibility influences plant reproduction and therefore fruit yield, and has been studied intensively in some other plant species (de Nettancourt, 1997). In olive, self-incompatibility (SI) can become a problem when a cultivar is planted in an isolated area, where other olive pollen is not available (Ateyyeh *et al.*, 2000; Cuevas and Polito, 1997). Cross-pollination can enhance fertilisation as shown by reports of higher fruit set (Cuevas *et al.*, 2001; Cuevas and Polito, 1997), and lower production of shot berries, which are small and usually seedless olive fruits (Griggs *et al.*, 1975; Sibbett *et al.*, 1992). Fruit set after cross-pollination relies on compatibility between recipient and polleniser trees. In some cases, pollenisers may fail to improve olive fruit set because of incompatibility (Cuevas and Polito, 1997; Lavee and Datt, 1978).

In some cases, inconsistent results are reported about the classification of the same cultivar as self-compatible or self-incompatible. For example Manzanillo is considered to be a self-compatible cultivar by some authors (Androulakis and Loupassaki, 1990; Sibbett *et al.*, 1992), but mainly self-incompatible by others (Cuevas and Polito, 1997; Griggs *et al.*, 1975; Lavee and Datt, 1978). Leccino is reported to be highly self-incompatible (Bartolini and Guerriero, 1995; Ugrinovic and Stampar, 1996), but pollen tube penetration of the ovule, and satisfactory fruit set following self-pollination have also been reported (Bartolini and Guerriero, 1995; Bartolini *et al.*, 2000). The reasons for this include the effect of local environmental conditions on self-incompatibility (Griggs *et al.*, 1975), and on the genetic background of the trees used, as there is confusion about the names of cultivars grown around world (Bartolini *et al.*, 1994; and Mekuria *et al.*, 1999).

Diallel matrix designs are frequently used to investigate SI (Sedgley, 1994). These involve controlled pollinations, including selfing and reciprocal crossing. In olives, however, there are no reports where diallel crossing has been applied, despite a number of investigations into self- and cross-compatibility (Ateyyeh *et al.*, 2000; Cuevas *et al.*, 2001; Griggs *et al.*, 1975). The

situation is further complicated as olive produce both perfect (complete) and staminate flowers (Brooks, 1948; Griggs *et al.*, 1975). The ratio appears to differ in different cultivars as well as under varying environmental conditions, such as insufficient winter chilling (Martin, 1990).

There are about 1500 olive cultivars, with 2700 synonyms (Bartolini *et al.*, 1999), but only a few of these, together with their corresponding crosses, have been examined for their sexual compatibility. In Australia, about 200 cultivars are grown commercially (Burr, 1999), and limited knowledge is available on their self- and cross-fertility. Pendolino has been regarded as being compatible with a number of other cultivars and therefore it has been planted as a common polleniser (Booth and Davies, 1996). Frantoio is considered to be self-compatible, and so has been planted as a single cultivar without the concern of possible low yields (Bini, 1984; Sharma *et al.*, 1976). However, detailed investigations have not yet been conducted into pollination under Australian environmental conditions.

Frantoio, Kalamata, Manzanillo, Pendolino, and Picual are five popular cultivars that are widely cultivated in Australian olive plantings, some for table olives and others for oil (Booth and Davies, 1996). Consequently, their self- and cross-compatibility is important for Australian growers. This research aimed to determine the self- and cross-compatibility of and between these cultivars to provide compatibility information to be used as a reference for the Australian olive industry. Some investigations were also conducted on the cultivar SA Verdale.

4.2 Materials and methods

4.2.1 Climatic conditions

The experiments were conducted in the Adelaide area of South Australia (Latitude: 34°32'–35°17' S, Longitude: 138°30' – 138°53' E) during the 2000 flowering season. The climatic data before and during flower opening, and the long-term averages (1929 – 2001) for the corresponding period, were collected (Figs. 4.1, 4.2). Although flowering lasted from late October to early November, the climatic conditions for winter (July and August) are also included because winter chilling plays important role in floral initiation and the production of staminate flowers (Martin, 1990).

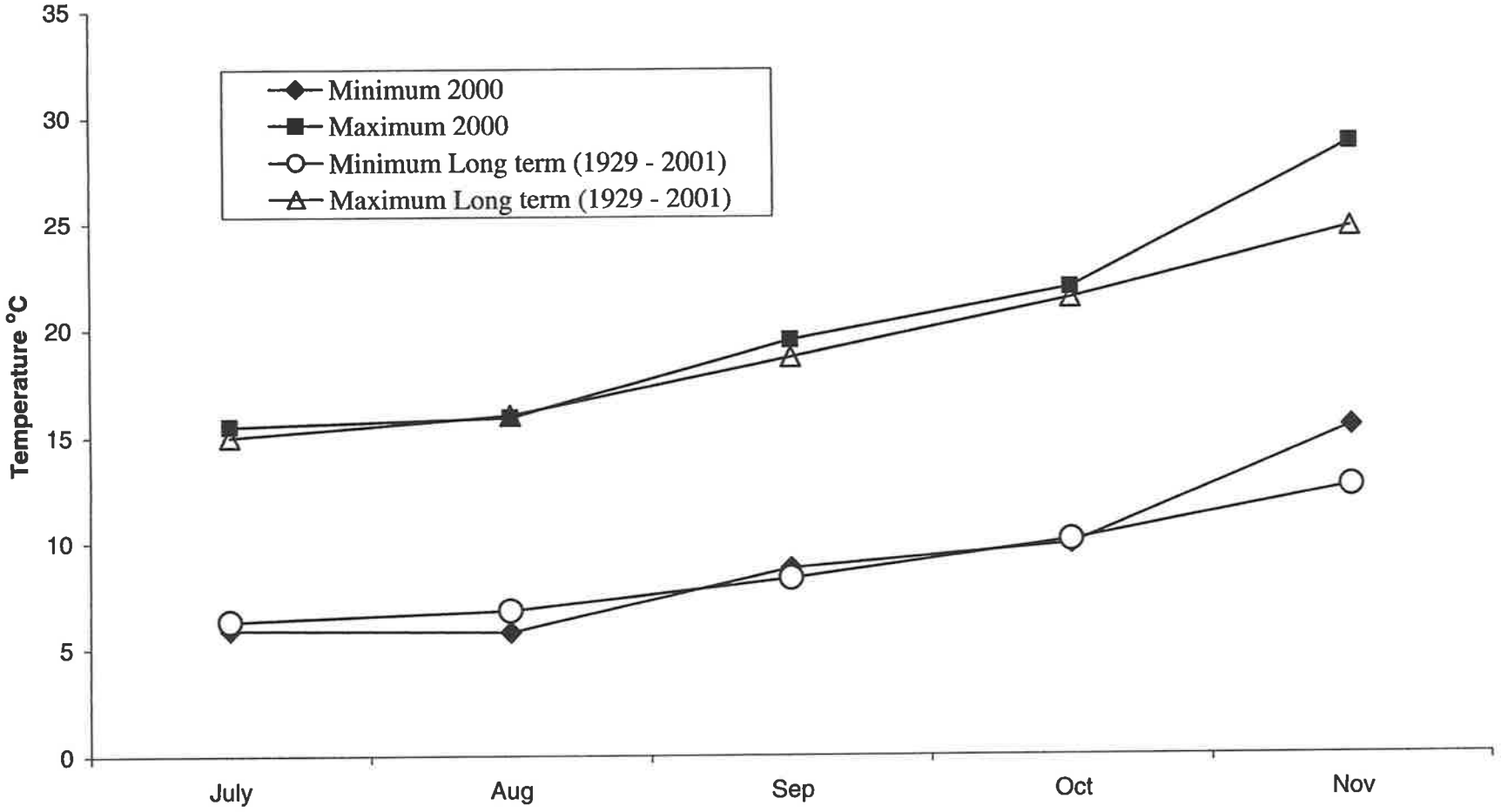

Fig. 4.1 Monthly means of daily maximum and minimum temperatures in the Adelaide area of South Australia from July to December for the year 2000 compared to the long term averages. Data were collected from Parafield Airport (34°48'S, 138°38'E) (Australian Bureau of Meteorology).

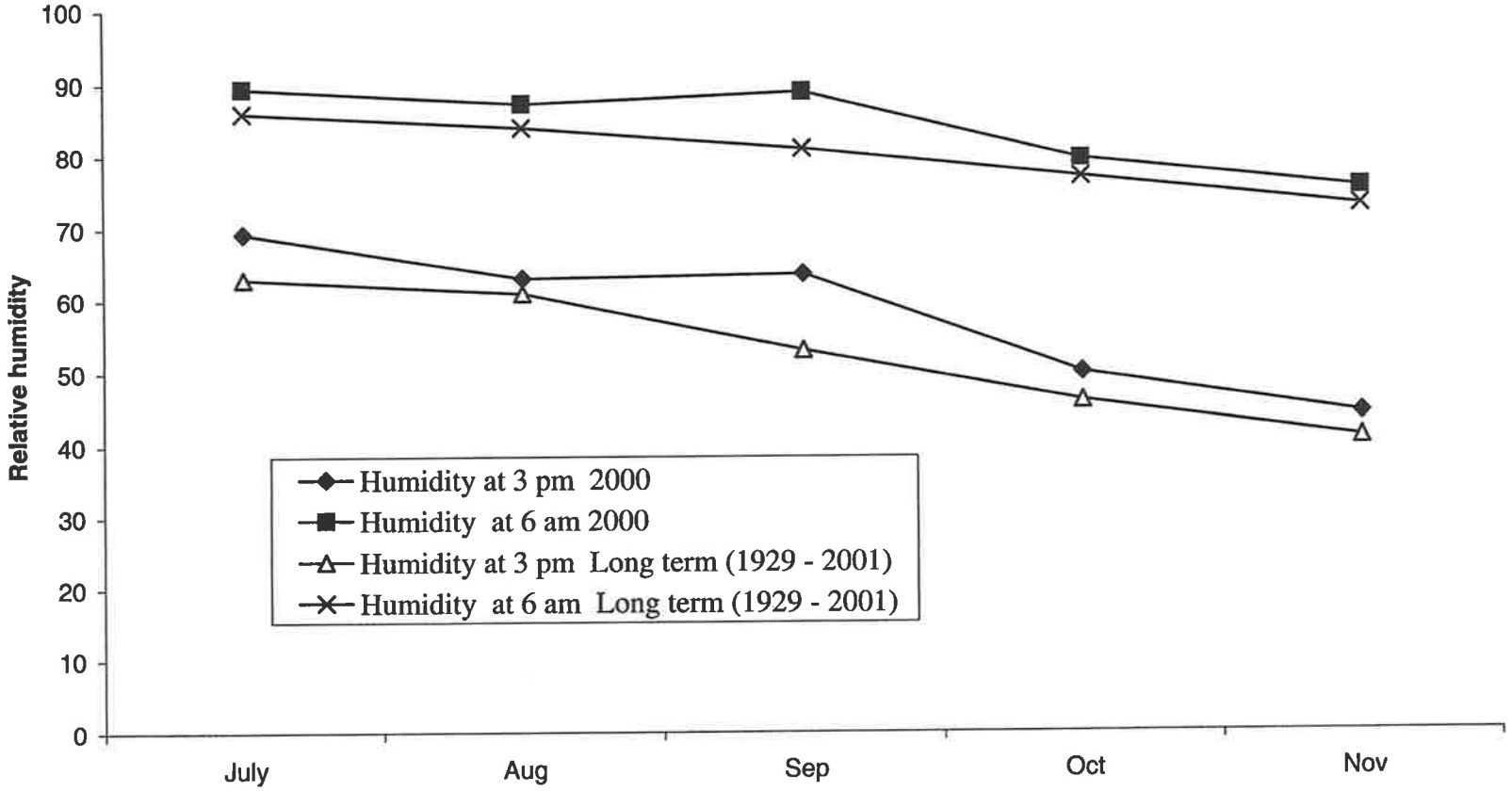

Fig. 4.2 Comparison of Adelaide monthly means of relative humidity at 6 am and 3 pm from July to December for the year 2000 compared to the long term averages. Data were collected from Parafield Airport (34°48'S, 138°38'E) (Australian Bureau of Meteorology).

Climatic conditions were similar at all sites before and during flower opening. For SA Verdale, three trees at six sites were investigated for male sterility and pistil receptivity (Table 4.1).

4.2.2 Trees

Six cultivars were involved in the 2000 pollination experiment: Frantoio, Kalamata, Manzanillo, Pendolino, Picual, and SA Verdale. For Frantoio, Kalamata, Manzanillo, Pendolino, and Picual, three trees were used of each cultivar, each tree being considered as a replicate. When the flowering stage of a cultivar did not overlap the flowering stage of others, either as male or female parents, another three trees of the same cultivar (genetically identical) were selected as substitutes. The locations and ages of the trees are listed in Table 4.1.

Prior to pollination, the identities of the trees used were confirmed by DNA fingerprinting as described by Mekuria *et al.* (1999). The standards used for identification were 'Frantoio', 'Manzanillo de Sevilla', 'Pendolino' and 'Picual' from the Olive World Collection, Centro de Investigacion y Desarollo Agrario, Cordoba, Spain, 'Kalamata' from Consiglio Nationale delle Ricerche, Instituto di Ricerca Sulla Olivicultura, Perugia, Italy, and 'Verdale' from Foundation Plant Material Service, University of California, Davis, California, USA. Genetic similarity and clustering analysis was carried out as stated in 2.2.3.4.

4.2.3 Inflorescence structure and sex ratio

During the full bloom stage, ten inflorescences per tree were used to determine the number of flowers per inflorescence, and the sex ratio of flowers, for Frantoio, Kalamata, Manzanillo, Pendolino, and Picual. Three trees were investigated, each tree as a replicate. The inflorescences were randomly chosen from the top, south, north, west, and east of the tree, two for each location.

Flowers with both well-developed anthers and a pistil were considered as complete. Staminate flowers were recorded if the flowers had either no or only remnants of pistils. There were no pistillate flowers that lacked anthers. The sex ratio was calculated from the ratio of complete flower number to total flower number, where the number of complete flowers represented the number of females and number of total flowers represented the number of males.

Table 4.1 Location and age of olive trees investigated for pollination study

	Location								
Cultivars	Virginia	Angle Vale	Roseworthy	Gumeracha	Willunga Hill	Lonsdale	Waterloo Corner	Two Wells	age (year)
Frantoio	Tree 1,2,3				Tree 4,5,6				6
Kalamata	Tree 1,2,3				Tree 4,5,6				6
Manzanillo		Tree 1,2,3		Tree 4,5,6					3
Pendolino			Tree 4,5,6	Tree 1,2,3					3
Picual			Tree 1,2,3						3
SA Verdale		Tree 1,2,3	Tree 4,5,6		Tree 7,8,9	Tree 10,11,12	Tree 13,14,15	Tree 16,17,18	6-12

4.2.4 Pollen viability

4.2.4.1 Pollen grain staining

Pollen grains were collected by enclosing the inflorescences in white paper bags. Bags were put in place before the flowers opened, and the branch was shaken on the day the flowers were in bloom. Pollen grains were transferred to a 0.5 mL Eppendorf tube for experimentation.

Pollen grains were placed on a glass slide and immersed in a drop of fluorescein diacetate (FDA, 2 mg/mL in acetone) and incubated for 10 min. After covering with a cover slip, the sample was viewed under a fluorescence microscope with excitation at 450-490 nm wavelength and emission at 520 nm (Pinney and Polito, 1990). Five fields of view of pollen grains on slides were randomly chosen for counting. All pollen grains with bright fluorescence were counted as viable. Viability percentages were determined using approximately 100 grains.

4.2.4.2 Pollen *in vitro* germination

A pollen germination medium was prepared using Bacto-agar (0.8%), sucrose (15%), 100 ppm boric acid, and 60 ppm tetracycline (Pinney and Polito, 1990). Pollen grains were sprinkled over the surface of the medium in 15 x 60 mm Petri dishes and allowed to incubate for 24 hours before counting. Pollen was considered to have germinated if the pollen tubes extended to a length equal to at least twice the diameter of the grain. Germination percentages were determined using five randomly chosen fields of approximately 100 grains.

4.2.5 Pollination experiments

4.2.5.1 Pistil receptivity

Pistil receptivity was tested on SA Verdale, an early flowering cultivar in South Australia, using Kalamata pollen. Forty flowers on SA Verdale were emasculated immediately after the flowers opened, and pollen was applied to 10 pistils at each of 0, 3, 7, and 14 d. Seven days after the pollen was applied, the pistils were collected, and pollen tube germination on the stigma, and growth in the stigma and style, were observed after staining with de-colourised aniline blue (Martin, 1959) under a Zeiss photomicroscope (Axiophot) equipped with a filter set of exiter filter 395-440, interference beam splitter FT 460, and barrier filter LP 470.

4.2.5.2 Pollination experimentation

A 5 x 5 diallel matrix design was applied to the 2000 pollination experiments. Twenty flowers were used per cross and three matrices were made as replicates. Pollination methods and fluorescence microscopy were applied as described previously (see Chapter 2).

4.2.6 Data collection

The number of pollen grains on the stigma, pollen tubes on the stigma, pollen tubes in the upper and lower style, and pollen tubes penetrating an ovule were recorded. Pollen was considered to have germinated if the pollen tube extended to a length equal to at least twice the diameter of the grain.

4.2.7 Data analysis

One-way ANOVA was employed for the analysis of all data obtained for flower number per inflorescence and pollen viability for the cultivars investigated, using MINITAB (Version 13.20, Minitab Inc.). Confidence intervals of the means were applied to determine the significance of differences between cultivars and/or combinations.

Pollen tube numbers in various parts of the pistil were analysed initially by the method described by Griffing (1956). The variability in the means was partitioned into general combining ability (gca), specific combining ability (sca), reciprocal effect (r), and experimental error, using DIAL (Ukai, 1989). Valid analyses using this software program can be made only if the data are normally distributed with constant variance. In the case of the diallel data, this assumption could not be made because of the small number of pollen tubes reaching the lower style, and the presence of incompatibility following cross pollination that resulted in a high number of zeros in the data.

To overcome this problem, the analysis was limited to the number of pollen tubes in the lower style. Because the number of pollen tubes in this region was rarely more than 1, the presence of at least one pollen tube was allocated '1', and the absence of pollen tubes was allocated '0'. A binary matrix was developed for the diallel cross data, and this was analysed as a logistic regression using a generalized linear model (GLM) assuming a binomial distribution and a logit link (Lefort and Legisle, 1977).

The probability of a flower having a pollen tube in the lower style can be calculated using:

$$\text{Logit}\,(p_{ij}) = \log\left(\frac{p_{ij}}{1-p_{ij}}\right) = \mu + (Female)_i + (male)_j + (Female{\cdot}Male)_{ij}$$

Where

$i = 1,\ldots,5$ corresponding to each cultivar as a female parent; $j = 1,\ldots,5$ corresponding to each cultivar as a male parent.

p_{ij} is the probability that a flower has a pollen tube in the lower style ($0 < p_{ij} < 1$).

$1 - p_{ij}$ is the probability that a flower does not have a pollen tube in the lower style.

μ is the grand mean.

The probability that a flower has a pollen tube present in the lower style (p_{ij}) can be thought of as a long run expected value. This means that it approximates the proportion of flowers that have a pollen tube if the experiment was repeated many times. Values for p can be obtained for a particular cross by re-arranging the final predicted model. The importance of the various combinations of female and male is determined using a likelihood ratio test, which is distributed approximately according to the chi-squared analysis.

To detect differences in the presence of pollen tubes in various parts of the pistils between various combinations, one-way ANOVA was also applied to analyse pollen tube data using MINITAB (Version 13.20, Minitab Inc.).

4.3 Results

4.3.1 Comparison of climatic conditions

The monthly means of daily maximum and minimum temperatures during the flowering period for the area under study are shown in Fig. 4.1 together with the long-term averages. Temperatures from July to October matched those of the long-term averages, whereas the maximum and minimum temperatures in November were 4.0°C and 2.8°C higher than the long-term averages, respectively.

The monthly means of daily relative humidity at 6:00 am and 3:00 pm during the flowering period are shown in Fig. 4.2 together with the long-term average. Relative humidity for July,

August, October, and November matched those of the long-term averages, whereas in September the means were 7.9% and 10.7% higher than those of the long-term average for 6:00 am and 3:00 pm, respectively.

4.3.2 Identification of the cultivars

The DNA fingerprints of the trees used were identical to those for 'Frantoio', 'Manzanillo de Sevilla', 'Pendolino' and 'Picual' from the Olive World Collection, Centro de Investigacion y Desarollo Agrario, Cordoba, Spain, 'Kalamata' from Consiglio Nationale delle Ricerche, Instituto di Ricerca Sulla Olivicultura, Perugia, Italy, and Verdale from Foundation Plant Material Service, University of California, Davis, California, USA, respectively (Fig. 4. 3).

4.3.3 Inflorescence structure and sex ratio

The inflorescence of the olive is a panicle, with flowers distributed along the rachises in an opposite pattern. Flower number per inflorescence varied from 12 to 45 for the cultivars investigated. The results of the statistical analysis showed that numbers of flowers per inflorescence were significantly different among the cultivars. Kalamata and Pendolino had the highest numbers of flowers per inflorescence, whereas Picual had the lowest. Frantoio and Manzanillo were intermediate (Table 4.2).

Each of the cultivars investigated showed a different level of sex ratio (Table 4.2). Complete flowers were predominant in Frantoio, Manzanillo, and Pendolino. In contrast, staminate flowers were more frequent in Kalamata and Picual. The highest ratio of complete to staminate flowers was 0.87 : 1 for Frantoio, and the lowest was 0.23 : 1 for Kalamata.

There were no significant differences between the three different trees examined for each cultivar (data not shown).

4.3.4 Pollen viability

Pollen viability was investigated prior to each controlled pollination. The results for both FDA (Fig. 4.4 A) and *in vitro* germination (Fig. 4.4 B) are shown in Fig 4.5.

The results from FDA staining showed that Frantoio had the highest pollen viability of 78.7%, and this was significantly higher than for the other four cultivars at $P < 0.05$. Kalamata and Picual had about the same viability, and were intermediate between Frantoio, and Manzanillo

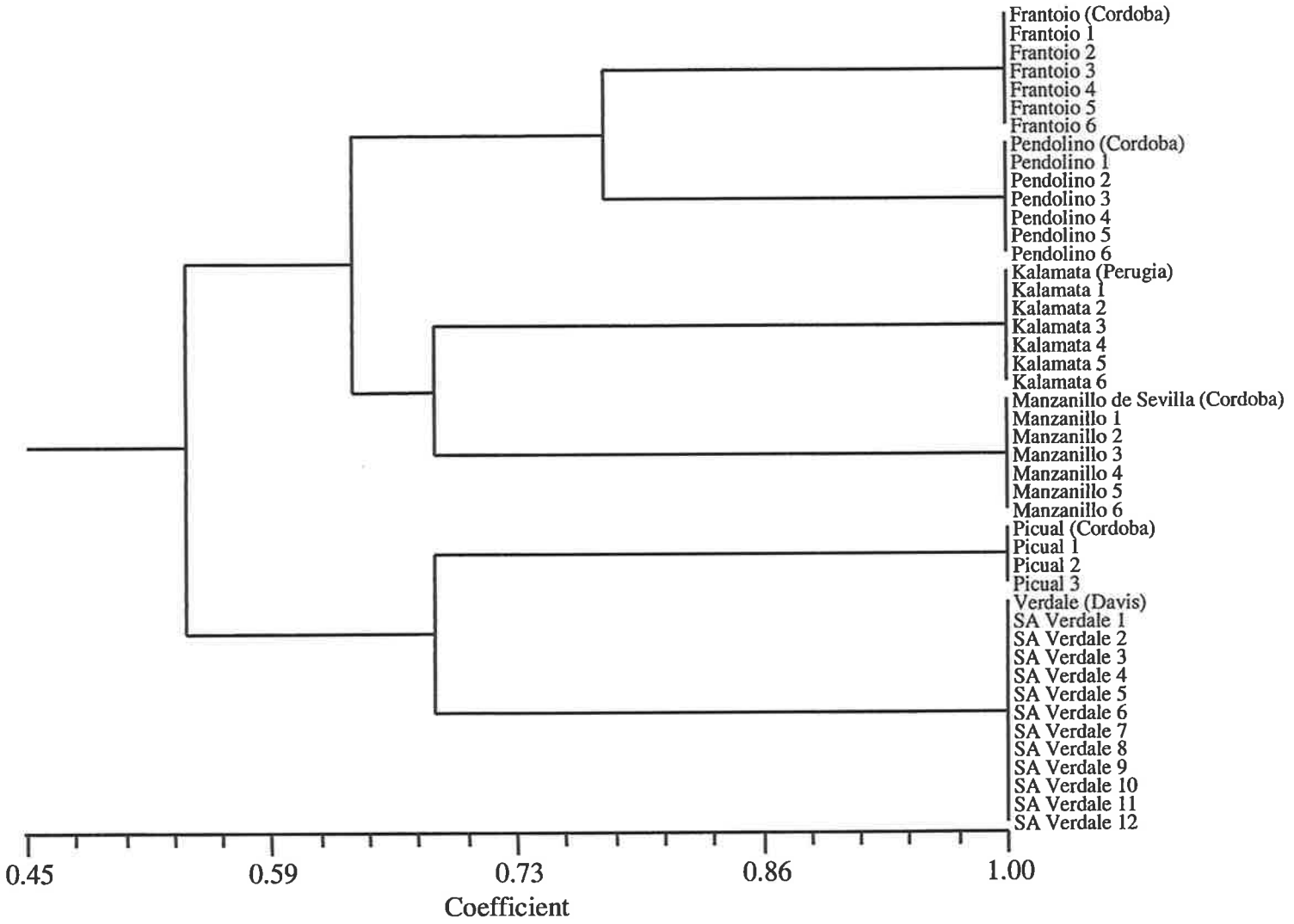

Fig. 4.3 Dendrogram of the trees used for pollinations compared with standard cultivar samples. Genetic similarities among all pairs of individuals were estimated using the hierarchical distance method of simple matching coefficient with NTSYS-pc (Exeter software, NY, USA). Cluster analysis was performed on the estimated similarity using the unweighted pair group method with arithmetic average (UPGMA), and the SAHN algorithm.

Table 4.2 Flower numbers per inflorescence and sex ratio of olive cultivars

Cultivars	Flowers/ inflorescence		Complete flowers (%)		Sex ratio (Female : Male)
Frantoio	21.1	b*	86.6	a*	0.87 : 1
Kalamata	28.3	a	22.9	b	0.23 : 1
Manzanillo	18.4	b	80.6	a	0.81 : 1
Pendolino	25.9	a	85.4	a	0.85 : 1
Picual	14.6	c	29.3	b	0.29 : 1

* Within the column, mean values followed by different letters are significantly different at the 0.05 probability level.

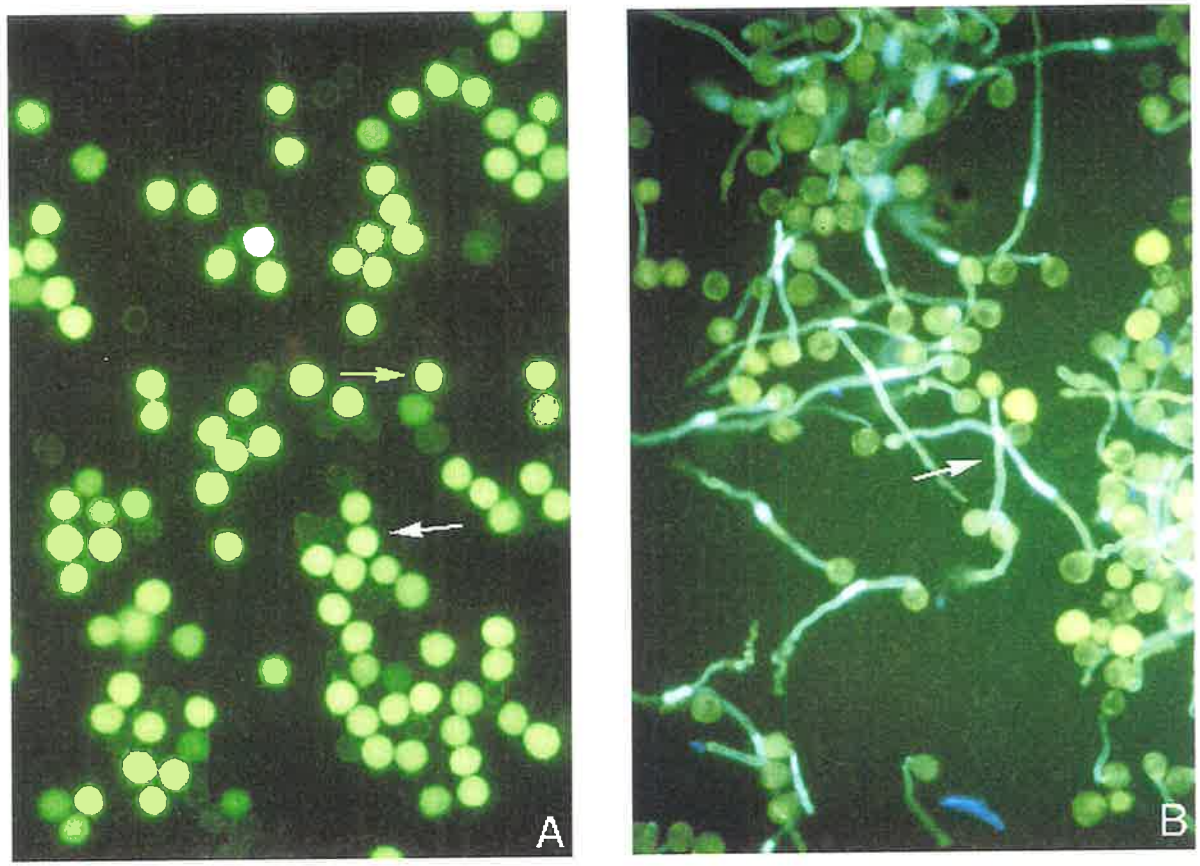

Fig. 4.4 Olive pollen viability test (Frantoio). A. FDA staining and B. *in vitro* pollen germination. Arrow shows viable pollen grain (A) and pollen tube (B)

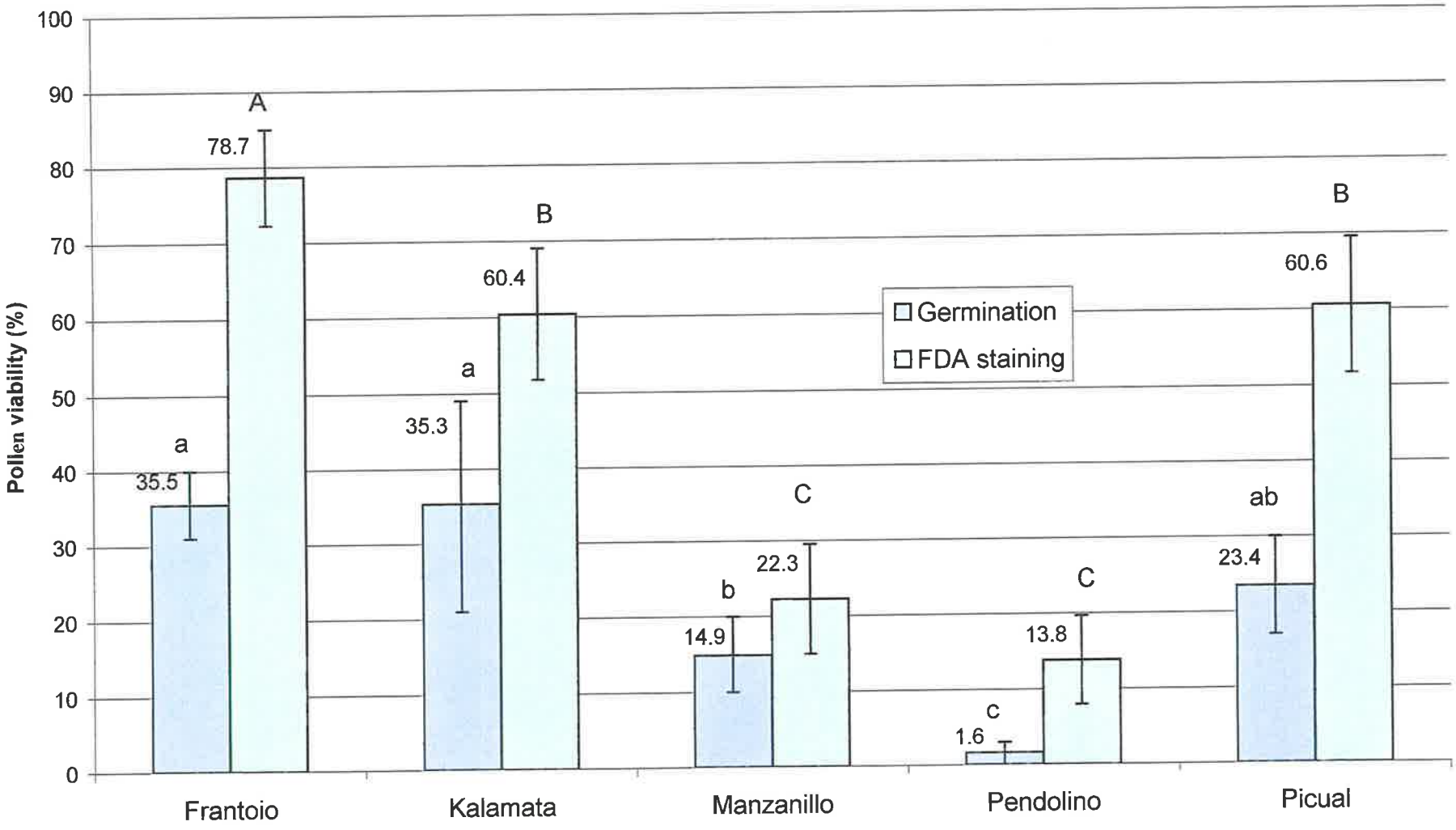

Fig.4.5 Pollen viability in olive cultivars compared by FDA staining and *in vitro* germination. Columns with the same letter, upper case for FDA staining, and lower case for *in vitro* germination, are not significantly different. Bars represent 95% confidence interval. Values are the means of pollen viability percentage.

and Pendolino. Manzanillo and Pendolino had the lowest pollen viability at 22.3% and 13.8% respectively. The results for the *in vitro* germination tests were always lower than the FDA staining method. However, the viability was still highest for Frantoio, intermediate for Kalamata and Picual, and lowest for Manzanillo and Pendolino, and the coefficient of determination for the two methods was 0.86 (Fig 4.6).

4.3.5 Male sterility

SA Verdale was a cultivar considered for the 2000 pollination experiment. However, this cultivar was male sterile for most of the flowering season. The anthers were white or pale yellow and there were no pollen grains inside, or shed from, the anther loculus. Almost at the end of the bloom period, some flowers started to produce yellow anthers with pollen, but pollen viability was only 5.06% as measured by FDA staining. Six orchards were inspected around Adelaide, South Australia, at Roseworthy, Angle Vale, Two Wells, Waterloo Corner, Lonsdale and Willunga Hill, and the results were identical at each site. Picual was substituted for SA Verdale in the diallel matrix.

4.3.6 Stigma receptivity

Pollen germination on the stigma was 12.9% when Kalamata pollen was applied to the stigma of SA Verdale on the day of flower opening (Fig. 4.7). On days 3 and 7 after pollination, the germination was significantly lower at 10.5% and 9.3% respectively. On day 14, the stigmas were shrivelled indicating that the stigma had already lost its receptivity. All subsequent pollinations were conducted on the day of flower opening.

4.3.7 Pollen tube growth on the stigma and in the pistil

4.3.7.1 On stigma

The germination of pollen grains among the crosses is shown in Fig 4.8. The highest germination, 27.7%, was produced by pollen of Frantoio on stigmas of Pendolino. Manzanillo and Picual pollen on stigmas of Frantoio produced 23.5% and 23.4% germination respectively. Pendolino and Picual pollen produced about 3% germination on stigmas of Kalamata and Manzanillo, as did self pollen on Frantoio and Pendolino. Self-pollination of Picual, Manzanillo, and Kalamata produced germinations of 12.4, 19.2, and 18.3% respectively, and the values for Manzanillo and Kalamata were significantly higher than these for Picual, Frantoio, and Pendolino.

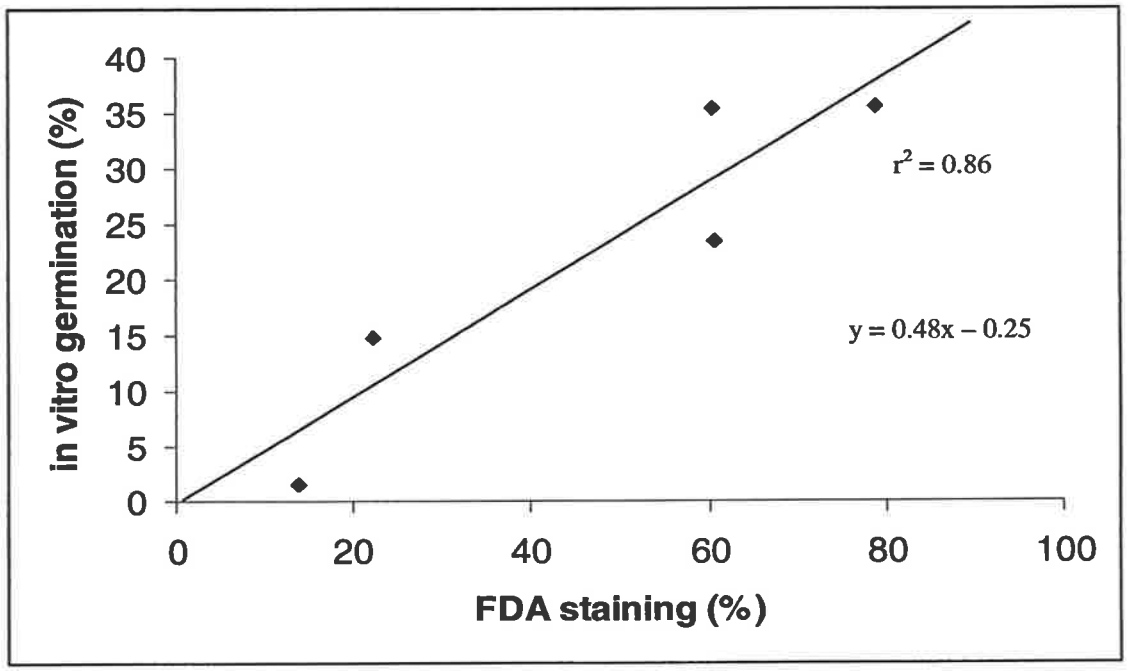

Fig 4.6 Regression of *in vitro* germination of pollen against FDA staining to determine pollen viability of olive cultivars

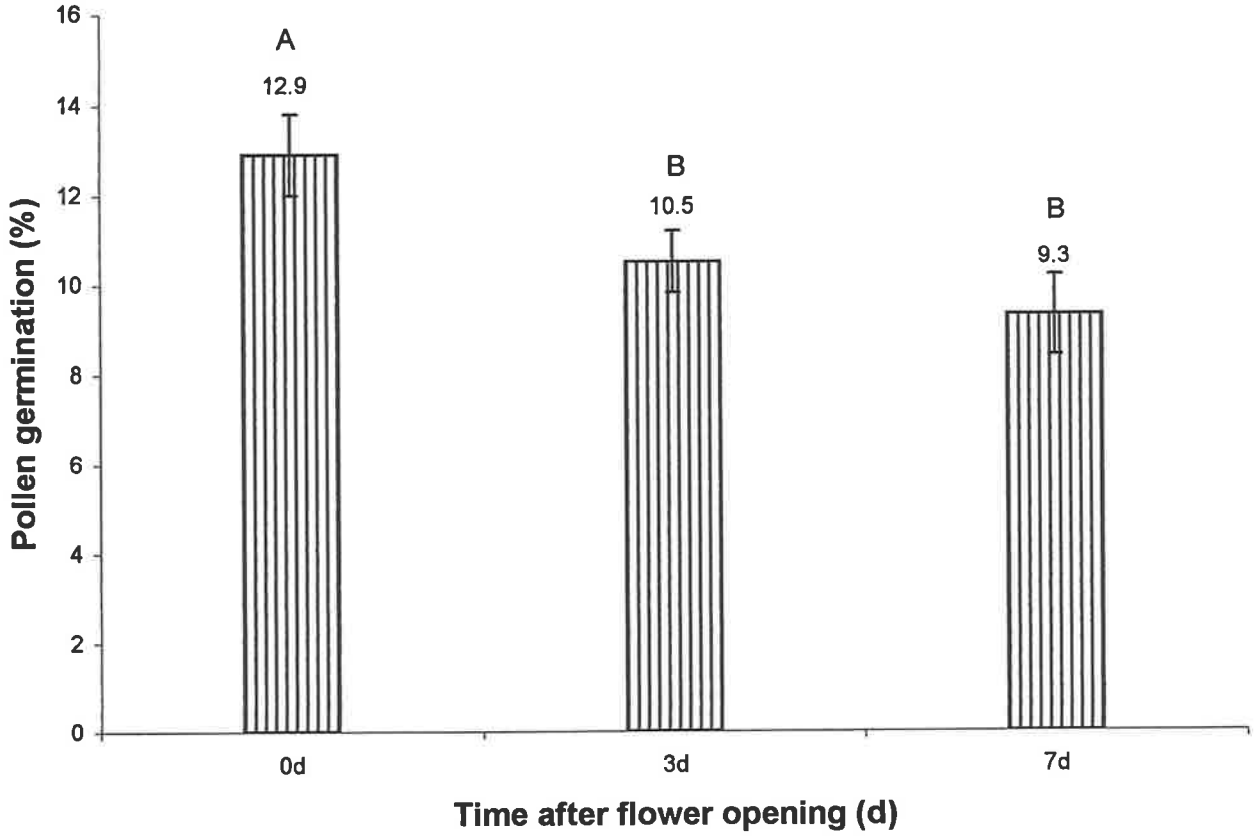

Fig. 4.7. Kalamata pollen was applied to the stigmas of SA Verdale at flower opening and at 3, 7, and 14 days later. Seven days after the pollen was applied, pistils were harvested, stained with aniline blue, and pollen tubes counted. No pollen tubes were observed when pollen was applied 14 days after flower opening. Columns with the same letter are not significantly different. Bars represent 95% confidence interval. Values are the means of pollen germination percentage.

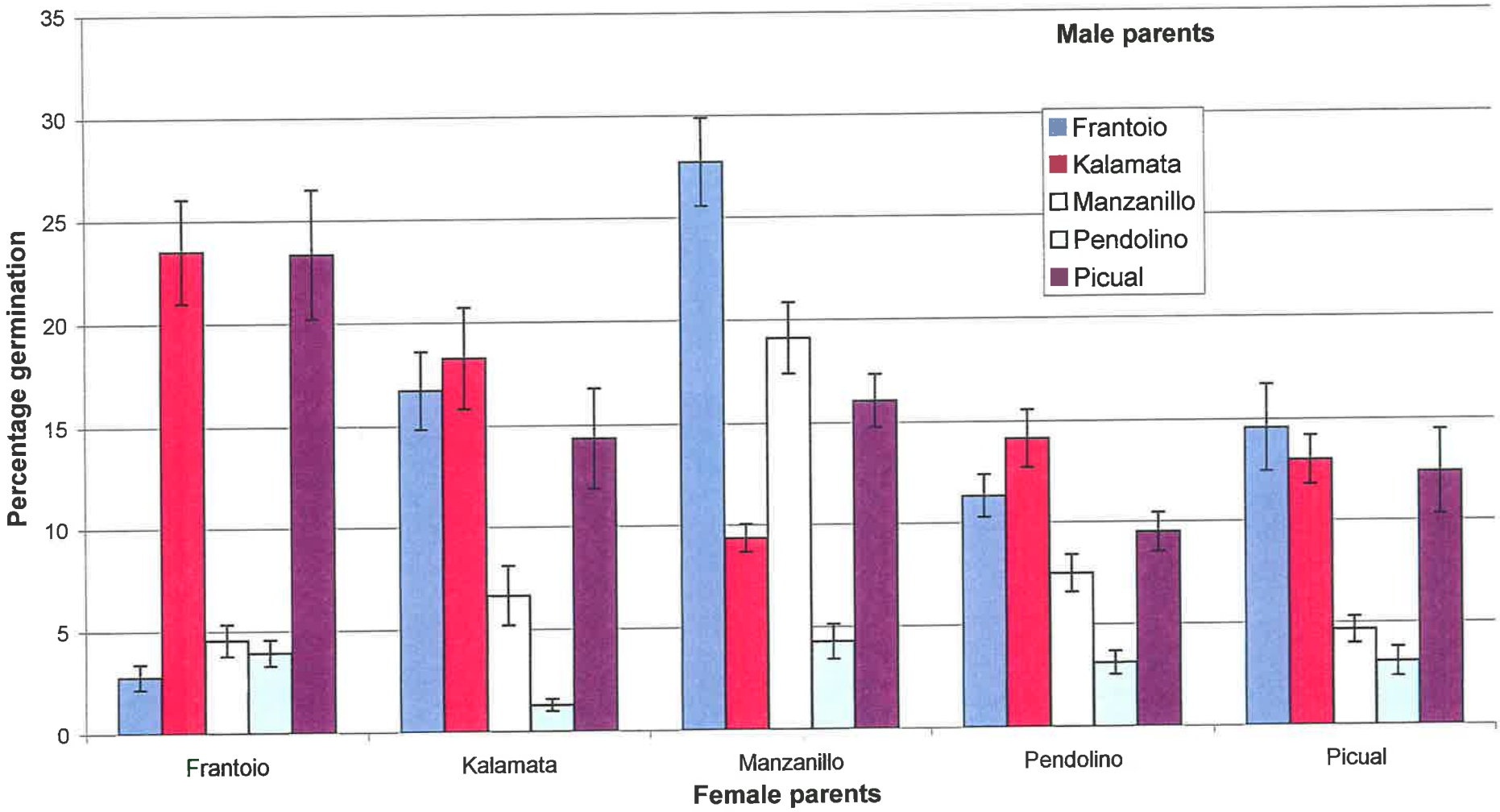

Fig 4.8 Results of diallel cross between five olive cultivars showing pollen germination on the stigma. Bars represent 95% confidence interval.

When the germination of pollen grains was compared between male parents, regardless of female parents, the average was highest for Frantoio, and lowest for Pendolino (Table 4.3). When the cultivars were considered as female parents, the results showed that the stigma of Pendolino had the highest numbers of pollen grains germinating, Frantoio and Kalamata were intermediate, and Manzanillo and Picual had the lowest.

Comparison between pollen germination on the stigma with pollen viability showed that the ranking was similar, with Frantoio the highest and Pendolino the lowest (Table 4.4). The germination of Manzanillo pollen ranked higher on the stigma than in the viability test.

4.3.7.2 In upper style

Generally, the number of pollen tubes in the upper style was much lower than the number of germinated pollen grains on the stigma. Usually, most of the pollen tubes arriving at the upper style from the stigma were blocked at a point just below the junction between stigma and style. The results showed that less than 30% of pollen tubes grew down to the upper style. Among those, Frantoio x Picual and Frantoio x Kalamata had the greatest pollen tube numbers in the upper styles. The average number of pollen tubes varied from 2.4 to 13.4 per pistil.

All self-pollinations produced significantly lower pollen tube numbers in the upper style than cross pollinations, even though Manzanillo and Kalamata selfs had high germination on the stigma (Fig. 4.8). There were significant gca, sca and reciprocal effects (Table 4.5).

4.3.7.3 In lower style

In comparison with the number of pollen tubes in the upper style and on the stigma, tube numbers in the lower style declined further. It was found that although many pollen tubes arrived at the upper style, only one or rarely two arrived at the lower style. Also, not all individual pistils showed pollen tubes in the lower style, and therefore, the average pollen tube number in the lower style was mostly less than 1. The cross of Frantoio x Picual was an exception, however, which had 1.1 pollen tubes on average in the lower style, indicating that two or more pollen tubes were reaching the ovary with a relatively high frequency. In this cross, up to four pollen tubes were observed in the lower style.

Table 4.3 Comparison of means of pollen germination percentages on the stigma between cultivars both as male and female parents

Cultivars	As male parents		As female parents	
	Pollen germination (%)	Significance	Pollen germination (%)	Significance
Frantoio	14.6 ± 1.2	a*	11.6 ± 1.4	b*
Kalamata	11.9 ± 0.8	b	11.5 ± 1.1	b
Manzanillo	11.0 ± 1.1	b	9.5 ± 0.9	bc
Pendolino	7.7 ± 0.8	c	15.5 ± 1.1	a
Picual	12.0 ± 1.2	b	9.1 ± 0.6	c

* Within the column, mean values followed by different letters are significantly different at the 0.05 probability level.

Table 4.4 The rank of cultivars for pollen viability and germination on stigma (pooled for all cultivars).

Rank	Pollen viability		Pollen germination on stigma (pooled)		
1	Frantoio	a*	1	Frantoio	a*
2	Kalamata	b	2	Kalamata	b
2	Picual	b	2	Picual	b
3	Manzanillo	c	2	Manzanillc	b
3	Pendolino	c	3	Pendolino	c

* Within the column, mean values followed by different letters are significantly different at the 0.05 probability level.

Table 4.5 Analysis of variance of diallel cross of five olive cultivars based on pollen tubes occurring within upper style, lower style, and ovule

Source of variation		Degrees of freedom	Mean Square	Significance
Replicates (upper style)		2	796.25	P > 0.1
(lower style)		2	6.87	P > 0.1
(ovules)		2	0.00	P > 0.1
General combining ability	(upper style)	4	61515.42	P < 0.01
	(lower style)	4	917.63	P < 0.01
	(ovules)	4	42.71	P < 0.01
Specific combining ability	(upper style)	5	5793.07	P < 0.01
	(lower style)	5	12.24	P < 0.01
	(ovules)	5	5.51	P < 0.01
Reciprocal (upper style)		10	7909.85	P < 0.01
(lower style)		10	12.30	P < 0.01
(ovules)		10	3.65	P < 0.01
Error (upper style)		38	438.44	
(lower style)		38	3.59	
(ovules)		38	0.63	
Total (upper style)		59		
(lower style)		59		
(ovules)		59		

4.3.7.4 In ovule

Ovule penetration by a pollen tube always occurred via the micropyle and generally in only one of the four ovules. For the combinations that showed pollen tube penetration, ovules in only 5 to 30% of ovaries were penetrated. Frantoio had a significantly high percentage of ovules penetrated by Pendolino and Picual pollen tubes.

4.3.8 Self- and cross-compatibility

Analysis of the data from the diallel cross showed that there was significant specific combining ability (sca) and general combining ability (gca) amongst the cultivars and combinations respectively. Also, significant reciprocal effects were found, as measured by pollen tube growth in both the upper and lower styles, as well as by pollen tube penetration in the ovules (Table 4.5).

From the logistic regression it can be concluded that there is a significant interaction between male and female ($p < 0.001$). The predicted probabilities of flowers having pollen tubes in the lower style are presented in Table 4.6, and Fig 4.9. Although pollen tubes in the lower style are unlikely for cultivar Frantoio when self-pollinated, the probability of pollen tubes being found in the lower style is quite high when crossed with the other four cultivars regardless if it is used as female or male parent. The next most successful combination is the self-pollinated Manzanillo, with 22% of its flowers with pollen tubes in the lower style.

The combinations were ranked in order of fertility according to the mean of pollen tubes in the upper and lower style (Table 4.7). The order was similar for both upper and lower style, and Frantoio x Picual showed the highest fertility of all crosses. The selfs, except for Manzanillo x Manzanillo, and the crosses without Frantoio as either male or female parent, were infertile. Manzanillo selfs showed similar pollen tube numbers in the upper style as the other selfs, but significantly more in the lower style (Table 4.7).

4.4 Discussion

4.4.1 Inflorescence number and sex ratio in olive cultivars

This study identified differences in numbers of flowers per inflorescence, and sex ratio, for the olive cultivars Frantoio, Kalamata, Manzanillo, Pendolino, and Picual. Such differences have

Table 4.6 Predicted proportion of pollen tubes in the lower style

Female parents	Male parents				
	Frantoio	Kalamata	Manzanillo	Pedollino	Picual
Frantoio	0.00	0.85	0.60	0.67	0.90
Kalamata	0.62	0.07	0.02	0.00	0.03
Manzanill	0.63	0.00	0.22	0.00	0.00
Pendolino	0.57	0.00	0.00	0.00	0.00
Picual	0.63	0.02	0.05	0.02	0.05

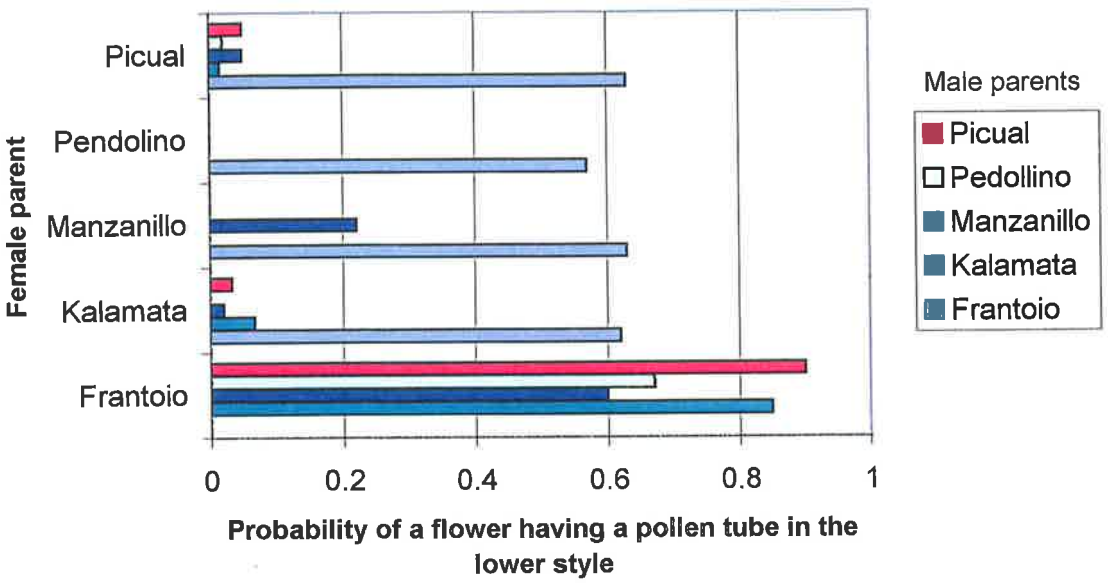

Fig 4.9 Interaction plot of the probability of flowers having pollen tubes in the lower style for each combination

Table 4.7 Olive crosses in order of decreasing fertility based on pollen tube numbers in the upper and lower style. Within a column, mean values followed by different letters are significantly different at the 0.05 probability level (ANOVA results by MINITAB 13.20)

Pollen tube in upper style			Pollen tube in lower style		
Female parent	Male parent	Significance	Female parent	Male parent	Significance
Frantoio x	Picual	a	Frantoio x	Picual	a
Frantoio x	Kalamata	b	Frantoio x	Kalamata	b
Manzanillo x	Frantoio	c	Frantoio x	Manzanillo	b
Kalamata x	Frantoio	d	Kalamata x	Frantoio	b
Picual x	Frantoio	e	Picual x	Frantoio	b
Pendolino x	Frantoio	e	Manzanillo x	Frantoio	b
Frantoio x	Manzanillo	ef	Frantoio x	Pendolino	b
Frantoio x	Pendolino	f	Pendolino x	Frantoio	c
			Manzanillo x	Manzanillo	d
Manzanillo x	Manzanillo	g			
Kalamata x	Kalamata	g	Kalamata x	Kalamata	e
Kalamata x	Manzanillo	g	Picual x	Picual	e
Picual x	Pendolino	g	Picual x	Manzanillo	e
Manzanillo x	Picual	g	Kalamata x	Picual	e
Picual x	Kalamata	g	Picual x	Kalamata	e
Picual x	Picual	g	Kalamata x	Manzanillo	e
Picual x	Manzanillo	g	Picual x	Pendolino	e
Kalamata x	Picual	g	Manzanillo x	Picual	e
Pendolino x	Kalamata	g	Pendolino x	Kalamata	e
Manzanillo x	Kalamata	g	Manzanillo x	Kalamata	e
Pendolino x	Picual	g	Pendolino x	Picual	e
Pendolino x	Manzanillo	g	Pendolino x	Manzanillo	e
Frantoio x	Frantoio	g	Frantoio x	Frantoio	e
Manzanillo x	Pendolino	g	Manzanillo x	Pendolino	e
Pendolino x	Pendolino	g	Pendolino x	Pendolino	e
Kalamata x	Pendolino	g	Kalamata x	Pendolino	e

not been reported previously. Differences in flower number per inflorescence between cultivars are reported to be mainly genetic, whereas nutrients and environmental conditions contribute to inflorescence induction and growth (Bouranis *et al.*, 1999; Poli, 1986).

Staminate flowers are common on olive trees (Brooks, 1948; Lavee *et al.*, 1996). In this study, sex ratios were found to be different among the cultivars. The staminate character may be influenced by environmental conditions (Bouranis *et al.*, 1999; Brooks, 1948) and therefore results need to be collected over several years to identify the amount of variability in this character. Staminate flowers can provide pollen to pollinate other flowers, but fail to be pollinated because of the lack of pistil. If the ratio of staminate to complete flowers is too high in a cultivar, the percentage of the flowers that produce fruits may be very low. No correlation was found between flower number per inflorescence and sex ratio.

4.4.2 Partial male sterility in SA Verdale

Male sterility has been reported in the olive, and several phenotypes, differing in the stage of pollen abortion are involved (Besnard *et al.*, 2000; Donini *et al.*, 1982; Iannotta *et al.*, 1999; Villemur *et al.*, 1984). SA Verdale is a cultivar that originated from Verdale in France and was subsequently propagated in South Australia. There are no reports of male sterility in the cultivar Verdale although many genetically different accessions were found by Mekuria *et al* (1999). Therefore male sterility in SA Verdale may be derived from a mutation, or may be the result of an environmental effect of the South Australian climate. Male sterility in SA Verdale was not detected in the 1999 pollination experiment, but was found to be male sterile again in 2001, so it seems likely that the main cause is environmental. Therefore, it is not recommended that SA Verdale be planted as a polleniser in orchards particularly in South Australia.

4.4.3 Pollen viability

Pollen viability was assessed using FDA staining and *in vitro* germination. Compared to the results from FDA, viability assessed by *in vitro* germination was much lower. The reliability of *in vitro* germination tests has been questioned by Lavee and Datt (1978). Since *in vitro* germination was positively correlated to FDA staining, as revealed by regression analysis (r^2=0.86, $P < 0.05$), and both were related to pollen germination on the stigma, an appropriate correction can be made. Recently, an improvement to the *in vitro* pollen germination technique was made by adding olive oil to the culture media, and this resulted in greater pollen germination (Ateyyeh *et al.*, 2000).

4.4.4 Pollen germination on stigma

Pollen grains from different cultivars showed different germination percentages on the stigma of the same cultivar. This can be attributed partly to pollen viability as evidenced by the similarity in the rankings between germination on the stigma and pollen viability. However, the genetic potential of the pollen to germinate on a particular stigma also plays an important role in pollen germination (Lavigne *et al.*, 1999; Pfahler *et al.*, 1997). For example, Manzanillo pollen had better performance than was expected according to its viability compared to that of other cultivars. Similarly, female genetic variation is also essential for pollen germination, as shown by the stigmatic receptivity and compatibility demonstrated by each cultivar. In the olive cultivars investigated, the stigmas of Pendolino gave the highest pollen germination, whereas Picual gave the lowest. This effect is probably related to pollen-pistil interactions on the stigma (Herrero and Hormaza, 1996; Stead *et al.*, 1979).

4.4.5 Pollen tube attrition in olives

In the olive cultivars examined, the number of pollen tubes declined markedly in the pistil as the distance between stigma and ovary decreased. Pollen tubes converged towards the central part of the style and only one, or rarely two, pollen tubes were able to grow down to the ovary. This explains the fact that olive fruit usually contain one or rarely two seeds in its stone, even though four ovules are produced in the ovary. Similar pollen tube attrition (Cruzan, 1989) has also been reported in the Oleaceae species *Phillyrea angustifolia* (Vassiliadis *et al.*, 2000).

In spite of this strong pollen tube attrition, the cross Frantoio x Picual was often found to have more than one pollen tube passing the middle of the style. The maximum found was four pollen tubes in the lower style. It was not investigated whether, in Frantoio x Picual, more double seeded fruits were produced than for other crosses.

4.4.6 Compatibility in olives

The olive cultivars investigated in this experiment showed significant variance caused by general and specific combining ability and significant reciprocal effects with regard to pollen tube growth in both upper and lower styles. A similar effect has been reported for macadamia cultivars (Sedgley *et al.*, 1990). Pollen tube growth was significantly lower for self-pollination compared to compatible cross-pollination. This result confirmed the presence of self-incompatibility in olives, as widely reported (Ateyyeh *et al.*, 2000; Caruso *et al.*, 1993; Lavee

and Datt, 1978). Among the five cultivars investigated, Frantoio showed the best combining ability with the other cultivars as both male and female parent. This reveals that Frantoio can be a general polleniser as well as a good recipient for all other four cultivars. The other four cultivars, however, showed significantly low cross-compatibility in both directions compared to Frantoio. Thus, appropriate pollenisers need to be chosen for those cultivars so as to attain reasonable pollination in orchards.

Frantoio has been reported as a self-compatible cultivar (Bini, 1984; Sharma *et al.*, 1976). However, results from self-pollination in the present study showed that it was SI. Although Frantoio pollen showed the highest overall germination on the stigmas of the other cultivars, and the highest pollen viability, as well as good stigmatic receptivity to foreign pollen, its germination was only 2.8% after self-pollination. In addition, pollen tube growth was not observed in the style. Since Frantoio was highly compatible with the other four cultivars either as a male or female parent, this excludes the possibility that experimental error led to the failure of pollen tube growth after self-pollination. The difference in self-pollination found in this study compared to other reports may be due to the following reasons: 1. the trees used in other reports may be genetically different from the trees used in this trial. Therefore, genetic variation may be responsible for the compatibility divergence. This is unlikely, as the DNA fingerprinting of the Frantoio used in this study matched that in the Italian collection at CO.RIPR.OL, Pescia; 2. The climatic conditions in Australia may not be identical to those in the countries where the previous research was carried out (Booth and Davies, 1996), and there is ample evidence of environmental effects on pollination success (Androulakis and Loupassaki, 1990; Fernandez and Gomez, 1985; Lavee and Datt, 1978; Sedgley, 1994).

There are also inconsistent reports with regard to SI of Manzanillo, ranging from self-incompatible (Caruso *et al.*, 1993; Cuevas and Polito, 1997; Griggs *et al.*, 1975; Lavee and Datt, 1978), through partially self-incompatible (Dimassi *et al.*, 1999) to self-compatible (Androulakis and Loupassaki, 1990; Sibbett *et al.*, 1992). In South Australian environmental conditions, Manzanillo showed significantly low fertility compared to the compatible cross-combinations but higher than other selfed cultivars and incompatible crosses, according to the results of pollen tube growth in lower style. Therefore, Manzanillo can be regarded as a partially self-incompatible cultivar.

Cross-pollination generally enhances fruit set of olives because of the presence of SI (Cuevas *et al.*, 2001; Ghrisi *et al.*, 1999; Griggs *et al.*, 1975). However, some cultivars are found not to be cross-compatible (Cuevas and Polito, 1997; Griggs *et al.*, 1975; Lavee and Datt, 1978; Sharma *et al.*, 1976; Singh and Kar, 1980). In this study, Kalamata, Manzanillo, Pendolino, and Picual showed cross incompatibility. This suggests that each of these four cultivars should not be used as pollenisers for the others, and therefore another compatible cultivar, such as Frantoio, should be planted together with them in orchards. Cross-incompatibility between Kalamata, Manzanillo, Pendolino, and Picual suggests that they may share a common SI allele.

The results reported here follow controlled hand pollination of perfect flowers, and do not take into account the fact that large variation was observed on the sex ratio and pollen fertility of the cultivars. These factors would also be expected to impact on grove yield. There is evidence of environmental effects on pollination in olives and other woody crops (Lavee and Datt, 1978; Martin, 1990; Sedgley, 1994), and therefore the results may include genotype-environment interactions on sex ratios, pollen viability, and self-incompatibility. Maximum and minimum temperatures and relative humidity, were compared between the year under study with the long-term average, and temperatures from July to early November, which encompass the period from floral initiation to anthesis, did not deviate appreciably from the long-term (73 yr) averages. The relative humidity was higher than the average for September, and although this may have affected some aspects of floral development, anthesis occurred later than this and at the same time in all five cultivars. Therefore, it is likely that the results obtained for self- and cross-compatibility presented in this study are representative all of the cultivars for most seasons in the Adelaide area.

4.4.7 Mechanism of self-incompatibility in olives

The majority of pollen tubes ceased growth in stigmatic tissue when incompatibility occurred in this study. Thus, pollen-pistil recognition occurs first in the stigma. This recognition site determines whether the pollen tubes can extend further into the pistil or not. This is mainly governed by the genotype of pollen and pistil (de Nettancourt, 1977; de Nettancourt, 1997). If the initial recognition is one of compatibility, a second recognition occurs after the pollen tubes reach the middle part of the style. Many pollen tubes can go beyond the stigmatic tissue in a compatible pollination, but only one, or rarely two, pollen tubes were able to pass the centre of the style and penetrate to the ovary and ovule. Pollen tube competition appears to be involved in the reaction. The point where the pollen tubes compete in the style forms a bottleneck of

pollen tube growth. It is possible that vigorous pollen tubes are able to grow further whereas less competitive ones stop at this point. Hence in the olive pistil, there are two sites that are responsible for stopping pollen tube growth. The stigma is one point of inhibition, while the style is the other. This is confirmed by observations in the cultivar Nabali Baladi (Ateyyeh *et al.*, 2000).

While genetic investigations have not been conducted on olive SI, the gametophytic system of SI has been proposed for Manzanillo (Cuevas and Polito, 1997), Moraiolo (Bini and Lensi, 1981), and Nabali Baladi (Ateyyeh *et al.*, 2000). Other plants that exhibit gametophytic SI are mostly characterised by wet stigma and binucleate pollen, as is the case in olive (Heslop-Harrison and Shivanna, 1977). The incompatible pollen germinates successfully on the surface of the stigma, but growth of the pollen tube is arrested during growth (Newbigin *et al.*, 1993).

4.4.8 Possibility of low fertilisation rate and high fruit set

In olives, good yields may be produced by as low as 1% fruit set (Griggs *et al.*, 1975). It seems that as long as 1% of ovules can be fertilised after adequate pollination, there is the possibility that fruit load can be satisfactory for industry requirements. If so, cultivars with flowers that showed 2% or more pollen tube penetration of ovules in this study may not perform as incompatible combinations in the field. However, according to Cuevas and Polito (1997), postzygotic incompatibility may be involved in Manzanillo self-pollination, and fruit drop is reported to be severe in olives (Griggs *et al.*, 1975; Lavee *et al.*, 1999; Martin, 1990; Sharma *et al.*, 1976). Therefore, higher rates of fertilisation may be needed to obtain ample fruit set. In this case, further study is essential to determine the minimum fertilisation rate (or pollen penetration rate) required for adequate fruit set, allowing for fruit drop, especially in crosses that show low fertilisation to see whether fertilised ovules will drop even if only few are fertilised.

4.4.9 Effect of cross-incompatibility on data analysis

Diallel cross designs can be applied to tests of SI, as well as genetic studies (Griffing, ~~1957~~1956; Fuss *et al.*, 1991; Sedgley, 1990). The data can be analysed to detect the effect of both general and specific combining abilities, and the efficiency of reciprocal crosses. The results allow predictions to be made on various combinations. According to Griffing (1957), the assumption for the analysis is that the data need to be normally distributed, and self-pollinations are ignored because SI may lead to bias. In this experiment, however, the

distribution of the data was not normal because of cross-incompatibilities between the four cultivars. Only Frantoio is compatible with the other four cultivars, and this led to a large number of zeros in the data. Therefore, standard diallel software failed to give a correct indication concerning the combinations that produced highest pollen tube growth in the pistil. This was overcome by applying logistic regression to a binary matrix developed from the data of pollen tube numbers occurring in the lower style to estimate the probability that a pollen tube will reach the lower style.

4.4.10 Further studies

More information needs to be collected on the combinations conducted in this study. The results were mainly based on the observations of one season, so the same experiments are needed over several years to confirm the results presented. Moreover, more cultivars are needed to be included in the studies to expand the knowledge of sexual compatibility between olive cultivars, especially in the Australian environment. Cross-compatibility of Pendolino, in particular, needs to be investigated with more cultivars, as it is regarded in the industry as a polleniser for other cultivars.

CHAPTER FIVE

Fruit Set

5.1 Introduction

Fruit set in olives is low, ranging from 1.0-16.5 % (Griggs *et al.*, 1975; Lavee *et al.*, 1999; Martin, 1990; Sharma *et al.*, 1976), despite the production of many flowers. The variation is probably associated with alternate bearing (Cuevas and Rallo, 1990; Cuevas *et al.*, 1994a; Villemur *et al.*, 1984), usually with one year 'on' and the next year 'off'.

The reasons for the low fruit set include: 1. Internal competition for nutrients between flowers and leaves. Thinning experiments indicate the competitive nature of the abscission of ovaries and fruits in the olive (Rallo and Barranco, 1986). The shortage of nutrient supply in the flowers or fruits causes abscission of ovaries before and after fertilisation (Bouranis *et al.*, 1999; Rallo *et al.*, 1990; Rallo and Fernandez, 1985; Rallo *et al.*, 1981; Rapoport and Rallo, 1990; Rapoport and Rallo, 1991a; Rapoport and Rallo, 1991b). 2. High proportion of staminate flowers. Olive trees show variable sex expression in the flowers (Bouranis *et al.*, 1999; Brooks, 1948; Lavee *et al.*, 1996). Staminate flowers are not able to set fruits, and therefore, fruit set percentage is low in trees that show this character. 3. Severe environmental conditions, such as water deficit and high temperature. Water deficiency leads to high fruit drop (Hartmann and Panetsos, 1962; Inglese *et al.*, 1996), and irrigated treatments under a rain-fed climate often show an increase in fruit yield (Patumi *et al.*, 1999). High temperature inhibits flower set. It was reported that fruit set in 'Manzanillo' olive was completely inhibited at 30°C constant temperature (Cuevas *et al.*, 1994b). 4. Inadequate or ineffective pollination. Adequate pollination may lead to the fertilisation of the egg and as a result stimulation of ovary expansion. Insufficient or incompatible pollen sources are responsible for poor fertilisation, and subsequently low production of olive fruits (Rallo *et al.*, 1990; Rapoport and Rallo, 1991a; Sibbett *et al.*, 1992). 5. Short ovule longevity. After pollination, pollen tube growth may be slow in some circumstances, such as in the case of self-pollination. Therefore, ovule longevity is critical in determining the level of fertilisation (Rapoport and Rallo, 1991a; Villemur *et al.*, 1984). The longer the viable ovule lasts, the higher the level of fertilisation.

Low fruit set is a disadvantage for both olive breeders and for commercial growers. This experiment was designed to examine the fruit set percentages of various crosses, the pattern of

fruit fall and possible relationship between fruit set and seed set in order to compare artificial hybridisation with natural pollination.

5.2 Materials and methods

5.2.1 Trees

Trees of cultivars Frantoio, Kalamata, Manzanillo, and Verdale were used in this experiment as either male or female parents, and all trees were over five years old (Table 5.1). These cultivars were selected because they are commercially important in Australia.

5.2.2 Crosses

Controlled pollination was employed in October and November, 1999, using the method described in Chapter 2. Crosses were made between Frantoio x Kalamata, Kalamata x Frantoio, Kalamata x Manzanillo, Manzanillo x SA Verdale, and SA Verdale x Manzanillo.

5.2.3 Data collection

Following pollination, flowers were completely sealed with paper bags. The bags were removed one month later and the numbers of fruit set were counted. Fruit numbers were recorded every month thereafter until the fruits were mature, and the percentage fruit set was calculated for each cross.

For each mature fruit, the flesh was removed, and the seeds allowed to dry for 1 week. The seed coats (endocarp) were then cracked and removed using a vise. Seeds with both endosperm and embryo were recorded as set, whereas empty seeds indicated a failure of seed development. Seed set percentage was calculated.

The length of each fruit and the stone within it were measured, and used to investigate possible relationships with fruit size and seed set.

5.3 Results

5.3.1 Fruit set

Fruit development continued about 5 months after pollination. The numbers of flowers pollinated, and fruits retained at different stages in all the crosses, are shown in Table 5.2.

Table 5.1 Olive cultivars used to examine fruit set

Cultivars	Number of trees	Male parent	Female parent	Tree age (year)	Location
Frantoio	3	-	+	5	Willunga Hill
Kalamata	6	+	+	5	Virginia, Willunga Hill
Manzanillo	7	+	+	20	Two Wells
SA Verdale	2	+	+	20	Two Wells

-: not used. +: used

Table 5.2 Fruit set number and percentages following pollination

Cross	Numbers of flowers pollinated	Months after pollination 1	2	3	4	5
Frantoio x Kalamata	3058	191	174	168	164	162
		(6.2%*)	(5.7%)	(5.5%)	(5.4%)	(5.3%)
Kalamata x Frantoio	2623	90	65	56	50	49
		(3.4%)	(2.5%)	(2.1%)	(1.9%)	(1.9%)
Kalamata x Manzanillo	2408	1806	243	181	153	127
		(75.0%)	(10.1%)	(7.5%)	(6.4%)	(5.3%)
Manzanillo x SA Verdale	840	119	39	30	28	28
		(4.2%)	(4.6%)	(3.6%)	(3.3%)	(3.3%)
SA Verdale x Manzanillo	165	131	12	7	3	3
		(79.3%)	(7.3%)	(4.2%)	(1.8%)	(1.8%)

*Percentage of fruit set

The initial percent fruit set varied from 79.3% for SA Verdale x Manzanillo to 3.4% for Kalamata x Frantoio. Fruit abscission was evident as early as one month after pollination, and by two months, the highest fruit set of any cross was only 10.1%. Between two and five months after pollination, fruits retained declined to between 1.8% and 5.3%.

As shown in Table 5.2 and Fig. 5.1, reciprocal crosses produced different results. Fruit set of the cross between Frantoio x Kalamata was 5.3% by 5 months after pollination, whereas Kalamata x Frantoio produced 1.9% fruit set at the same time. In addition to the different fruit set percentages in reciprocal crosses between Manzanillo and SA Verdale, fruit abscission patterns were different. For the cross Manzanillo x SA Verdale, the fruit drop was 95.8% by the end of one month after pollination, while for SA Verdale x Manzanillo, the drop was only 20.7%. The peak abscission was delayed for one month for SA Verdale x Manzanillo compared to its reciprocal cross. At maturity, the fruit set percentages varied from 1.8-5.3%, with the highest for Frantoio x Kalamata, and the lowest for SA Verdale x Manzanillo.

5.3.2 Seed set

The numbers of seeds obtained are shown in Table 5.3. For Frantoio x Kalamata, 122 seeds were produced, which was the highest number amongst all five crosses. This cross produced the highest percentage seed set (4.0%), but the second highest percentage of seed per fruit (75.3%). The reciprocal cross Kalamata x Frantoio, produced only 0.8% seed set and 44.9% seeds per fruit, and the number of seeds harvested was 22. Manzanillo x SA Verdale, for which 24 seeds were harvested out of 28 fruits and 840 flowers, produced 2.9% seed set and 85.7% seeds per fruits. The latter was the highest in all the crosses. For the cross SA Verdale x Manzanillo, the seed set was 1.2% and seeds per fruit was 66.7% ranking in the middle of all crosses for both. For Kalamata x Manzanillo, which produced 127 fruits and percentage fruit set of 5.3% (Table 5.2), there were no seeds formed, indicating the failure of ovule development after pollination.

5.3.3 Size of fruits and stones

As shown in Table 5.4, the mean lengths of fruits and stones for the cross between SA Verdale x Manzanillo were the largest of all crosses, and those of the reciprocal cross were second largest. For Kalamata x Frantoio and Frantoio x Kalamata the values were smaller at 20.6 and 16.3 mm, and 19.2 and 15.0 mm respectively. Except for Kalamata x Manzanillo, the ratios of fruit size to stone size were constant for all crosses, ranging from 1.26 ~ 1.28 : 1. For the cross

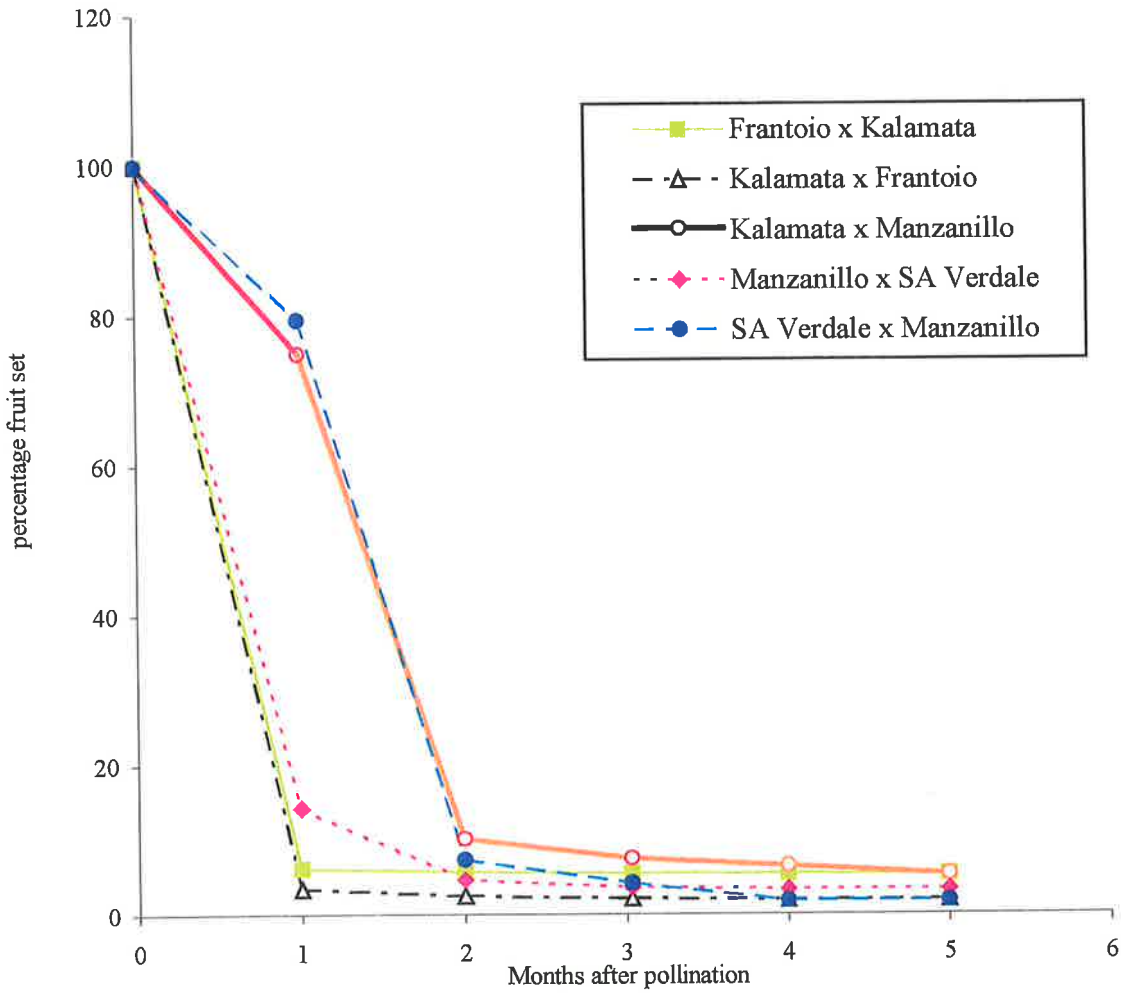

Fig. 5.1 Abscission pattern of olive ovaries from different crosses following controlled pollination.

Table 5.3 Seed set and percentage compared with flowers pollinated and fruits harvested

Cross	Flower	Final fruit numbe	Seed	Seed set (%)	Seed/fruit (%)
Frantoio x Kalamata	3058	162	122	4.0	75.3
Kalamata x Frantoio	2623	49	22	0.8	44.9
Kalamata x Manzanillo	2408	127	0	0.0	0.0
Manzanillo x SA Verdale	840	28	24	2.9	85.7
SA Verdale x Manzanillo	165	3	2	1.2	66.7

Table 5.4 Size of mature fruits and stones in different olive cultivar

Cross	Mean fruit length (mm)	Mean stone length (mm)	Length ratio (fruit : stone)
Frantoio x Kalamata	19.2 ± 3.4	15.0 ± 2.8	1.28 : 1
Kalamata x Frantoio	20.6 ± 4.9	16.3 ± 4.7	1.26 : 1
Kalamata x Manzanillo	15.5 ± 3.2	10.6 ± 3.0	1.46 : 1
Manzanillo x SA Verdale	22.1 ± 2.1	17.2 ± 1.7	1.28 : 1
SA Verdale x Manzanillo	25.3 ± 0.0	20.0 ± 0.0	1.26 : 1

Kalamata x Manzanillo, this ratio was 1.46 : 1. This difference is due to the relatively smaller stone length of this cross compared to the others.

5.4 Discussion

Olive fruit set declines soon after full bloom because of flower and ovary abscission (Cuevas *et al.*, 1995; Marco *et al.*, 1990; Rallo *et al.*, 1990; Rallo and Fernandez, 1985; Rapoport and Rallo, 1991a; Rapoport and Rallo, 1991b). Flowers of open or cross-pollinated populations started abscission earlier than self or unpollinated populations, indicating that onset of ovary expansion triggers the shedding of ovaries (Rallo *et al.*, 1990; Rapoport and Rallo, 1991a). The abscission can be delayed for about 10 days for self and unpollinated populations but the fruit set is about the same at 30 days after full bloom.

In this experiment, the fruit set pattern of crosses between Frantoio x Kalamata, Kalamata x Frantoio, and Manzanillo x SA Verdale agree with data from other cross-pollinated populations (Rallo *et al.*, 1990; Rapoport and Rallo, 1991a). This may imply compatible and efficient pollination in the crosses. For Kalamata x Manzanillo and SA Verdale x Manzanillo, however, the pattern was similar to that of self or unpollinated populations. For Kalamata x Manzanillo, although the fruit set percentage was high, no seeds were found within the stones. This indicates that the ovule did not develop properly possibly due to the failure of fertilisation or to post-zygotic abortion. This is the case in unpollinated flowers and incompatible cross- or self-pollination. For SA Verdale x Manzanillo, however, the fruit set and seed set were 1.8% and 1.2% respectively. This suggests normal fertilisation for this cross, which is different from unpollinated flowers and incompatible cross- or self-pollination. However, due to availability, the number of flowers pollinated for the cross SA Vedale x Manzanillo was only 165. This is a relatively small sample population compared with 2408 flowers pollinated for Kalamata x Manzanillo. It is possible that the fruit drop pattern will differ if pollination was made with a larger number of flowers.

The present study showed that reciprocal crosses of Frantoio and Kalamata, and Manzanillo and SA Verdale produced different results of fruit drop pattern, final fruit and seed set, and percentage of seeds per fruit. It is usually assumed that the factors are controlled by the female parent. However, it is possible that xenia is operating in some olive cultivars. Further studies need to be carried out to confirm this by comparison between crosses using the same female parent but different male parents.

Although fruit and stone lengths vary in different crosses, the ratios of fruit to stone lengths were comparable in all crosses except for Kalamata x Manzanillo, which failed to produce seeds. This implies that normally developed fruits generally have a fruit-seed length ratio of 1.26-1.28 : 1 in all those crosses studied, unless the seed development fails. In Kalamata x Manzanillo, the larger ratio may indicate that the stone of unfertilised fruits develops slowly because of the failure of seed growth.

Rallo and Fernández (1985) noted that early fruit shedding due to competition between fruits counteracted an increase in the number of fertilised flowers. Hence, the use of fruit set, even in the early stages, to indicate response to pollination was questioned. In field practice, however, only about 1% of fruit set may result in adequate commercial crops (Griggs *et al.*, 1975). Therefore, fruit set has been used to determine appropriate pollenizers for olive cultivars (Bartolini and Guerriero, 1995; Cuevas and Polito, 1997; Cuevas and Rallo, 1990; Singh and Kar, 1980). In the present study, the fact that Kalamata x Manzanillo produced 5.3% fruit set but 0% seed set indicates that fruit set should not be used as an index for successful fertilisation and consequently the compatibility of pollination. Seed set produces a more accurate index of proper fertilisation in the ovule.

The cross of Kalamata x Manzanillo produced significantly smaller fruits and stones than other crosses, which was probably related to the lack of seeds. In plants, the absence of fertilisation results in either the drop of fruit or the cessation of carpel development (Chareonboonsit and Splittstoesser, 1984; Vivian-Smith and Koltunow, 1999). Seeds produce plant growth regulators (PGR) that stimulate the growth of the carpel. For the cross of Kalamata x Manzanillo, the lack of seeds in the fruit, possibly caused by incompatibility between pollen and pistil of the parents, may have resulted in the limited growth of fruit. This is reported to be common in orchards when the trees are not pollinated efficiently (Rapoport and Rallo, 1990).

CHAPTER SIX

Seed Germination

6.1 Introduction

Olive seeds generally show poor germination. A number of factors appears to be involved, including the mechanical resistance of the endocarp to embryo expansion (Crisosto and Sutter, 1985b; Sotomayor-Leon and Caballero, 1990), the dormancy imposed by seed coat (Lagarda and Martin, 1983), endosperm (Lagarda and Martin, 1983; Mitrakos and Diamantoglou, 1984) or embryo (Voyiatzis, 1995), and premature or over maturity harvest time of seeds (Lagarda *et al.*, 1983; Linan *et al.*, 1999). In some cases, germination can take up to three years or more (Sotomayor-Leon and Caballero, 1990). A high germination rate is essential for propagation of olive rootstocks, for breeding programs, and for conventional and molecular genetic studies.

Efforts have been made to improve olive seed germination, including harvesting olive fruits at an appropriate maturity stage (Lagarda *et al.*, 1983; Yuce, 1979), removing the endocarp (Crisosto and Sutter, 1985; Sotomayor-Leon and Caballero, 1990), and pre-chilling seeds at about 10°C before germination (Voyiatzis, 1995). The optimum germination capacity of olive seeds is reported to occur at the green stage of fruit development, before dormancy begins (Lagarda *et al.*, 1983).

Removal of the hard endocarp, even at the hard green fruit stage, can improve germination (Crisosto and Sutter, 1985a) but olive seeds still do not germinate readily. Voyiatzis and Porlingis (1987) and Voyiatzis (1995) germinated olive seeds and embryos after storage at 10°C and found that this treatment improved germination compared to storage at 20°C. A similar finding was reported by Brhadda *et al.* (2000). The present study aimed to develop a method that would result in rapid germination of olive seeds and subsequent high seedling recovery.

6.2 Materials and methods

6.2.1 Materials

Fruits of *Olea europaea* L. cv. Corregiola, turning from green to purple on a 3-year-old tree, were harvested at Kangaroo Island (Latitude 35° 45' S, Longitude 136° 35'E) on 8 July, 1999. After removal of the flesh, the stones were washed three times in tap water and left to air-dry at room temperature (20-22°C) for one week. Seeds were released from the endocarp by

cracking the stones with a vice. Rotted and poorly developed seeds (~ 28%) were discarded and only well-developed seeds were used for further treatments. Seeds were tested for germination either immediately after release from the endocarp, or after 10 months storage at room temperature in the dark. Seed germination was regarded as successful when the radicle emerged from the seed.

6.2.2 Germination treatments

The following seed germination treatments were applied.

6.2.2.1 Treatment 1

Two hundred and eleven freshly prepared seeds were imbibed in sterilised deionised water for 24 h, surface-sterilised using Milton solution (Procter and Gamble Australia Pty Ltd, 1% available chlorine) for 15 min with shaking at 60 oscillation per minute (opm) and then rinsed twice in sterilised deionised water. The seeds were placed on ½ MS salts (Murashige and Skoog, 1962) solidified with 0.8% Bacto agar in 250 mL polyethylene jars, 10 seeds per jar and incubated at 10 ± 0.5°C in the dark.

6.2.2.2 Treatment 2

Fifty seeds that had been stored at room temperature for 10 months were imbibed in distilled water for 24 hr, then washed in running water for three weeks to break the dormancy (Sotomayor-Leon *et al.*, 1994), surface-sterilised using Milton solution for 15 min with shaking at 60 opm and then rinsed twice in sterilised deionised water. The seeds were divided equally into five 250 mL polyethylene jars containing one sheet of Whatman No.1 filter paper moistened with distilled water. The jars were placed at room temperature in the dark.

6.2.2.3 Treatment 3

Fifty seeds that had been stored at room temperature for 10 months were imbibed in distilled water for 24 hr, surface-sterilised using Milton solution for 15 min with shaking at 60 opm and then rinsed twice in sterilised deionised water. The seeds were divided equally into five 250 mL polyethylene jars containing ½ MS medium solidified with 0.8% Bacto agar and left at room temperature in the dark.

6.2.2.4 Treatment 4

Fifty seeds that had been stored at room temperature for 10 months were imbibed in distilled water for 24 hr, surface-sterilised using Milton solution for 15 min with shaking at 60 opm and

then rinsed twice in sterilised deionised water. The seeds were divided equally into five 250 mL polyethylene jars containing ½ MS medium solidified with 0.8% Bacto agar and germinated at 10°C in the dark.

For each treatment, germination was recorded at three and four weeks after the seeds were placed in the germination treatments.

6.2.3 Seedling growth

After four weeks, seedling vigour was examined by transferring 57 germinated seeds from treatment 1 to 75 mm pots containing ½ peat and ½ perlite, one seed in each. The remaining jars were transferred from 10°C to room temperature for one week in a 16h : 8h light : dark regime, and then a further 26 germinated seeds were transferred to pots. All pots were placed in a fogger at 20°C in a glasshouse, and seedling growth was observed.

6.2.4 Data analysis

The data from different treatments were analysed by chi squared test using the following formula (de Fossard, 1976):

$$\chi^2 = \sum \left[\frac{(observed - \exp ected)^2}{\exp ected} \right]$$

The null hypothesis was that the treatments for seed germination had no effect on the germination of each treatment.

6.3 Results

6.3.1 Germination treatments

The χ^2 values were 26.56 and 28.47 for three and four weeks respectively. Since both χ^2s were greater than 11.3, the value necessary for significance at the 0.01 probability level, the null hypothesis was rejected, i.e., there was a significant effect of treatment on seed germination at both three and four weeks (Table 6.1).

6.3.1.1 Treatment 1

Some seeds started germination two weeks after incubation at 10°C (Fig. 6.1). The germination of freshly prepared seeds incubated at 10°C on agar containing ½ MS salts was 77% and 84% after three and four weeks respectively (Table 6.1. and Figs. 6.2 and 6.3).

Table 6.1 Seed germination number and percentage under different treatments. The treatments are significantly different based on the chi square test ($P<0.01$).

	Fresh seeds	Stored 10 months RT**		
Treatments	10°C ½ MS*	RT Filter paper	RT ½ MS	10°C ½ MS
Total Number Seeds	211	50	50	50
Germinated (3 wk)	163	18	12	41
Percentage	77%	36%	24%	82%
Germinated (4 wk)	178	19	15	47
Percentage	84%	38%	30%	94%

* MS salt medium (Murashige and Skoog, 1962)

** RT: room temperature (20-22°C)

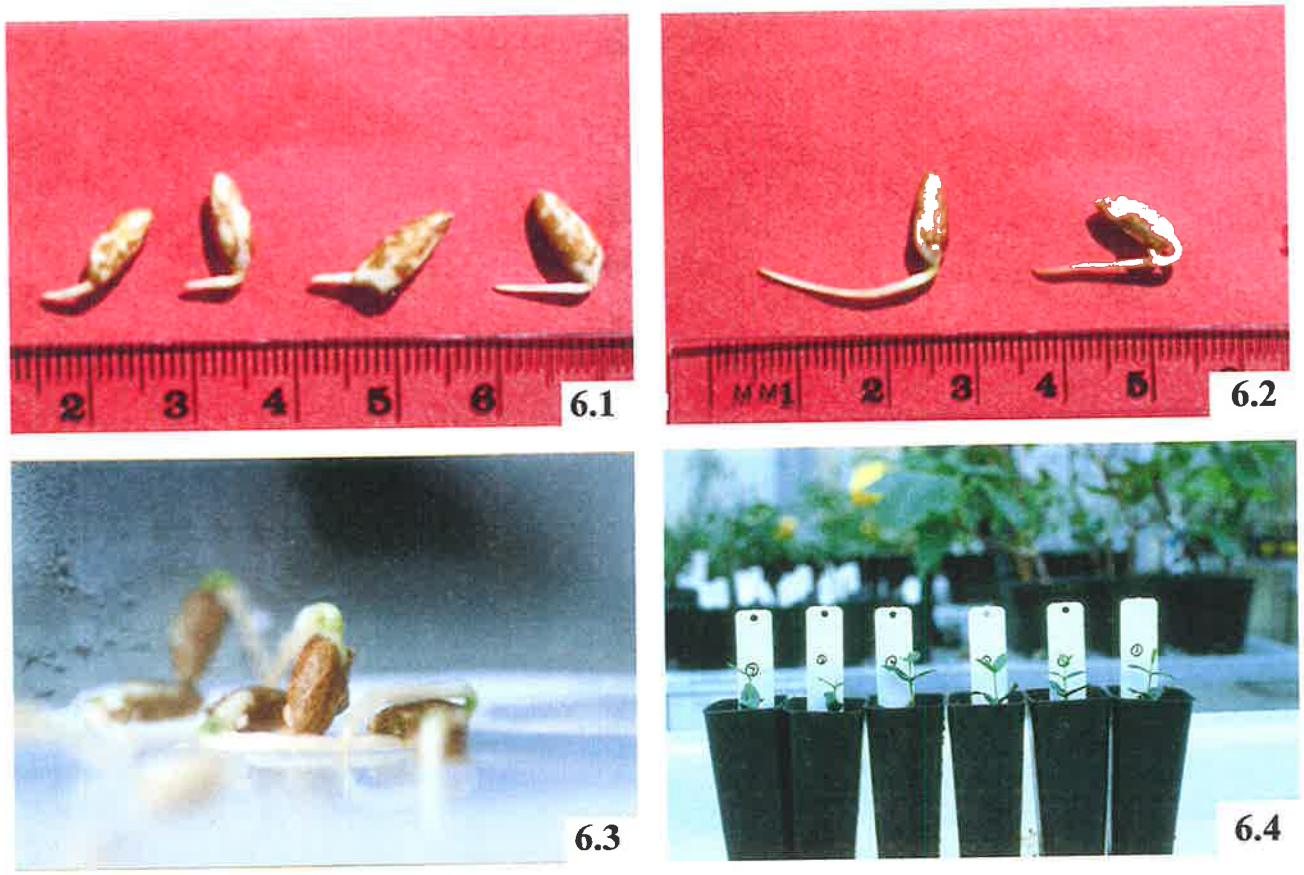

Fig. 6.1 Germinated seeds after two weeks in treatment 1. **Fig. 6.2** and **Fig. 6.3** Germinated seeds after three weeks in treatment 1. **Fig. 6.2**. shows the radicle and **Fig. 6.3** shows the seeds on ½ MS medium. **Fig. 6.4** Seedlings at 8 weeks after transfer to potting medium.

6.3.1.2 Treatment 2

The germination of seeds stored at room temperature for 10 months and then imbibed for 3 weeks before placing on moist filter paper at room temperature was 36% and 38% after three and four weeks respectively (Table 6.1).

6.3.1.3 Treatment 3

The germination of seeds stored at room temperature for 10 months and incubated at room temperature on agar containing ½ MS salts was 24% and 30% at three and four weeks respectively.

6.3.1.4 Treatment 4

Germination of seeds stored for 10 months and then incubated at 10°C on agar containing ½ MS salts was 82% and 94% at three and four weeks respectively.

6.3.2 Seedling growth

Of the 57 germinated seeds transferred to soil in pots, the first shoot emerged above the soil at 4 days, and by three weeks approximately 77% of the shoots had emerged, and by six weeks all of the shoots emerged. For the 26 germinated seeds transferred to pots after being placed at room temperature under light for one week, the seedlings were already about 10 mm high and ready to be transferred to pots. All seedlings showed vigorous growth in pots (Fig. 6.4).

6.4 Discussion

Olive seeds germinate better at 10°C than at room temperature. Seeds exposed to 10°C for four weeks on agar containing ½ MS salts reached greater than 80% germination compared to only 30% when incubated at room temperature. For seeds previously stored at room temperature for 10 months, and then incubated at 10°C on agar containing ½ MS salts, the germination was 94%. This germination percentage is satisfactory for the propagation of olives from seeds in order to obtain rootstocks, for sexual cross progeny for breeding, or for conventional and molecular genetic studies.

It has been demonstrated that pretreatment of seeds by storage at low temperature can improve germination in many plants, possibly by overcoming the dormancy imposed by endosperm, seed coat, or embryo (Araki and Washitani, 2000; Del Tredici, 1987; Finneseth, *et*

al., 1998; Masuda *et al.*, 1999; Reddy, 1988). The temperature of the treatment may vary from 2 to 15°C for different species. In olives, a pre-chilling treatment of seeds was effective in improving the germination of cvs. Manzanillo (Lagarda *et al.*, 1983), Khondrolia Khalkidikis (Voyiatzis and Porlingis, 1987), and Chalkidikis (Voyiatzis, 1995), but not of cv. Moraiolo (Lambardi *et al.*, 1994). The failure of Lambardi *et al.* (1994) to find an effect of chilling may be because the chilling time was too short, only 5 or 15 days, or that seeds of cv. Moraiolo are insensitive to chilling.

Olive seeds are usually germinated under higher temperatures than 10°C, which has been regarded only as a pre-chilling temperature for breaking dormancy (Lambardi *et al.*, 1994; Voyiatzis, 1995; Voyiatzis and Porlingis, 1987). In Corregiola, however, the seeds started germination at 10°C and reached a high level after only four weeks. The result indicates that the chilling treatment at 10°C was not only effective for breaking seed dormancy but the temperature is also suitable for seed germination. In this study, fruits were collected when the colour of the exocarp was turning from green to purple. It is possible that this may assist seed germination at 10°C, as ease of germination has been related to time of harvest (Lagarda *et al.*, 1983; Legesse, 1993; Yuce, 1979). The genetic background may also be responsible for different requirements in germination temperature.

The dormancy of olive seed may decline gradually during storage (Istanbouli and Neville, 1977), so germination may be improved after storage. This was confirmed in Corregiola. The fresh seeds gave maximum germination of 84% after four weeks germination at 10°C, whereas the germination rate reached at 94% after 10 months storage.

The germination percentage for seeds incubated at room temperature on moist filter paper was slightly higher than that for seeds placed on agar containing ½ MS salts at room temperature. This effect may be due to the prior treatment of imbibition for three weeks with frequent changes of water. Since it has been reported that improved germination resulted when seeds were subjected to running water for 30 days (Sotomayor-Leon *et al.*, 1994).

Seeds that were placed on agar containing ½ MS salts for four weeks and then transferred to potting medium consisting of ½ peat and ½ perlite grew vigorously, and this combination of treatments resulted in a high proportion of seedlings being produced in a relatively short period of time.

CHAPTER SEVEN

Development of Molecular Linkage Maps

7.1 Introduction

Molecular linkage maps based upon DNA markers are widely recognised as essential tools for genetic research in many species. The maps can be used for analysing and selecting complex traits (Tanksley *et al.*, 1989) and studying individual genes that control expression of polygenic traits (Tanksley, 1993). Molecular marker assisted selection (MAS) (Lande, 1991) is potentially useful for olive selection and breeding, since seedlings may be preselected for traits of importance before the trees mature. This may save much cost and time for field maintenance of the plants and evaluation of the interested traits of interest.

The construction of genetic maps requires the selection of an appropriate mapping population, calculation of pairwise recombination frequencies of molecular markers in the population, establishment of linkage groups, estimation of map distances, and determination of map order (Staub *et al.*, 1996). Based on partial linkage data, the genome size of the plant species analysed can be estimated using the moment method of Hulbert *et al.* (1988).

Molecular markers that have been developed and applied to the assessment of olive germplasm include RAPD (Fabbri *et al.*, 1995; Mekuria *et al.*, 1999), AFLP (Angiolillo *et al.*, 1999), RFLP (Besnard and Berville, 2002; de Caraffa *et al.*, 2002), and SSR (Carriero *et al.*, 2002; Cipriani *et al.*, 2002; Rallo *et al.*, 2000; Sefc *et al.*, 2000). These can be utilised to construct linkage maps, such as for the offspring of a cross between the cvs Leccino and Dolce Agogia (Baldoni *et al.*, 1999).

RAPD-PCR has been widely used in the identification of olive cultivars (reviewed by Wunsch and Hormaza, 2002), assessment of genetic diversity (Mekuria *et al.*, 2002a), and development of molecular markers (Hernandez *et al.*, 2001a; Mekuria *et al.*, 2001). RAPD fragments show extensive polymorphism in olive germplasm and this suggests that the technique is valuable for its application to more broad investigations in olive such as linkage mapping.

SSRs (or microsatellites) have been used extensively in the investigation of genetic diversity and molecular mapping in the last decade, and they are becoming the preferred technique in plant species (Gupta *et al.*, 1999; Carriero *et al.*, 2002; Cipriani *et al.*, 2002; Rallo *et al.*, 2000;

Sefc *et al.*, 2000). Many primer sequences are published and available for use. However, at the start of this research program, SSR primers were not available for olive, and therefore microsatellite sequences were identified in an $(AC)_n$ enriched library prepared from the genomic DNA of cv. Kalamata.

Genetic linkage maps have been constructed in many economically important tree crops based on the strategy of using a pseudo-test cross (Weeden, 1994). These include apple (Conner *et al.*, 1997), almond (Viruel *et al.*, 1995), chestnut (Casasoli *et al.*, 2001), cocoa (Crouzillat *et al.*, 1996), eucalypt (Thamarus *et al.*, 2002), pine (Nelson *et al.*, 1993; Plomion *et al.*, 1995), and tea (Hackett *et al.*, 2000). In olive, one preliminary molecular map has been reported using the F_1 pedigree from a cross between cvs. Dolce Agogia and Leccino (Baldoni *et al.*, 1999). However, the linkage information was limited, as only 9 and 2 linkage groups, respectively, were obtained for the parents. More comprehensive linkage maps are needed for olive in order to locate important morphological traits on the genome and thus to facilitate marker assisted selection.

The aims of the present study were:

1. to generate progeny from a cross between Kalamata and Frantoio to be used as a mapping population

2. to identify microsatellite sequences in one of the parents, namely cv. Kalamata

3. to construct a genetic linkage map based on RAPD, SCAR, and SSR markers.

7.2 Materials and Methods

7.2.1 Plant material

Based on previous work (Mekuria *et al.*, 1999), olives are considered to be highly heterozygous, and therefore the pseudo-testcross strategy was applied to generate a segregating population. Four crosses were made between four cultivars, using the combinations of Frantoio x Kalamata, Kalamata x SA Verdale, SA Verdale x Manzanillo, and Manzanillo x SA Verdale. Only one of these crosses, Frantoio x Kalamata, produced enough offspring to be useful in the construction of a linkage map. The maternal parent, Frantoio, is an oil cultivar that originated from Italy, and the paternal parent, Kalamata, is a table olive cultivar that originated from Greece. These two cultivars are different in terms of their morphological

characters, such as small leaves and fruits for Frantoio, and large leaves and characteristically-shaped fruits for Kalamata.

7.2.2 Generation of the F_1 population

Controlled pollinations were conducted in 1999 according to the methods described in Section 2.2.1. Fruits were harvested at the mature stage, and after removing the flesh, the seeds were chilled at 10°C (Section 6.3.1.1) and then germinated on a solidified medium containing 1/2 MS salts. The seedlings were transplanted into 5 cm pots containing Nu Earth Potting Mix soil (Nu Earth, Adelaide, Australia), placed in a fogger for 6 weeks, and then transferred to a glasshouse for six months. After 3 months of hardening in a shade house, 104 seedlings were planted in the field at the Roseworthy Campus of the University of Adelaide (34°31'S, 138°41'E, mean rainfall of 440 mm per annum). The distance between trees was 4 m. Weed, pest and disease control, irrigation, fertigation, and pruning were applied when necessary. Weeds were controlled by spraying with Basta (glufosinate ammonium) at a rate of 1 : 100 in water. Irrigation and fertigation were conducted during summer when the rainfall was low; irrigation occurred every four days at a rate 8 mm per irrigation. Each tree received 2.5 g of nitrogen per week.

7.2.3 DNA isolation, PCR amplification and gel electrophoresis

7.2.3.1 DNA isolation

DNA was extracted from the young leaves of parent trees, and from the leaves of the seedlings after they reached at least 60 cm in height. The extraction followed the micro extraction protocol described in Section 2.2.3.1.

7.2.3.2 PCR amplification

RAPD-PCR was conducted as described in Section 2.2.3.2. For SCAR and SSR markers, the procedure was applied as follows. The amplifications were performed in a 20 μL volume containing 40 ng of genomic DNA, 1.5 mM $MgCl_2$, 0.25 μM of each primer, 200 μM of each dNTP, 1 x PCR buffer (10 mM Tris HCl, pH 8.3, 50 mM KCl, 0.001% (w/v) gelatine) and 1 unit *Taq* DNA polymerase (Invitrogen Australia Pty. Ltd., Mt. Waverly, Victoria). A negative control tube containing all PCR reagents except for DNA was added in each run to check for DNA contamination.

DNA amplifications were performed in a 96 well thermocycler (Programmable Thermal Controller, MJ Research Inc., USA) under the following conditions: an initial denaturation at 95°C for 2 min followed by 34 cycles of 30 s at 95°C, 45 s at appropriate annealing temperature (cf. Table 7.2. 7.5), 1 min at 72°C, with a final extension step of 10 min at 72°C. A touch down program (Don *et al.*, 1991) was used for some of the microsatellite primers using the following conditions after the first step of denaturation at 94°C for 2 min: 94°C for 45 s, annealing for 45 sec (64°C for the first cycle, and reducing by 0.5°C for the next 20 cycles until the temperature reached 55°C, followed by another 24 cycles with annealing temperature at constant 55°C), 72°C for 45 s, with a final extension step of 10 min at 72°C. An alternative program was trialled in which the annealing temperature of the first cycle was 59°C, followed by a reduction to 50°C.

7.2.3.3 Gel electrophoresis

DNA fragments were separated on 1.75% agarose gels, as described in Section 2.2.3.3, for RAPDs, SCAR, and the preliminary screening of SSR primers.

For mapping analysis, amplified SSR fragments were separated on polyacrylamide gels as described by Collins and Symons (1993). Gel electrophoresis was carried out using a vertical 16 x 13 cm gel running apparatus. One mm thick gels were prepared using 8% polyacrylamide (Accugel™ 40% 19 : 1 acrylamide : bisacrylamide, Kimberly Research, Atlanta., Georgia) containing 7 M urea and 1 x TBE. Five µL/mL of 10% ammonium persulphate (APS) and 1.2 µL/mL of N,N,N',N'- tetramethylethylenediamine (TEMED) (Sigma Chemical Co. St. Louis, USA) were added as polymerisation catalysts. A shark's tooth comb, with teeth at intervals of 3 mm, was used to make the wells for loading samples. One lane on each gel contained 3 µL of pUC19 DNA restricted with Hpa II (DMW-P1, GeneWorks Pty. Ltd, Adelaide, Australia) to aid in the interpretation of band homology between gels.

Three µL of PCR reaction was mixed with 1.5 µL of 6 x formamide loading dye (95% deionised formamide, 10 mM EDTA, pH 8.0, 0.02% each of xylene cyanol FF and bromophenol blue) (Collins and Symons, 1993), heated for 5 min at 95°C, and chilled on ice for 5 min before loading. Gels were allowed to run for about 3 hr at 20 mA depending on the length of the amplified fragments.

After electrophoresis, two different staining methods were applied. 1. Ethidium bromide staining method: gels were stained with ethidium bromide (0.5 µg/mL) for 10 min and

destained with milliQ water for 5 min with mild shaking. The gel was visualised under UV light at a wave length of 254 nm. The images were captured either digitally by using the program Tekcap (version 1.0. Tekram Corporation, 1998) or by printout using Mitsubishi video copy processor P68E (Mitsubishi Electric Corporation, Japan). Digital images were converted to bitmap (BMP) format using PaintShop Pro (version 5.00, Jasc Software Inc, 1998). 2. Silver staining method (Bassam *et al.*, 1991): Gels were gently shaken in 10% acetic acid for 20 min. After three washes each of 2 min with distilled water, the gels were stained with a silver solution (0.2 gm $AgNO_3$ and 300 µL of 38% formaldehyde (HCOH) in 200 mL of water) for 30 min. After a rapid rinse with water (20 sec), gels were developed in cold (4°C) alkaline solution (12 gm anhydrous $NaCO_3$, 600 µL of 30% HCOH, and 800 µL of NaS_2O_3 (1 mg/mL) in 400 ml of water) until bands were of required intensity. The reaction was then terminated with 10% acetic acid. The gels were backed onto 3 MM Whatman paper and dried on a gel drier at 80°C for 1 hr.

7.2.4 Identification of parent trees

Prior to pollination, the identity of each of the parent trees was confirmed by DNA fingerprinting using RAPD markers. Six optimised decamer primers were used, GC01, GC05, GC18, GC20, OPZ11, and OPZ13 (Mekuria *et al.*, 1999). Their sequences are shown in Table 7.1. DNA extraction, PCR amplification, and gel electrophoresis were conducted following the protocol described previously (Section 2.2.3.3). The data were analysed according to the method illustrated in Section 2.2.3.4.

7.2.5 Screening of RAPD primers

Sixty decamer primers of kits OPA, OPB, and OPC designed and synthesized by Operon Technologies Incorporated (Alameda, California) were screened using DNA from the two parents, and a sub-population of 10 individuals, as PCR amplification templates. Primers that amplified at least one clear polymorphic band were chosen for large-scale PCR amplifications with the whole population.

Acronyms for the markers were assigned as follows: the first two letters represent the name of the company (Operon), the following letter refers to the particular kit (e.g. A, B, or C), the next two digits refer to the particular primer within a kit, and the digits following the hyphen represent the size of the marker in base pairs. For example, OPA01-590 is a fragment amplified by primer OPA01 that consisted of 590 bp.

Table 7.1 Sequences of RAPD primers used for fingerprinting parent trees

Primers	Sequence (5'→ 3')	G + C (%)	Sources*
GC01	CAGGCCCTTC	70	1
GC05	AGGGGTCTTG	60	1
GC18	AGGTGACCGT	60	1
GC20	GTTGCGATCC	60	1
OPZ11	CTCAGTCGCA	60	2
OPZ13	GACTAAGCCC	60	2

* 1: GeneWorks Pty. Ltd, Adelaide, Australia
2: Operon Technologies Incorporated (Alameda, California)

7.2.6 Development of SSR markers

7.2.6.1 Construction and screening of an enriched library for SSRs

Genomic DNA digestion Genomic DNA, prepared from leaves of Kalamata, was digested with the restriction enzyme *Bsp* 143I (*Sau* 3A1) (GeneWorks Pty. Ltd, Adelaide, Australia) (5'- G $^{?}$GATC - 3') in 50.0 µL containing 1 x universal buffer (33 mM Tris acetate, pH 7.8, 65 mM potassium acetate, 10 mM magnesium acetate, 4 mM spermidine, 0.05 mM dithioerythritol), 5 µg bovine serum albumin (BSA, Fraction V, Sigma), 2.0 µg DNA, and 25 U *Bsp* 143I, and incubated at 37°C for 3 hr. The efficiency of the digestion was examined by running 5 µL of the mixture on 1.75% agarose in 1 x TBE and staining the gel with ethidium bromide.

Ligation of linkers to digested DNA fragments Oligonucleotide linkers were ligated to each end of the restriction fragments to enable PCR amplification to be carried out during the following procedures. The linkers were prepared in a volume of 4 µL by combining 70 pmoles each of BSP 24 (5'- AGC ATC TCT CCA GCC TCT CAC CGA G -3') and BSP 12 (3'- AGT GGC TCC TAG -5') (synthesized by GeneWorks Pty. Ltd, Adelaide, Australia), heating at 90°C for 2 min, and letting the mixture cool to room temperature for 5 min to allow the linkers to anneal. The resulting linker was as follows:

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BSP 24: AGC ATC TCT CCA GCC TCT CAC CGA G
BSP 12:                         A GTG GCT CCT AG
```

A ligation mix was prepared in a volume of 10 µL, containing 1 x ligation buffer (Promega: 30 mM Tris-HCl, pH 7.8, 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP), 3 U of T_4 DNA ligase (3U/µL) (Promega), and 4 µL of the annealed linkers. This ligation mix was then added to the digested DNA (final volume = 55 µL) and incubated at 37°C for 3 hr.

Amplification of ligated DNA The restriction fragments, with linkers attached, were amplified using BSP 24 as the primer. PCR reactions were performed in a volume of 100 µL containing 5 µL of DNA/linker/ligation mix, 1.5 mM $MgCl_2$, 0.20 µM BSP 24, 200 µM of each dNTP, 1 x PCR buffer (10 mM Tris HCl, pH 8.3, 50 mM KCl, 0.001% (w/v) gelatine) and 5 U of *Taq* DNA polymerase. The reaction mixture was incubated at 72°C for 5 min to endfill the BSP 12 portion of the linkers, and then subjected to a PCR profile consisting of denaturation at 95°C for 2 min, followed by 35 cycles of 95°C for 15 sec, 55°C for 15 sec, and 72°C for 15 sec. The final step was extension for 5 min at 72°C. The efficiency of the amplification was examined by

running 5 μL of the mixture on 1.75% agarose in 1 x TBE and staining the gel with ethidium bromide.

Capture of microsatellite sequence Streptavidin-coated magnetic beads (Dynabeads® M-280 Streptavidin, Dynal A.S., Oslo. Norway) were prepared by suspending 0.5 mg of the beads (sufficient to bind 200 pmoles of biotinylated oligonucleotide) in 400 μL of TBST solution (0.1 M Tris-HCl, pH 7.5, 0.15 M NaCl, 0.1% Tween 20) with gentle vortexing, centrifuging the suspension for 1 min at 15,000 x g, and removing the supernatant. The washing was repeated once, and the washed beads were used in the following steps.

To minimise non-specific binding of olive DNA to the magnetic beads, the beads were blocked by incubating in 100 μL of blocking solution at 50°C for 30 min with occasional mixing. The blocking solution consisted of 30 μL of 5 x HSB (4.5 M NaCl, 0.15 M PIPES, pH 6.8, 37.5 mM Na_2EDTA), 20 μL of 50 x Denhardt's solution (1% Ficoll 400 (Pharmacia), 1% PVP, and 1% BSA), 30 μL of 25% dextran sulphate, 5 μL of 10% SDS, 10 μg of salmon sperm DNA (pre-heated at 95°C for 5 min) , and 15 μL H_2O.

The remaining 95.0 μL of PCR products amplified from the restriction fragments were made to 5 x SSC, by adding 32.5 μL of 20 x SSC (3 M NaCl, 0.3 M sodium citrate, pH 7.0) and 2.5 μL of H_2O, to maximise the hybridisation of DNA fragments to the probe. The solution was denatured at 95°C for 5 min and cooled on ice immediately. One μg of biotinylated probe $(AC)_{12}$ (GeneWorks Pty. Ltd, Adelaide, Australia) was added and the mixture was incubated at 50°C for 2 hr with occasional mixing. The blocked beads were added, and the mixture incubated at 50°C for 1 hr with occasional mixing. The mixture was centrifuged at 15,000 x g for 1 min, and the supernatant was removed. Weakly bound DNA fragments were removed by 3 washes of 400 μL of TBST, followed by 3 washes of 400 μL of 0.2 x SSC containing 0.1% SDS. Finally, the beads with their attached AC-enriched fragments were suspended in 100 μL of TLE (10 mM Tris-HCl pH 8.0, 0.1 mM EDTA), incubated at 95°C for 5 min and stored at –20°C.

Amplification of captured sequences The AC-enriched fragments were amplified by PCR, using BSP 24 as a primer. The reaction was performed in a volume of 100 μL containing 8 μL of beads with captured DNA, 1.5 mM $MgCl_2$, 0.20 μM BSP 24, 200 μM of each dNTP, 1 x PCR buffer and 5 U of *Taq* DNA polymerase. The PCR program consisted of an initial denaturation at 95°C for 2 min, followed by 34 cycles of 30 s at 95°C, 45 s at 55°C, 1 min at

72°C, with a final extension step of 10 min at 72°C. The efficiency of the amplification was examined by running 8 μL of the mixture on 1.75% agarose in 1 x TBE and staining the gel with ethidium bromide.

Cloning of the $(AC)_n$ enriched DNA sequences The enriched DNA sequences were cloned using the TOPO™ TA Cloning® Kit (Invitrogen, California, USA). The PCR products were ligated to the vector, pCR®2.1-TOPO, by mixing 1 μL of fresh PCR products with 1 μL of the vector, 1 μL of salt solution, and 3 μL of sterile water. The reagents were gently mixed, incubated for 5 min at room temperature, and placed on ice. Two μL of the TOPO cloning reaction was gently mixed into a vial of TOPO10F' One Shot™ cells, followed by incubation on ice for 30 min, heat shock at 42°C for 30 sec, and then incubation on ice for a further 2 min. 250 μL of SOC medium (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, and 20 mM glucose) was added to the vial, and the cells allowed to recover at 37°C for 1 hr with gentle horizontal shaking.

Luria-Bertaini (LB) medium (10% tryptone, 5% yeast extract, 10% NaCl, pH 7.0), containing 100 μg/mL of ampicillin, was prepared in 9 cm Petri dishes. Just before use, the plates were coated with a mixture of 16 μL of 5-bromo-4-chloro-3-indolyl-beta-D-galactoside (X-gal, 50 mg/mL in N, N'-dimethylformamide) and 33 μL of isopropylthio-β-D-galactoside (IPTG, 25 mg/mL in water), and allowed to dry at 37°C for 30 min. The plates were spread with 100 μL of cells and incubated overnight (16 hr) at 37°C, and inspected for blue/white colonies. IPTG is used together with the chromogenic substrate X-gal to determine *lac* gene expression. Blue colonies are those without insertion of foreign DNA into the vector, and which therefore show IPTG induced β-galactosidase activity.

LB plates containing 100 μg/mL of ampicillin were labelled on the base with a grid of 9 x 9 mm. 141 large, white colonies were transferred to identical grid positions on duplicate plates and incubated at 37°C overnight. One of the duplicate plates was stored at 4°C, and colonies from the other duplicate plate were transferred to nitrocellulose transfer membrane.

7.2.6.2 Colony lift and hybridisation of the colonies with an AC_{12} probe

Hybond™-N+ (Amersham Pharmacia Biotech) nitrocellulose membrane was placed over the colonies with gentle pressure, and left for 5 min. The membrane was lifted, without sliding, and placed successively, colony side up, on 3MM paper (Whatman Paper Ltd., England) soaked in 1% SDS/5mM EDTA, lysis solution (0.5 M NaOH/1.5 M NaCl), neutralizing

solution (1 M Tris HCl, pH 8.0/1.5 M NaCl), and 2 x SSC. In each case, the membranes were allowed to remain in contact with the appropriate paper for 5 min. Membranes were air-dried for 15 min, baked in an oven for 2 hr at 80°C, and stored at room temperature.

Membranes were soaked in 2 x SSC and transferred to hybridisation bottles with the DNA side facing inwards. Three membranes could be placed in one bottle. The pre-hybridisation solution consisted of 3.0 mL of 5 x HSB buffer, 2.0 mL of 50 x Denhardt's solution, 3.0 mL of 25% dextran sulphate, 0.5 mL of 10% SDS, and 1.5 mL of MQ water. 100 μL of autoclaved salmon sperm DNA (10 mg/mL) was heated at 95°C for 10 min. and added to the pre-hybridisation solution at 55 °C and the solution was then added to the hybridisation bottle containing the membranes, and pre-hybridisation was allowed to proceed at 55°C for 3 hr.

The probe was "end-labelled" by mixing 24 pmoles of $(AC)_{12}$ oligonucleotide, 24 pmoles of γ-^{32}P-ATP (GRA-32U, NEN®, 111 TBq/mmole, PerkinElmer Pty Ltd, Boston, USA), 2.4 μL of 10 x T_4 buffer 'No ATP' (5 M Tris HCl, pH 7.5, 1 M $MgCl_2$, 500 mM DTT, 10 mM spermidine) and 1.0 μL of T4 polynucleotide kinase (PNK1, GeneWorks Pty. Ltd., Adelaide, Australia) (10U/μL), incubating at 37°C for 30 minutes, and cooling on ice.

Following the pre-hybridisation, the probe was heated at 95°C for 5 min, cooled on ice for 5 min, added to the hybridisation bottle, and hybridisation was allowed to proceed at 55°C overnight.

Excess label was removed by rinsing the bottle with 50 mL of 2 x SSC/0.1% SDS at room temperature. A further 50 mL of 2 x SSC/0.1% SDS at 55°C was added and the bottle placed on the rotor in the hybrisation oven at 55°C for 10 min. The membranes were then transferred to a plastic tray and washed successively, for 10 min intervals, with two lots of 400 mL of 2 x SSC/0.1% SDS at 55°C, and two lots of 400 mL of 0.2 x SSC/0.1% SDS at 55°C. The membranes were dried on paper tissue, wrapped in thin plastic film, and exposed to X-ray film (Fujifilm, Super RX) for 5 hr and developed.

7.2.6.3 Small scale preparation of plasmid DNA

Thirty five DNA samples on the membranes produced a strong, positive signal on the X-ray film, indicating likely $(AC)_n$ repeats. These samples were located relative to the positions of the colonies on the grids placed on the Petri dishes, and the corresponding colonies on the duplicate plates stored at 4°C were used to prepare plasmid DNA. Each colony was

transferred into a 10 mL screw cap tube containing 1.5 mL of LB broth made to 100 μg/mL with ampicillin, and incubated at 37°C overnight with vigorous shaking on an Orbital Mixer Incubator OM25 (ADELAB Scientific, Adelaide, Australia). The culture was poured into a 1.5 mL Eppendorf tube, centrifuged at 4,600 x g for 5 min, and the supernatant was removed . The pellet was loosened by vortexing briefly and resuspended in 90 μL GET (50 mM glucose, 10 mM EDTA, pH 8.0, 25 mM Tris HCl, pH 8.0), and completely dispersed by vigorously vortexing the tube. 180 μL of freshly prepared lysis buffer (0.2 M NaOH, 1% SDS) was added and mixed gently by inversion 6 times. 130 μL of 3 M potassium acetate, pH 4.6 was added and gently mixed again by inversion 6 times. The mixture was centrifuged at 17,500 x g for 15 min, and the supernatant transferred into a clean tube containing 2 μL of RNase A (10 mg/mL, DNase free) (AMRESCO®, Solon, Ohio, USA) and incubated at 37°C for 30 min.

After incubation, 400 μL of Tris-saturated phenol and 400 μL of chloroform were added, briefly vortexed for 10 sec, and centrifuged for 5 min at 17,500 x g. The supernatant was transferred into a new tube and the DNA was precipitated with 2.5 vol of ethanol (-20°C). After incubating for 10 min at room temperature, plasmid DNA was collected by centrifuging for 15 min at 17,500 x g, removing the supernatant, and washing the pellet with 100 μL 70% ethanol (-20°C). The tube was placed on a heating block at 50 °C for 3 min to remove remaining ethanol, and DNA was dissolved in 30 μL of 0.1 mM EDTA, pH 7.0, and stored at -20 °C.

The cloned DNA was analysed by cleavage with *Eco*RI using 1 μL of plasmid DNA, 1 μL of 10 x universal buffer, and 2 U of *Eco*RI (Boehringer, Mannheim, GmbH, Germany) in a volume of 10 μL. The mixture was incubated for 1 hr at 37 °C and separated by electrophoresis on 1.75% agarose in 1 x TBE buffer. The size of the insert was determined by comparison with a 100 bp ladder.

7.2.6.4 Preparation of cloned DNA for sequencing

PCR was performed on the plasmid DNA with the Dye Terminator Ready Reaction Mix supplied by PE Applied Biosystems, using either the M13 forward primer 5'-TGT AAA ACG ACG GCC AGT-3', or the M13 reverse primer 5'-CAG GAA ACA GCT ATG ACC (Invitrogen, California, USA). Amplification was performed in a volume of 20 μL, containing 1.5 μL of the plasmid DNA (about 200 ng), 1.9 pmoles of the appropriate primer, and 4.0 μL of Dye Terminator Ready Reaction Mix using the following program: 25 cycles of 96°C for 10

s, 50°C for 5 s, and 60°C for 4 min. Sequencing was carried out with an ABI Model 3700 Capillary DNA Sequencer (Division of Molecular Pathology, Institute of Medical and Veterinary Science, Adelaide). In most cases, the elongation of DNA fragments rich in GT repeats was not successful, and the fragments were not fully sequenced. However, DNA fragments containing AC repeats were fully sequenced. Therefore, the sequencing of inserts with GT repeats was carried out with the alternative M13 primer. A dGTP BigDye™ Terminator RR mix (PE Applied Biosystems) was also trialled for GT rich DNA strands.

PCR products were mixed with 80 μL of 75% isopropanol by vortexing and the mix left at RT for 15 min. The DNA pellet was collected following centrifuging at 17,500 x g for 20 min. 250 μL of 75% isopropanol was added, the tubes vortexed briefly, centrifuged at 17,500 x g for 5 min, and the supernatant removed. The DNA samples were air-dried for 5 min and stored at -20°C.

7.2.6.5 Alignment of sequences and search of the GenBank database

The sequences obtained were aligned to test for duplication using Clustal X (Thompson *et al.*, 1997) and WebANGIS (http://www.angis.org.au/), and checked for homology against sequences in the GenBank database using the BLASTA program (http://www.ncbi.nlm.nih.gov/BLAST/).

7.2.6.6 SSR primer design and testing

The sequences flanking the microsatellite repeat regions were targeted for primer design using Oligos 9.0 (Institute of Biotechnology, University of Helsinki, Finland), and primer pairs were tested using Net Primer (PREMIER Biosoft International, California, USA). Primer design mainly followed the rules recommended by Innis and Gelfand (1991)

1. primers should be 17-28 bases in length
2. the GC composition should be more than 40%
3. the 3' end should be a G or C to increases efficiency of priming
4. T_ms should be higher than 50°C, with a maximum of 3°C difference between primer pairs
5. runs of three or more Cs or Gs should be avoided

6. 3'-ends of primers should not be complementary

7. primer self-complementarity should be avoided.

However, sometimes the GC content and melting temperature (T_ms) were compromised due to a high content of A and T in the flanking sequences

Primer pairs were synthesised by Genset Pacific Pty. Ltd (Lismore, Australia), GeneWorks Pty. Ltd (Adelaide, Australia), and Invitrogen Australia Pty. Ltd. (Mt. Waverly, Victoria, Australia) and tested against the parents, i.e., Frantoio and Kalamata. Only primers amplifying reproducible DNA fragments were used for mapping.

7.2.6.7 Test of published SSR markers

Sixteen published SSR markers from three different sources (Lefort *et al.* (1999), Sefc *et al.* (2000), and Cipriani *et al.* (2002)) were tested against Frantoio and Kalamata (Table 7.2). Those showing heterozygous PCR fragments in at least one cultivar were chosen for further analysis.

7.2.7 Test of SCAR markers

Sequence characterized amplified region (SCAR) markers were tested against the parents and a sub-set of the progeny. A peacock disease resistance marker of olive was developed by Mekuria *et al.*, (2002), a SCAR marker linked to high olive flesh/pit ratio by Hernandez *et al.* (2001a), and a self-incompatibility allele of almond by Channuntapipat *et al.* (2001). The primers were G7 (forward: 5'-CAT CAC CAC TCC ACT CCT CTC-3', and reverse: 5'-CAG CAT CTC CAT AAT CCT TTC-3'.), ScoeMS-2 (requested from the authors, forward: 5'-AGT GAT TTG ACA TGG AAA TGT GG-3', and reverse: 5'-CAT ATC ATG TAG ACG CGT AGA ACG-3'), and Con (forward: 5'-GTG CAA CAA TGG CCA CCG AC-3', and reverse: 5'-TAC CAC TTC ATG TAA CAA CTG AG-3'), respectively.

7.2.8 Analysis of morphological traits

Morphological traits expressed by the mapping population were analysed when the seedlings were 15 mth old. The shape of the leaves was classified as either ellipse or lanceolate, the colour of the upper side of the leave as either dark green or light green, the leaves as either straight or twisted, and the upper surface of leaves as either glossy or flat. These characters were recorded for the parents also.

Table 7.2 Published microsatellite markers tested for map construction

Markers	Primer sequences (5'→ 3')	$T_a(C^o)$	Length range (bp)	References
FEMSATL2	AGCAGCATTTATGAATGTTC ATCAACTGAAGATGACGACG	50	174 - 224	Lefort *et al.* (1999)
FEMSATL16	TTTAACAGTTAACTCCCTTC CAACATACAGCTACTAATCA	52	180 - 200	Lefort *et al.* (1999)
ssrOeUA-DCA1	CCTCTGAAAATCTACACTCACATCC ATGAACAGAAAGAAGTGAACAATGC	50	204-230	Sefc *et al.* (2000)
ssrOeUA-DCA3	CCCAAGCGGAGGTGTATATTGTTAC TGCTTTTGTCGTGTTTGAGATGTTG	50	228-250	Sefc *et al.* (2000)
ssrOeUA-DCA5	AACAAATCCCATACGAACTGCC CGTGTTGCTGTGAAGAAAATCG	50	195-211	Sefc *et al.* (2000)
ssrOeUA-DCA15	GATCTTGTCTGTATATCCACAC TATACCTTTTCCATCTTGACGC	50	242-266	Sefc *et al.* (2000)
UD099-006	TCAGTTTGTTGCCTTTAGTGGA TTGTAATATGCCATGTAACTCGAT	57	150-174	Cipriani *et al.* (2002)
UD099-008	AAAAACACAACCCGTGCAAT AAATTCCTCCAAGCCGATCT	57	160-170	Cipriani *et al.* (2002)
UD099-011	TGACTCCCTTTAAACTCATCAGG TGCGCATGTAGATGTGAATATG	57	105-132	Cipriani *et al.* (2002)
UD099-012	TCACCATTCTTAACTTCACACCA TCAAGCAATTCCACGCTATG	57	157-167	Cipriani *et al.* (2002)
UD099-014	TTCCCCTTATTCAATGTGAACC ACTGCAGTTTGGGAATCAAA	57	95-105	Cipriani *et al.* (2002)
UD099-019	TCCCTTGTAGCCTCGTCTTG GGCCTGATCATCGATACCTC	57	101-169	Cipriani *et al.* (2002)
UD099-031	TATCCTCTATGTGGCGATG TTGGTTAAAAGGATTGATACA	57	114-155	Cipriani *et al.* (2002)
UD099-035	AATTTAATGGTCACACACAC ATTGCGAAATAGATCTACGA	57	136-168	Cipriani *et al.* (2002)
UD099-043	TCGGCTTTACAACCCATTTC TGCCAATTATGGGGCTAACT	57	179-219	Cipriani *et al.* (2002)
UD099-044	AATTCCGACAAGTTGTGTGTG CACAGCACCCAACCAGATTT	57	128-150	Cipriani *et al.* (2002)

7.2.9 Analysis of hybridity of the pedigree

The hybridity of the F_1 population was investigated with the RAPD data using NTSYS-pc v 2.0 (Applied Biostatistics Inc. Setauket, NY) (Rohlf, 1998). Genetic similarities among all pairs of individuals, both parents and progeny, were estimated using the simple matching coefficient as the similarity coefficient. Cluster analysis was performed on the estimated similarities using the unweighted pair group method with arithmetic average (UPGMA) and the SAHN algorithm. Cophenetic matrices were derived from the dendrograms, and the goodness-of-fit of the clustering to the data matrix was calculated by comparing the original similarity matrices with the cophenetic matrices. Correlation coefficients of ≥ 0.9, < 0.9 or ≥ 0.8, < 0.8 or ≥ 0.7, and < 0.7 indicate very good, good, poor, and very poor fits respectively (Rolf, 1998).

Non-metric multidimensional scaling (nMDS) analysis was also performed, by extracting eigenvectors, computing the distances between individuals, and displaying the relationship in two dimensions (Rolf, 1998).

The hybridity of the population was also analysed using SSR markers. The genotypes of the offspring were investigated and compared with those of the parents. Progeny individuals whose alleles matched those that would be expected from a cross between 'Frantoio' and 'Kalamata' were considered as true hybrids.

7.2.10 Map construction

7.2.10.1 Generation of mapping data

For the RAPD data, markers are dominant, and those that segregated with a ratio of 1:1 or 3:1 were used for map construction. The presence of a band in the parents was assigned as 'ab' and the absence of a band was assigned as 'aa'. In the progeny, the presence of a band was assigned as 'ab', if one parent was heterozygous but another homozygous, 'b_' (representing 'bb' or 'ab') if both parents were heterozygous, and 'aa' if the band was absent.

For the microsatellite data, markers are codominant and different alleles at the same locus are recognisable, and therefore up to four alleles can be present in the population. They were assigned as 'a', 'b', 'c', and/or 'd', and corresponding genotypes were determined accordingly.

According to the two-way pseudo-testcross strategy, two separate data sets were organised, one for each parent. Testcross markers (Grattapaglia and Sederoff, 1994) were included only in one data set if the marker was present in the corresponding parent, whereas intercross markers (Verhaegen and Plomion, 1996) were included in both. The data sets were analysed using Joinmap 2.0 (Stam, 1993).

7.2.10.2 Segregation analysis

Four different types of segregation model were classified: testcross markers segregating at 1:1 and intercross markers segregating 3:1, 1:2:1 or 1:1:1:1. Each marker was tested for Mendelian segregation by the chi-square test ($\alpha = 0.05$) using the single locus analysis module, JMSLA.

7.2.10.3 Linkage analysis

Preliminary grouping of the markers was performed using the JMGRP command with a LOD score at 3.0 (Staub *et al.*, 1996) and the original file was split into separate files using JMSPL. The markers within the groups were analysed for pairwise linkages using the JMREC command with the LOD threshold set at 0.01, and the REC threshold at 0.499 (Jermstad *et al.*, 1998) and the recombination frequencies were estimated. The REC and LOD values were inspected using JMCIA and the distribution of the values was analysed.

7.2.10.4 Map construction for the parents

The linkage groups were ordered, and the genetic distances were calculated from the recombination fraction using Kosambi's mapping function (Kosambi, 1944) with the command JMMAP. The following parameters were employed: LOD threshold at 0.1, REC threshold at 0.49, ripple value of 3, jump threshold at 3, and triplet threshold at 7 (Jermstad *et al.*, 1998). The original map data were compiled for map drawing, and the map was drawn by MapChart 2.1 (Voorrips, 2002).

7.2.10.5 Generation of an integrated map based on the maps of the parents

The linkage groups were investigated to locate loci that were present in both parent maps, and the groups were allocated in pairs accordingly, one group of the pair from male parent and the other from female parent. The pairwise data files, with recombination values of the paired

groups, were integrated, and the integrated map was generated as above (Section 7.2.10.4) using the Joinmap command JMMAP.

7.3 Results

7.3.1 Identity of the parent trees

Analysis of the DNA fingerprints for Frantoio and Kalamata, obtained by RAPD-PCR, showed that the trees used were identical to those for Frantoio from the Olive World Collection, Centro de Investigacion y Desarollo Agrario, Cordoba, Spain, and Kalamata from Consiglio Nationale Delle Ricerche, Instituto di Ricerca Sulla Olivicultura, Perugia, Italy (Fig. 7.1).

7.3.2 RAPD markers

Out of 60 decamer RAPD primers, 32 were chosen for mapping, and these generated 300 markers for the whole population. The highest number of bands (19) was generated by OPC06, and the lowest (4) by OPB03, OPB19, OPC12, and OPC16. The average number of bands per primer was 9.4 (Table 7.3). Primer OPA11 generated the most polymorphic bands, three in Frantoio and six in Kalamata. In contrast, primer OPB03 did not give any polymorphic bands, although 4 bands were amplified. Of the 19 bands generated by OPC06, 6 were polymorphic, whereas of the 14 bands produced by OPA11, 9 were polymorphic.

Markers that were polymorphic between the parents were not necessarily polymorphic in the progeny, and vice versa. There were 181 of these markers in the population, 63 in Frantoio, 54 in Kalamata, 61 present in both parents, and three that were absent in both. The average was 5.7 polymorphic bands per primer. After excluding them from the mapping data, 178 RAPD markers were used for linkage analysis.

7.3.3 SCAR markers screened

Three different SCAR markers were targeted, but only the peacock spot disease-resistance primers (G7 forward and reverse) produced a DNA fragment with the expected size of 700 bp (Mekuria, 2002). This fragment, named G7-700, was present in Frantoio, absent in Kalamata, and polymorphic in the mapping population. Hence, it was suitable for map construction. The G7 primers amplified four additional bands, with sizes of 1600, 720, 650, and 295 bp (named G7-1600, G7-720, G7-650, and G7-295, respectively). G7-1600 and G7-650 were

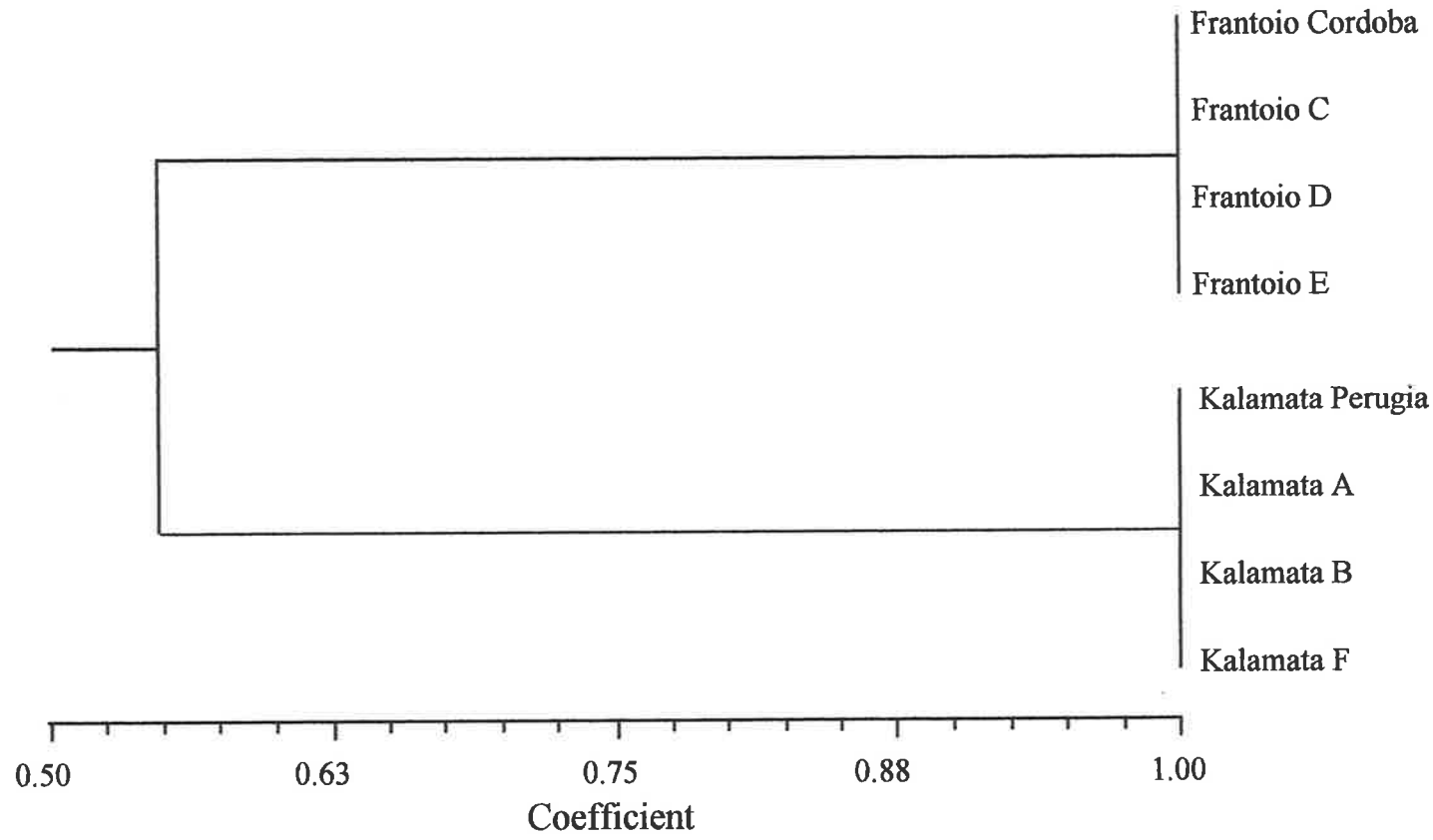

Fig. 7.1 Dendrogram of the trees used as parents of mapping population compared with standard olive cultivar samples using RAPD fingerprinting. The trees are identical to the respective standard cultivars. Frantoio Cordoba: a Frantoio sample collected from the Olive World Collection, Centro de Investigacion y Desarollo Agrario, Cordoba, Spain; Kalamata Perugia: a Kalamata sample collected from Consiglio Nationale Delle Ricerche, Instituto di Ricerca Sulla Olivicultura, Perugia, Italy

Table 7.3 Characterisation of RAPD primers against Frantoio and Kalamata

Primers	Sequences	No of bands only in Frantoio	No of bands only in Kalamata	No of poly-morphic bands	No of bands in both	Total No of bands
OPA01	5'-CAGGCCCTTC-3'	4	2	6	4	10
OPA04	5'-AATCGGGCTG-3'	4	3	7	2	9
OPA07	5'-GAAACGGGTG-3	4	0	4	8	12
OPA08	5'-GTGACGTAGG-3'	2	1	3	8	11
OPA09	5'-GGGTAACGCC-3'	1	2	3	5	8
OPA10	5'-GTGATCGCAG-3'	2	1	3	3	6
OPA11	5'-CAATCGCCGT-3'	3	6	9	5	14
OPA12	5'-TCGGCGATAG-3'	3	1	4	3	7
OPA13	5'-CAGCACCCAC-3'	2	0	2	4	6
OPA15	5'-TTCCGAACCC-3'	1	2	3	8	11
OPA20	5'-GTTGCGATCC-3'	1	2	3	9	12
OPB01	5'-GTTTCGCTCC-3'	1	1	2	5	7
OPB03	5'-CATCCCCCTG-3'	0	0	0	4	4
OPB05	5'-TGCGCCCTTC-3'	2	2	4	2	6
OPB10	5'-CTGCTGGGAC-3'	1	1	2	8	10
OPB11	5'-GTAGACCCGT-3'	2	1	3	6	9
OPB15	5'-GGAGGGTGTT-3'	3	1	4	7	11
OPB17	5'-AGGGAACGAG-3	1	0	1	6	7
OPB18	5'-CCACAGCAGT-3'	3	3	6	10	16
OPB19	5'-ACCCCCGAAG-3'	0	3	3	1	4
OPB20	5'-GGACCCTTAC-3'	3	1	4	7	11
OPC01	5'-TTCGAGCCAG-3'	2	2	4	1	5
OPC03	5'-GGGGGTCTTT-3'	3	3	6	4	10
OPC04	5'-CCGCATCTAC-3'	3	5	8	9	17
OPC06	5'-GAACGGACTC-3'	2	4	6	13	19
OPC07	5'-GTCCCGACGA-3'	5	3	8	6	14
OPC08	5'-TGGACCGGTG-3'	3	2	5	5	10
OPC10	5'-TGTCTGGGTG-3'	3	4	7	2	9
OPC12	5'-TGTCATCCCC-3'	0	1	1	0	1
OPC16	5'-CACACTCCAG-3'	2	1	3	1	4
OPC19	5'-GTTGCCAGCC-3'	3	3	6	2	8
OPC20	5'-ACTTCGCCAC-3'	1	1	2	10	12
Total		70	62	132	168	300

monomorphic bands for Frantoio and Kalamata as well as for the mapping population, and were not considered for map construction. G7-720 was present in Kalamata, G7-295 was present in Frantoio, and both showed polymorphism in the population. Therefore, they were used for mapping analysis.

7.3.4 Microsatellite development and analysis

7.3.4.1 $(AC)_n$ enriched library and screening

AC repeat sequences from genomic DNA of Kalamata were enriched by the use of streptavidin-coated magnetic beads. After amplification by PCR, the fragments were found to vary from 170 to 4000 bp, with about 70% being less than 1000 bp (Fig. 7. 2). These fragments were cloned, and 141 out of 225 colonies examined (63%) produced a positive (white) reaction to X-gal/IPTG selection. 66 white colonies (29% of the total) showed positive hybridisation following colony blotting with an $(AC)_{12}$ probe labelled with ^{32}P (Fig. 7.3).

7.3.4.2 Characteristics of the microsatellite sequences

Out of the 66 positive colonies, 33 were recovered and sequenced, and 29 (88%) contained AC repeats. Four of the colonies were found to contain plasmid DNA with identical inserts, and when these were discarded, 86% of the sequenced plasmids contained $(AC)_n$ repeat sequences. Five of the microsatellite sequences obtained were perfect, 12 were imperfect, and eight were compound. The imperfect microsatellites were interrupted by sequences that varied from 2 to 7 bp, and within any one microsatellite, the same interrupting sequence was often present in several different positions (Fig. 7.4). The length of the sequences of repeat motifs varied from 24 to 228 bp (Table 7.4), and the distribution is listed in Table 7.5.

When the sequences of the inserts were aligned, it was found that R115, R116, R117, and R127 were identical, and similarly for R102 and R110. Partially homologous sequences were found for R025 and F137, and also for F120 and F122 (Fig. 7.5).

The sequences flanking the microsatellites obtained from four colonies showed high homology with GenBank sequences. R113 included a 76 bp sequence that was 92% homologous to "*Olea europaea* repetitive DNA, clone OLEU-828" (AJ243943), and "*Olea europaea* satellite DNA, clone pOLEU-900" (AJ271721), and an 84 bp fragment of F077 was 90% homologous to a completely different region of the same GenBank accessions. 230 bp of F049 was 86%

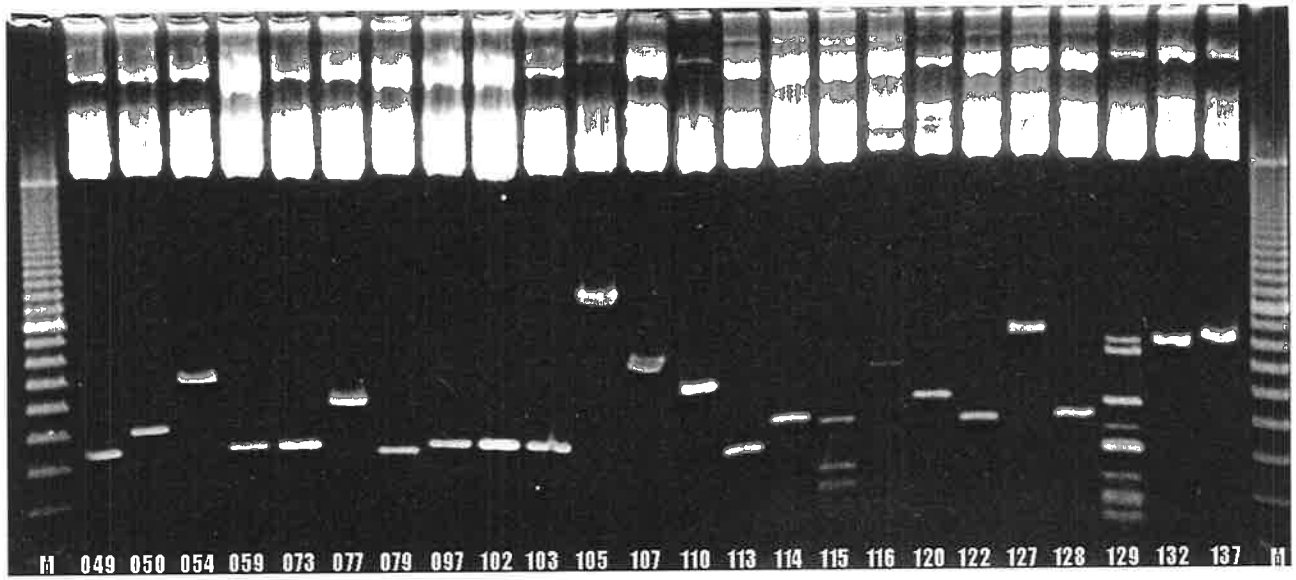

Fig. 7.2 Insert DNA after digestion of Kalamata DNA clones with EcoRI following AC repeat enrichment by streptavidin magnetic beads.

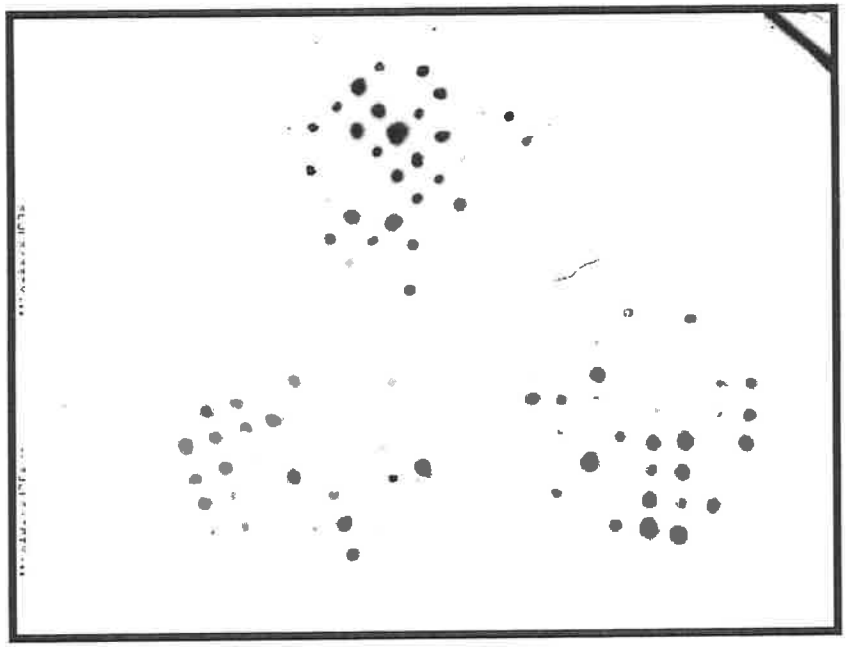

Fig. 7.3 Colony blotting: 141 Kalamata DNA clones hybridised with ^{32}P labeled $(AC)_{12}$, 66 colonies showing positive hybridisation.

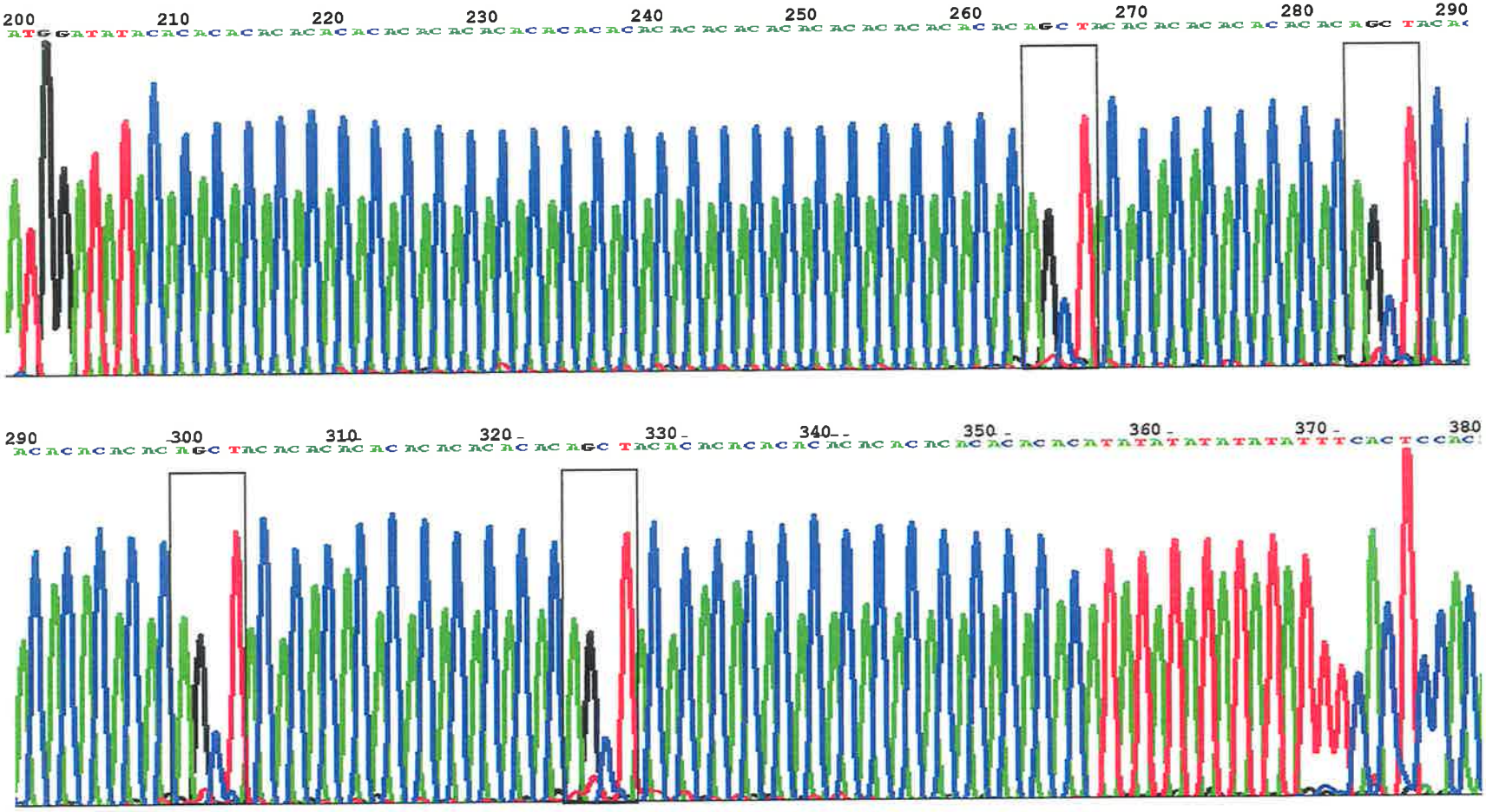

Fig. 7. 4 A chromatogram of an imperfect microsatellite sequence. The interrupting sequences (AGCT) showing in the rectangles are the same in four different areas.

Table 7.4 Length distribution of repeat mot

Sequence length (bp)	Number of sequences	Frequency (%)
0 - 19	0	0.0
20 - 59	9	34.6
60 - 99	6	23.1
100 - 139	4	15.4
140 - 179	4	15.4
180 - 219	2	7.7
220 - 259	1	3.8

Table 7.5 Primer sequences, repeat motif, annealing temperatures and fragment length of microsatellites identified in Kalamata

Marker	Primer sequences (5'→ 3')	Repeat motif	$T_a(C^o)$	Length (bp)
F003	CTCTCGTTATTCCTGTGTTGATG ACAGCCAAATTCTTCCATACC	$(AC)_{13}[TACCTA(AC)_2AACGTCC(AC)_7]_2(AC)_6$ $CTACC(AC)_{10}TACCTA(AC)_2AACGTCC(AC)_{34}$	55	301
R025	TTATAGATTGCATCTTCACTGG CGATTAACTTTTAATTGGCACTCG	$(AC)_{28}GC(AC)_3GC(AC)_{33}(A)_4$	50	215
R026	TTAAAATTACGAAAAGACATGC CATCTCCAGTAATATCTAATTCCTTG	$(AC)_{28}(AT)_6$	45	163
F035	AGGATGAGAAAAGTGGGAGATGG CGAACTACCTCATTGAGGCAGG	$(AC)_{16}$	50	120
R036	GACATACATACACACACACACA AATTAAATTGAAGCATACGTG	$(AC)_{47}TACAT(AC)_{45}$	45	216
F041	AAATTATGAGAAAATCAACAACG GGTGTTAGGAGGTCATTCGTG	$(AC)_{27}(AT)_6AG(AC)_6$	50	224
F049	CTGGGCTCCTTGAATCGTGG CGTAGTTCTTTATATGGTGTG	$(CT)_2AC_{18}$	45	118
R050	GTAATAGCCTTGTCCAAAGAGTTC GCTTATCTGATGCTTGATTTAGTG	$(AC)_{27}AT(AC)_2CTAT(AC)_{15}TCAG(AC)_{22}$	55	240
F054	AATTTATTATTGTACAGCGTTGCAC GCAATTTTACATACTACTTCCCAGTCC	$(CT)_2(AC)_{34}$	50	168
F073	GTAAGTAATCGTCTAAGACCCCAC CCGAATAAGCATCAATGATCCTGC	$T_5(AC)_{34}$	55	182
F077	CAAGAGATACAACTTAAAACCAACTGC ATCAAATTGAACTTTTGTGTGT	$(AC)_{39}A_4$	50	185

Table 7.5 continued

F097	GTCATAGATAATTCTAAAAGTAGGTG AACACTTGGTCCATTATCATTTCGCT	$(AC)_{12}CTG(TA)_2G(AC)_4C_2(AC)_2CGA(AC)_4CTG$ $(TA)_2G(AC)_3AG(AC)_3C_3A_2(AC)_{24}$	50	218
F103	GCAAAATGCCTATTTCTACG GGAGAGAGCGGATTGGTTGAGTGC	$(AC)_{40}A_4$	55	163
R105	TTACTGGTGACTTGTTAAGACG GGTTTAGAGATGTGTGTTTGTG	$(AC)_{17}$	45	185
R107	TGCCATTCTCTAATGTGATGC TTCCATTCCCCCGTTGAACC	$(AT)_2(AC)_{28}AGCT(AC)_8AGCT(AC)_6AGCT(AC)_{10}$ $AGCT(AC)_{14}(AT)_7$	45	339
R110	CCACAATTCCAACGGTCAAG ATCTTTGGGAGCCGCTATTAC	$(AC)_{21}AGT(AC)_5AGT(AC)_{12}AGTT(AC)_{10}AGT(AC)_4$	55	273
R113	GAATCAAATATCATCACACAC CCGATCGAGATTCAAT	$(ATC)_2(AC)_{21}(AT)_8$	45	271
F114	AAGTTCGGTTAATGTAGTCG AAAATCTCGCACAGGGCACC	$(AC)_{13}(AG)_2$	55	109
R115	CGAGTGCTACAATACCATTAGTACC GTTCTTCGTTTCTGGTTCGTG	$(AC)_3AT(AC)_{15}CCAGACAG(AC)_9AGTT(AC)_{10}$ $CTCACAT(AC)_2AG(AC)_{17}$	50	278
F120	TTGCTACAGTTTTGCGTGAGGTG TGTGTGTGTGTGTTTTTGTGT	$(TG)_2(TA)_3CT(AC)_3T(AC)_{15}A_4(AC)_6$	50	205
F122	TTTATATAGATGCCTACAGCGTGC CCACATTAATCATTATGCGT	$(TA)_3CT(AC)_3T(AC)_{13}(TA)_2$	50	131
R132	GCGTTCAAACTCCCTGCCAC ATTTGGATAAAAGAGCTTCCTTC	$(AC)_{13}AT(AC)_4A_3CAT_3(AC)_{11}(AT)_2(AC)_5AGAC$ $[(AT)_2(AC)_3]_2ATAC(AG)_2GC(AC)_{19}$	55	351
F137	TTATAGATTGCATCTTCACTGG TTAGACTTTTGAAACTTTTGACG	$(AC)_{12}$	50	119

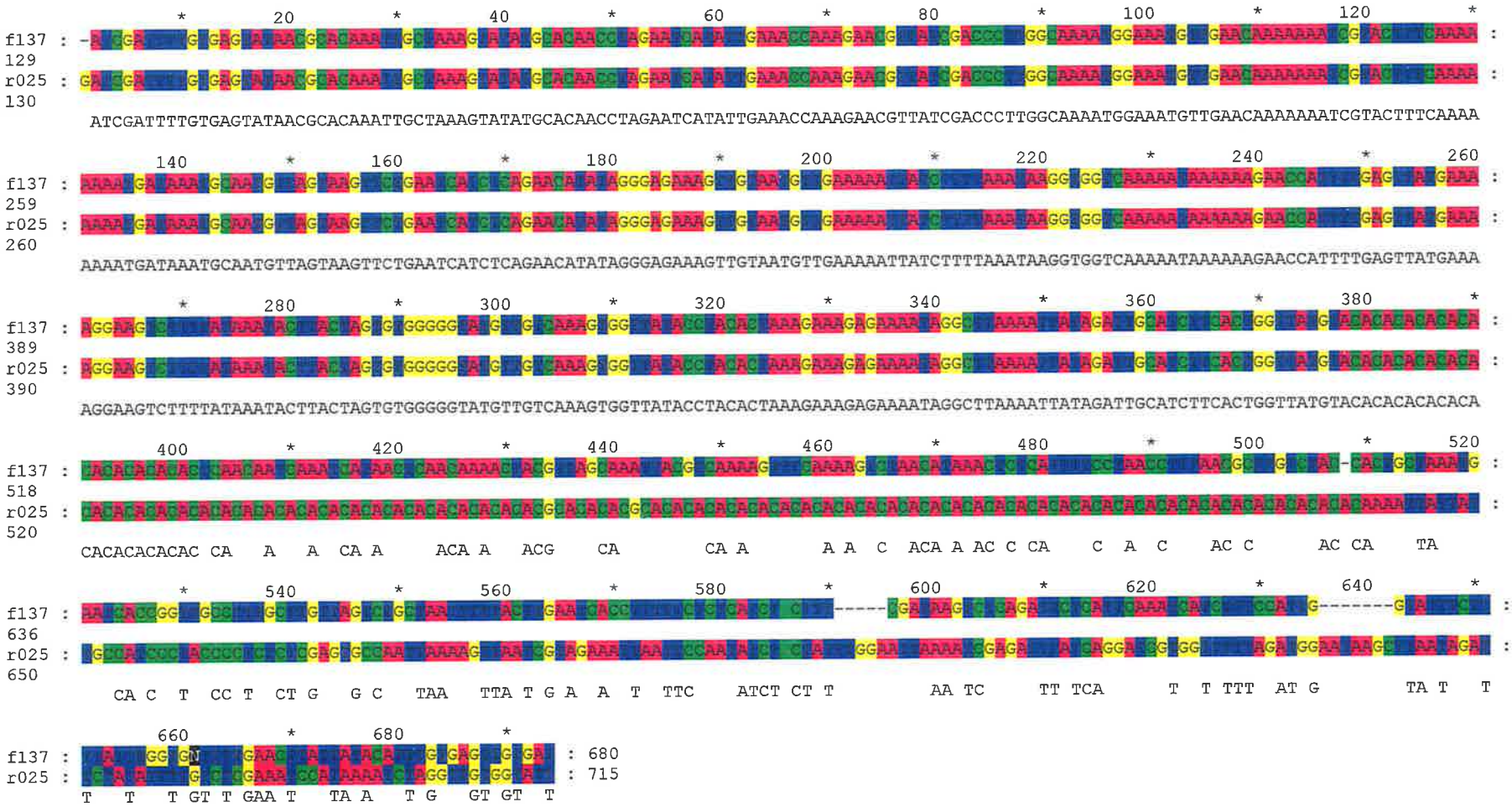

Fig. 7.5 Sequence alignment of microsatellites F137 and R025, showing the sequences are homologous on one side of AC repeats and heterologous on the other side.

homologous to a fragment of "*Olea europaea* RAPD genomic sequence, clone 212f" (AJ236558). 177 bp of F120 was 79% homologous to a fragment of "genomic sequence for *Arabidopsis thaliana* BAC F20B17 from chromosome I" (AC010793) and "*Arabidopsis thaliana* kinase, putative, predicted mRNA" ((NM_106613).

7.3.4.3 SSR primers and the microsatellite regions amplified

Six microsatellite sequences were discarded due to either duplication or absence of sufficient flanking sequence for primer design, and twenty-three primer pairs were designed. The sequences of these primers, and the lengths of the expected products are shown in Table 7.4.

Of the 23 primer pairs designed, twelve amplified DNA from both Frantoio and Kalamata, and four, i.e., F49, F54, R110, and R113, produced bands of the expected sizes.

7.3.4.4 PCR amplification using primers synthesised by different companies

Primer pairs F49, F54, R110, and R113 were each synthesised by three different companies, designated as I, II, and III. The three samples of each set of primers were used in separate PCR reactions with the same genomic DNA and identical thermal profiles. Primers received from providers I and III produced similar results, whereas those from provider II generated different bands for primers F49, R110, and R113 (Fig. 7.6). Therefore, provider II was not considered for primer synthesis in this experiment.

7.3.5 Analysis of published SSR markers

Six sets of primers developed from olive (Cipriani *et al.*, 2002; Sefc *et al.*, 2000), DCA3, UD006, UD011, UD019, UD043, and UD044 were tested. DCA3, UD006, UD011, and UD043 produced well-separated heterozygous bands that appeared in both parents and also segregated within the population. DCA3, UD011, and UD043 produced three alleles for the population, whereas UD006 produced four. Although UD019 produced three visible alleles, a null (non-amplifying) allele, which may arise from polymorphism in the priming sites (Jones *et al.*, 1998; Pemberton *et al.*, 1995), was detected by a mismatch between the parent and offspring genotypes when two homologous alleles were assumed. When the null allele was assumed to occur in Kalamata, the genotypes of the parents and offspring matched completely.

UD044 was reported by Cipriani *et al.*, 2002 to be a single locus marker, but two products of 125 and 149 bp were found for Frantoio, and one product of 147 bp for Kalamata. For

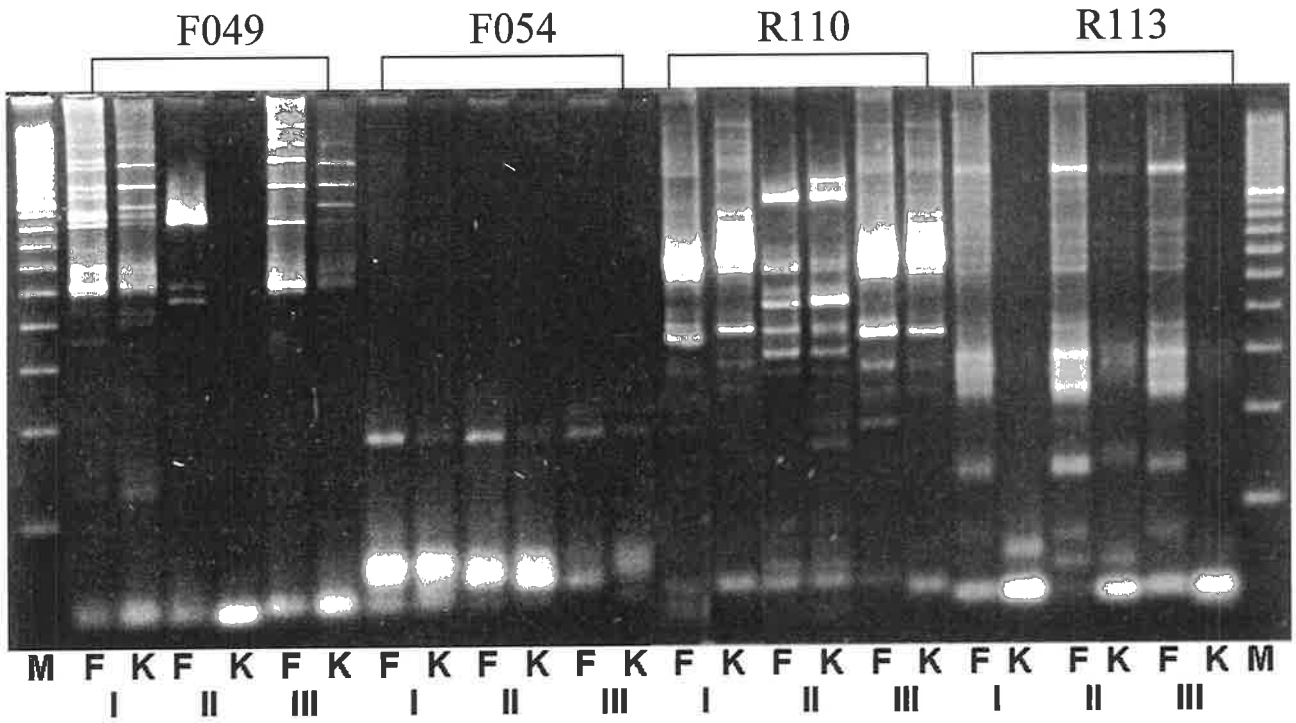

Fig. 7.6 Comparison of DNA amplification using primers synthesised by providers I, II, and III. Similar band patterns were produced by the same primers synthesized by Providers I and III which differed from those produced with Provider II primers. M: 100 bp marker; F: Frantoio; K: Kalamata. F049, F054, R110 and R113 are different primers for the amplification of microsatellites.

convenience, the alleles have been assigned the letters A, B and C. Using this nomenclature for the offspring, the band patterns observed were as follows: A, B, C, AB, AC, BC, and ABC. This configuration of genotypes for the offspring did not match the expectation of a single locus for either the case where homologous alleles were assumed for Kalamata (e.g. AC, BC) or where a null allele was assumed present in Kalamata (e.g. A, B, AC, BC). Therefore, A, B, and C were considered as three independent dominant markers for map construction and were named UD044-128, UD044-147, and UD044-149.

In addition, two sets of primers (FEMSATL2, and FEMSATL16) developed from *Fraxinus excelsior* (Lefort *et al.*, 1999), which belongs to the same family as olive, were tested but these did not produce any PCR products with either Frantoio or Kalamata.

In total, ten microsatellite markers were used for map construction.

7.3.6 Hybridity of the mapping population

No genetic differences could be distinguished between the three parent trees of Frantoio by a cluster analysis based on 300 RAPD markers, and a similar result was found for Kalamata. The similarity coefficient between Frantoio and Kalamata was 0.72, which was the lowest among all pairs of individuals. The highest similarity coefficient was 0.82 between progeny 76 and 88, and for the rest of the progeny, the similarity coefficients were between 0.72 and 0.82 (Fig. 7.7). The cophenetic correlation was 0.46 indicating that the clusters in the dendrogram could not be distinguished from random events. Since Frantoio and Kalamata were located on either side of the dendrogram, with all F_1 individuals between them, none of the progeny could be excluded from the cross between Frantoio and Kalamata.

MDS analysis confirmed the result shown by the dendrogram. The distances between Frantoio and Kalamata were greater than between the individuals, and all offspring were positioned between Frantoio and Kalamata (Fig. 7.8).

Further evidence of the hybridity of the progeny was provided by the SSR markers. The genotypes of all offspring matched the putative genotypes that could be expected if the parents were Frantoio and Kalamata.

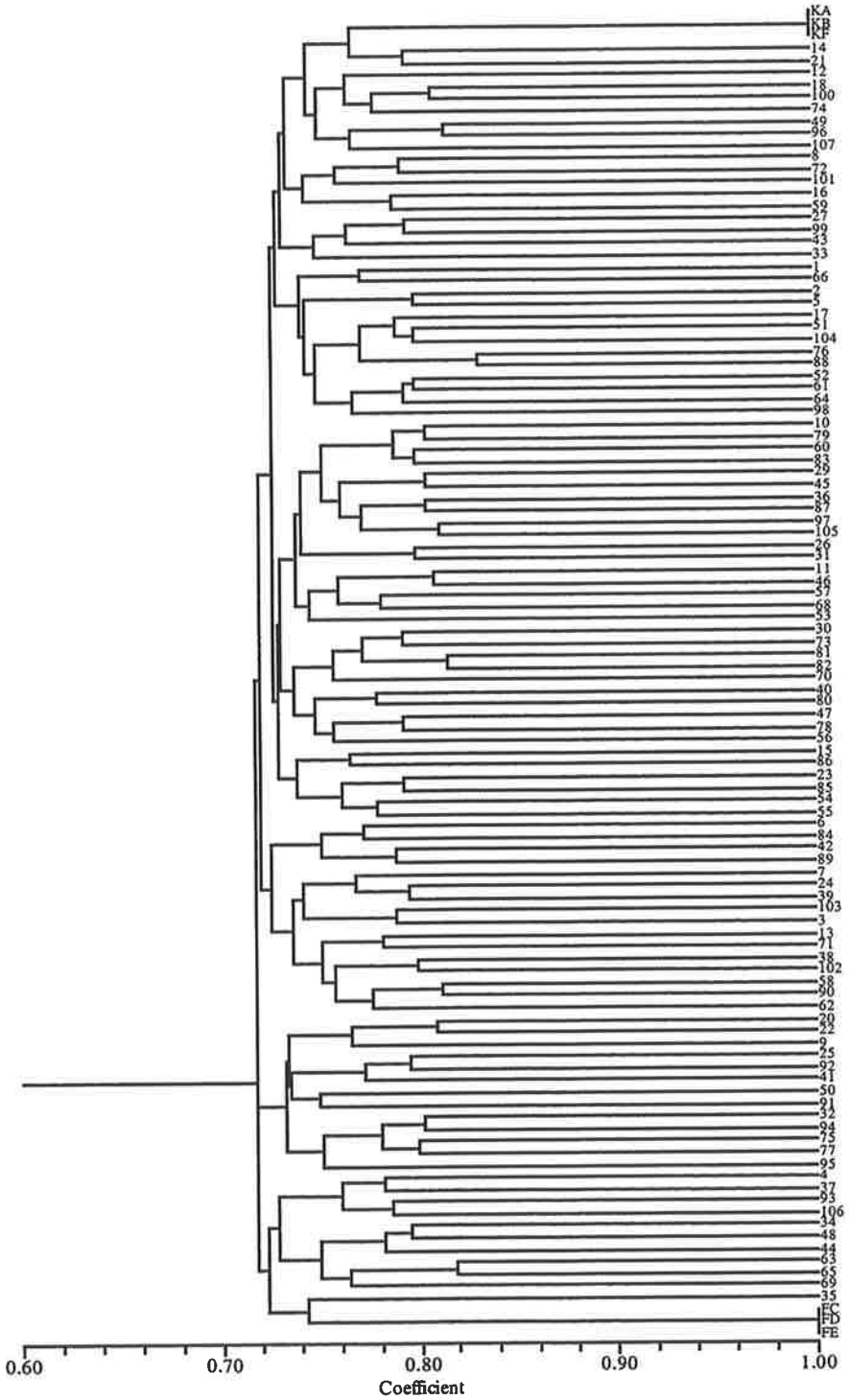

Fig. 7.7 Dendrogram of the parents and offspring trees used for map construction. Genetic similarities among all pairs of individuals were estimated using the hierarchical distance method of simple matching coefficient with NTSYS-pc (Exeter software, NY, USA). Cluster analysis was performed on the estimated similarity using the unweighted pair group method with arithmetic average (UPGMA), and the SAHN algorithm. F: Frantoio; K Kalamata.

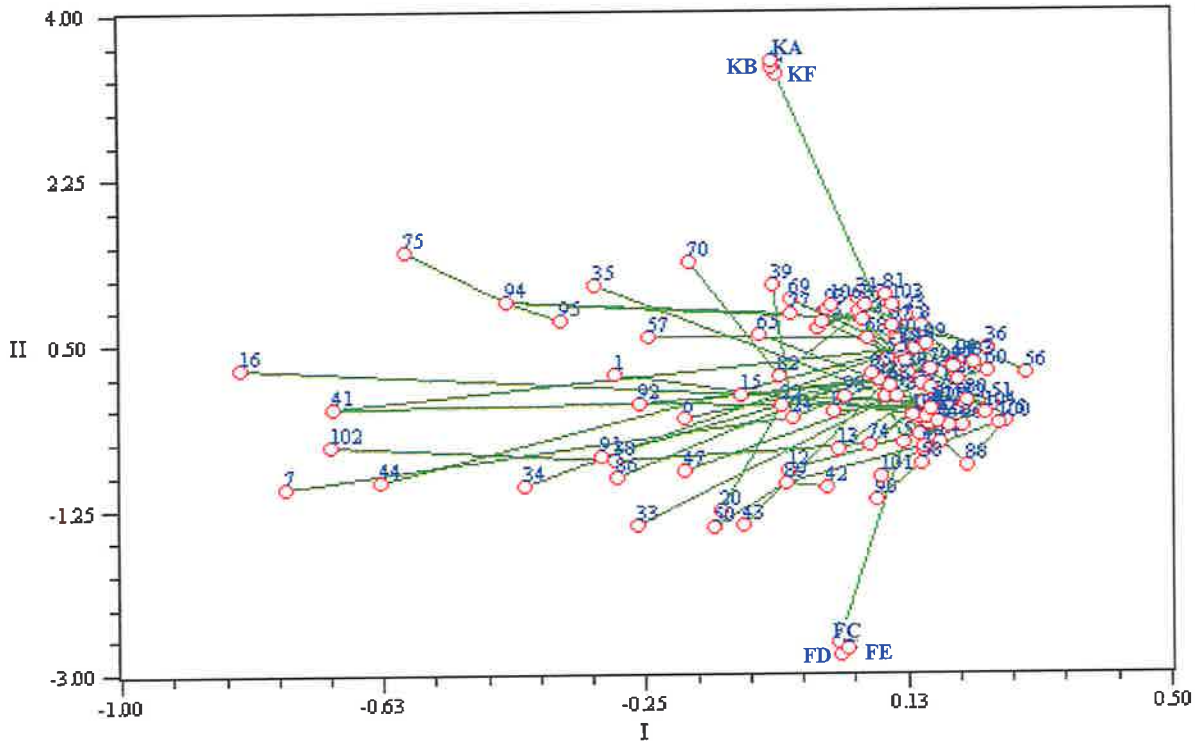

Fig. 7.8 Non-metric multi-dimensional scaling analysis of the RAPD data obtained from trees of Frantoio, Kalamata, and the F_1 population. The three trees of Frantoio (FC, FD, and FE) and Kalamata (KA, KB, and KF) were identical respectively, and the offspring individuals (1-18, 20-27, 29-66, and 68-107) were clustered between Frantoio and Kalamata. Note that Frantoio and Kalamata trees are distantly clustered from the offspring individuals

7.3.7 Segregation analysis of the loci

7.3.7.1 RAPD

A total of 178 RAPD markers were classified into two different groups on the basis of the segregation model (Table 7.6): (1) 117 loci that were heterozygous in one parent and homozygous null in the other segregated with a ratio of 1 : 1 (Fig. 7.9); 63 of these occurred in Frantoio and 54 in Kalamata, while 22 showed distorted segregation at the significance level of $\alpha = 0.05$; (2) 61 loci that were heterozygous in both parents and segregated in a 3 : 1 ratio (Fig. 7.9); 11 of these showed distorted segregation at the significance level of $\alpha = 0.05$.

7.3.7.2 SCAR markers

All three polymorphic SCAR markers, derived from the primers for peacock spot disease-resistance, were classified as a testcross configuration, two for Frantoio and one for Kalamata (Fig. 7.10). The χ^2 values at a significance level of $\alpha = 0.5$ indicated that markers G07-700 and G07-720 showed distorted segregation, whereas G07-295 segregated as 1 : 1.

7.3.7.3 SSR markers

All SSR markers segregated according to the ratios expected. Four segregated as 1 : 1, another four segregated as 1 : 1 : 1 : 1 (Fig. 7. 11), and one (with null allele) segregated as 1 : 2 : 1. No distorted ratios were detected at the significance level of $\alpha = 0.5$.

7.3.7.4 Morphological traits

Since the seedlings were in the juvenile stage, most of the agronomically important characters such as flowering time, fruit size, disease resistance, and oil quality, were not available for assessment. The leaf shape expressed by both parents was lanceolate and the upper side of the leaves was glossy. However, both of these characters segregated in the mapping population (Fig. 7.12). Leaf shape segregated as either straight or twisted, and the upper surface of the leaves was either glossy or flat, but the segregation ratios were significantly distorted from 3 : 1 at the level of $\alpha = 0.1$.

Table 7.6 Number and segregation types of markers used for map construction

Markers	Testcross (segregation 1:1) female	Testcross (segregation 1:1) male	Intercross (segregation 3:1, 1 : 2 : 1 or 1:1:1:1)	Total
RAPD	63	54	61	178
SCAR	2	1	0	3
SSR	3	1	5	9
Morphological traits	0	0	4	4
Total	**68**	**56**	**70**	**194**
Distorted (α=0.05)	11 (16.2%)	13 (23.2%)	13 (18.6%)	37 (19.1%)

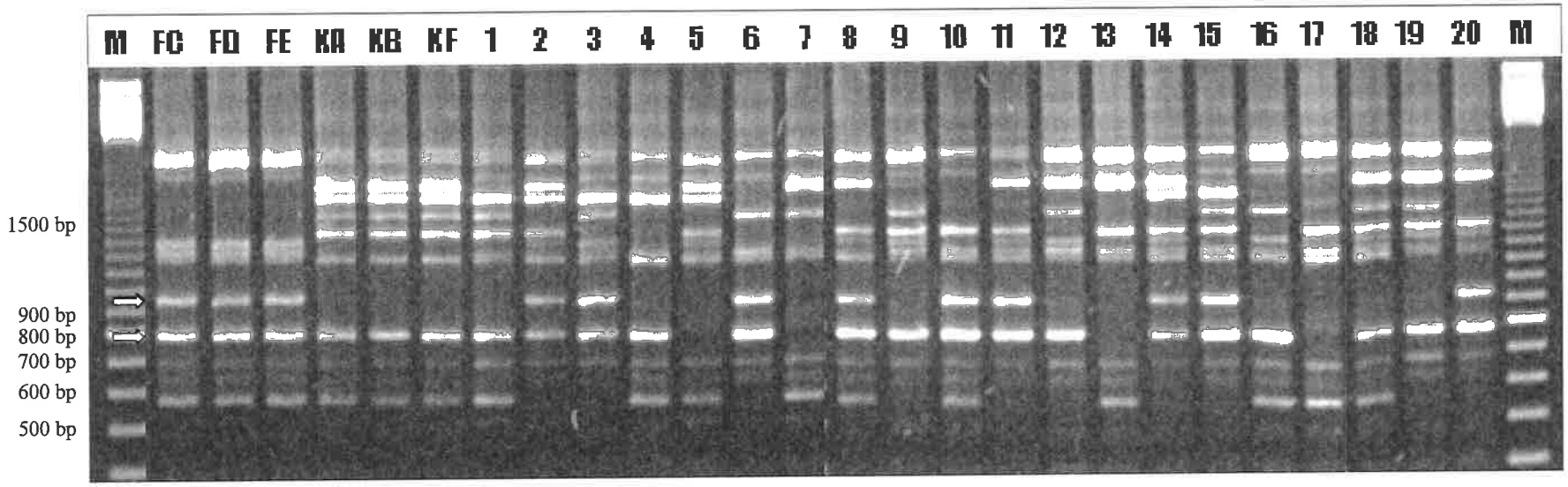

Fig. 7.9 RAPD markers generated by primer OPC10 segregate in the mapping population. Opc10-950 was a testcross marker segregating 1 : 1 that was heterozygous in Frantoio and homozygous null in Kalamata; OPC10-790 was a intercross marker that was heterozygous in both parents and segregated in a 3 : 1 ratio. M: 100 bp molecular standard; FC, FD, FE: Frantoio trees; KA, KB, KF: Kalamata trees; 1-20: F_1 progeny individuals.

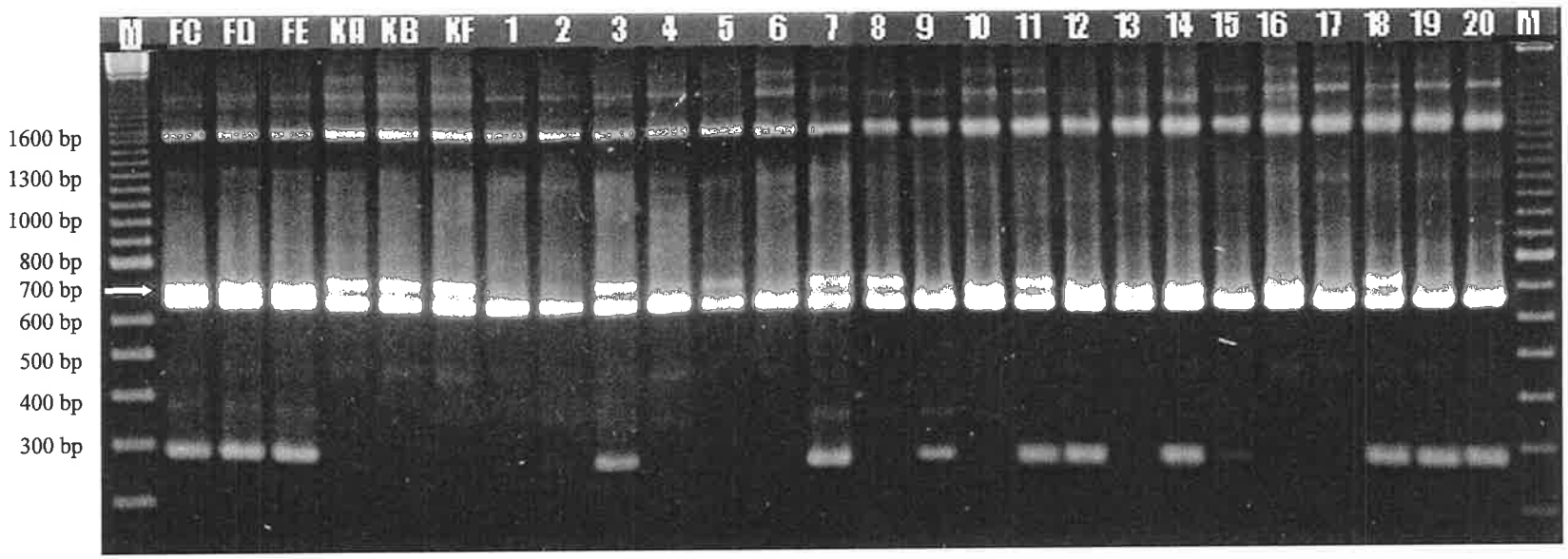

Fig. 7.10 SCAR markers generated by primer G07 segregating in the mapping population. All the polymorphic markers were testcross markers segregating 1 : 1. G07-295 and G07-700 (PDR) was heterozygous in Frantoio and homozygous null in Kalamata; G07-720 was heterozygous in Kalamata and homozygous null in Frantoio. M: 100 bp molecular standard; FC, FD, FE: Frantoio trees; KA, KB, KF: Kalamata trees; 1-20: F_1 progeny individuals.

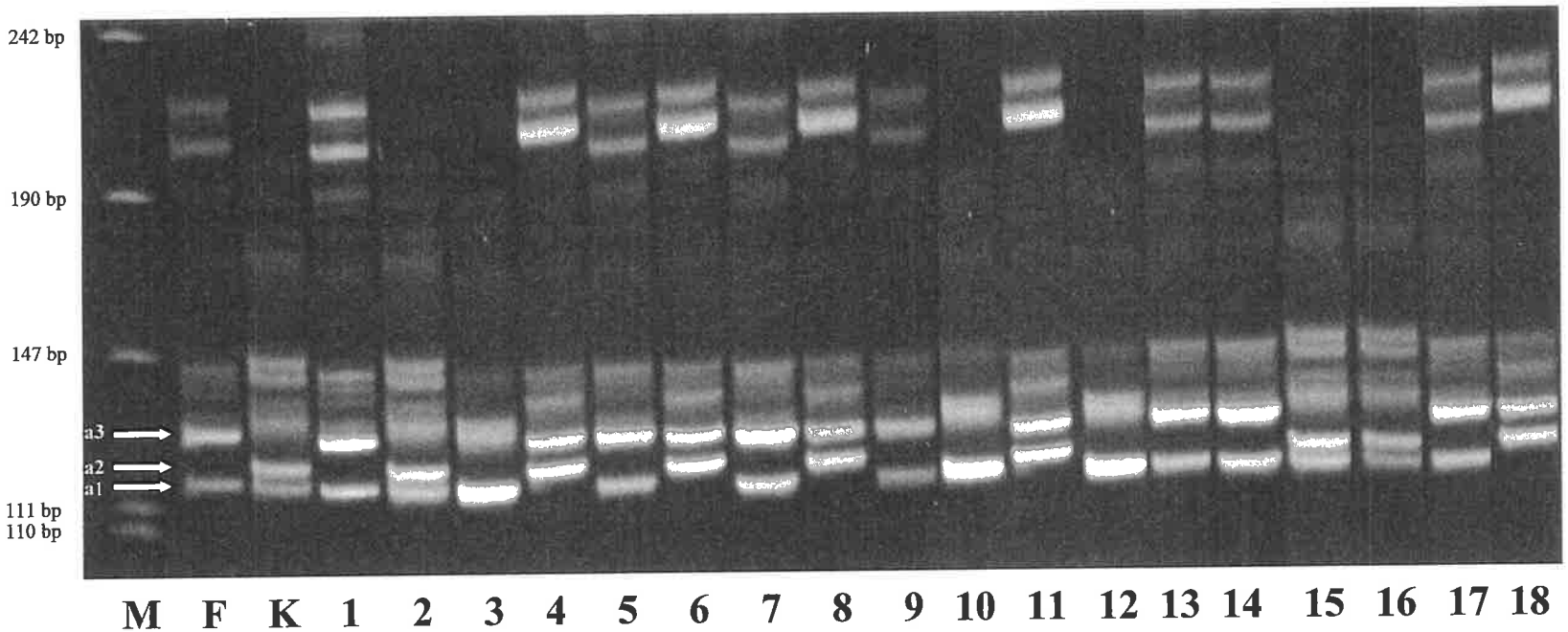

Fig. 7. 11 An SSR marker UD-11 showing three alleles present in the parents and segregated in the mapping population from a cross between Frantoio and Kalamata. M: molecular standard, pUC19 DNA restricted with Hpa II; F: Frantoio; K: Kalamata; 1-18: F_1 progeny individuals.

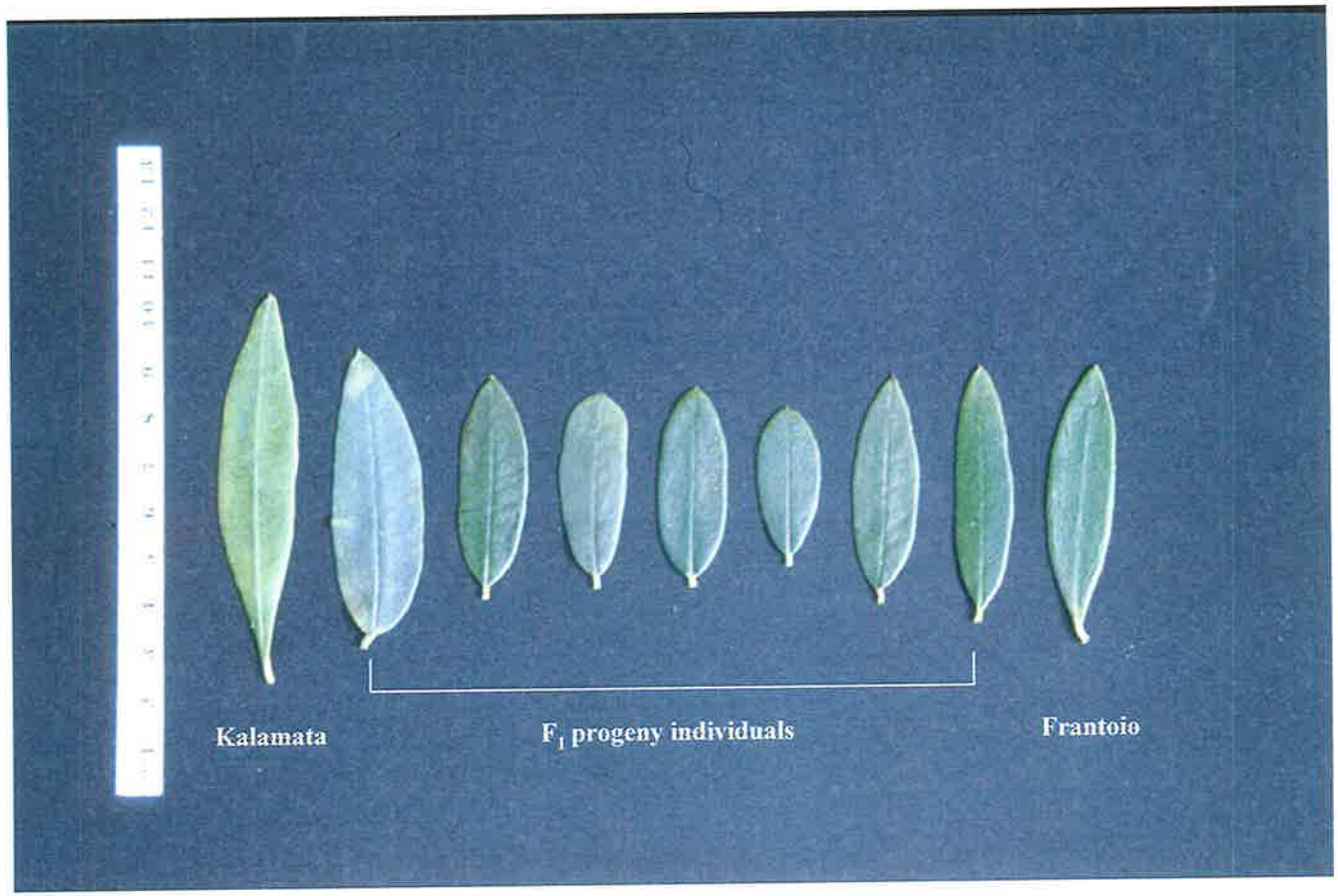

Fig. 7.12 The leaves of Frantoio and Kalamata, and their F_1 progeny individuals, showing polymorphism among the mapping population.

7.3.7.5 Informativeness of the population individuals

The analysis of Mendelian segregation and pairwise combinations of 8128 loci by the χ^2 test ($\alpha = 0.05$), using the single locus analysis module-JMSLA, showed that all 104 progeny were informative.

7.3.8 Map construction

A total of 194 markers were used to construct the linkage map (Table 7.6). Two separate maternal and paternal linkage maps were established using 138 and 126 test- and inter-cross markers, respectively. Marker grouping and ordering were performed using the module, JMGRP. The preliminary tests were carried out with different LOD thresholds (from LOD 2 to 5 at 0.5 increments) to group the markers. Both male and female markers appeared to be organised in the groups close to the haploid number for olive, n = 23, at the LOD of 3. Therefore, a minimum LOD score of 3.0 and a maximum recombination fraction of $\theta = 0.49$ were chosen as a good compromise between a reliable statistical criterion and the maximum number of linked markers.

In total, 152 markers were used to produce 27 linkage groups for Frantoio and 23 for Kalamata. The linkage groups for Frantoio cover 798 cM of the genome with 92 loci, and the average distance between loci is 12.3 cM. The linkage groups for Kalamata cover 759 cM of the genome with 89 loci, and the average distance between loci is 11.5 cM. After merging the maps of Frantoio and Kalamata, 15 major groups were obtained, which cover 879 cM of the genome with 101 loci, and the average distance is 10.2 cM (Table 7.7).

For the Frantoio map (Fig. 7.13), the maximum genetic distance was 36.0 cM between G07-700 and opa09-700, and the least maximum genetic distance was 0.0 cM, indicating the markers co-segregated in the offspring. Co-segregating markers were found in three linkage groups, Fra1 (opa08-510, opc06-990, and opc10-780), Fra9 (opa08-1200 and opb01-900), and Fra27 (opa04-1050 and opc20-970). The longest linkage group was Fra1 with 12 markers covering 116.4 cM of the genome, and the shortest was Fra27, which consisted of only one locus with two markers, opa04-1050 and opc20-970. Twelve linkage groups consisted of at least three markers, and 15 consisted of two. The average number of markers per linkage group was 3.4 covering 29.5 ± 19.8 cM.

Table 7.7 Statistics of linkage maps constructed

Maps	Length (cM)	No loci	Average distance between loci (cM)	Mean size/linkage group (cM)
Frantoio	798	92	12.3	29.5 ± 19.8
Kalamata	759	89	11.5	33.0 ± 28.4
Integrated	879	101	10.2	58.6 ± 41.9

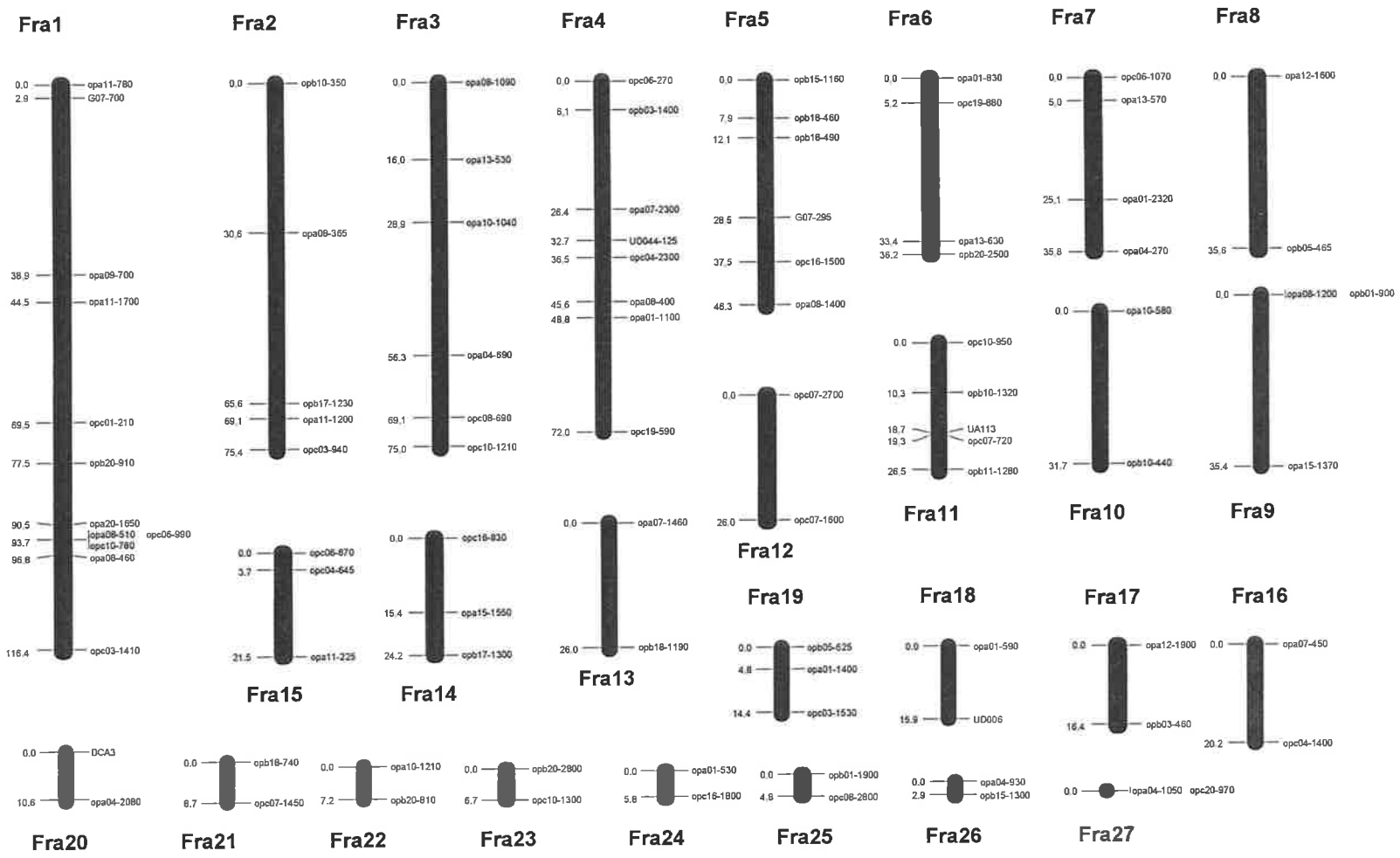

Fig. 7. 13 Frantoio linkage map consisting of 27 linkage groups

For the Kalamata map (Fig. 7.14), the maximum genetic distance was 45.3 cM between G07-720 and opc07-1750, and the minimum was 0.0 cM. Co-segregating markers were found in four linkage groups, Kal7 (opa08-1200 and opb01-900), Kal16 (opa04-2400 and opb19-2900), Kal20 (opa09-2700 and opa01-830), and Kal22 (opc06-990 and opc10-780). The longest linkage group was Kal1 with 12 markers covering 148.8 cM of the genome, and the shortest was Kal23, covering 2.8 cM. Fifteen linkage groups consisted of at least three markers, and eight consisted of two. The average number of markers per linkage group was 3.9 covering 33.0 ± 28.4 cM.

For the integrated map (Fig. 7.15), the maximum genetic distance was 35.5 cM between opa15-1370 and opa08-1200, and the minimum was 0.0 cM . Co-segregating markers were present in two linkage groups, FK2 (opc06-990 and opc10-780), and FK10 (opa08-1200 and opb01-900). The longest linkage group was FK1 with 12 markers covering 148.8 cM of genome, and the shortest was FK15 with two markers covering 8.2 cM. Fourteen linkage groups consisted of at least three markers, and one consisted of two markers. The average number of markers per linkage group was 6.7 covering 58.6 ± 41.9 cM. Thirty three of the markers were derived from Frantoio, 38 from Kalamata, and 30 were from both.

Of 37 distorted markers (Table 7.6), 23 were mapped. There were nine distorted markers in the Frantoio map. Two of these, opa11-780 and G07-700, were assigned to linkage group Fra1, two, opa08-365 and opa11-1200, to linkage group Fra2, and five, opc-19-590, opa08-1400, opb05-465, opa08-1200, and opc10-950, to Fra4, Fra5, Fra8, Fra9, and Fra11, respectively (Fig. 7.13). There were 19 distorted markers in the Kalamata map. Four of these, opb11-1030, opc10-1350, opb03-1900, and opc19-650 were assigned to linkage group Kal2, two, G07-720 and opc07-1750, to linkage group Kal3, three, opa20-1470, opc01-450, and opc01-370, to linkage group Kal 10, two, opa11-1200 and opa15-1200, to linkage group Kal11, two, opa08-1040 and opa11-1110, to linkage group Kal12, and five, opb15-2800, opa08-1200, opc07-1900, opb18-630, and opc19-710 to linkage groups Kal4, Kal7, Kal9, Kal13, and Kal14, respectively (Fig. 7.14). For the integrated map, 13 distorted markers were mapped to eight linkage groups (Fig. 7.15).

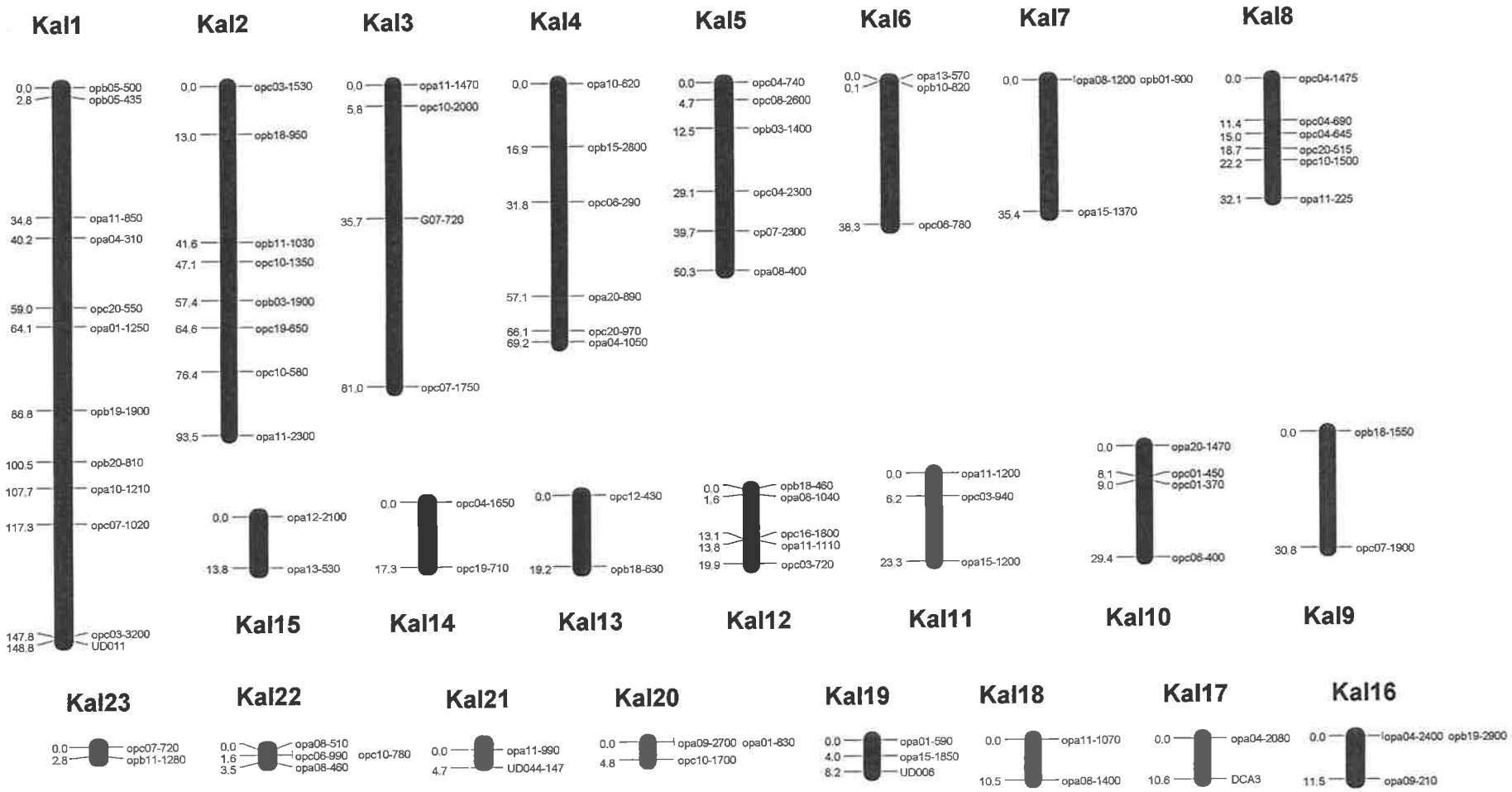

Fig. 7. 14 Kalamata linkage map consisting of 23 linkage groups

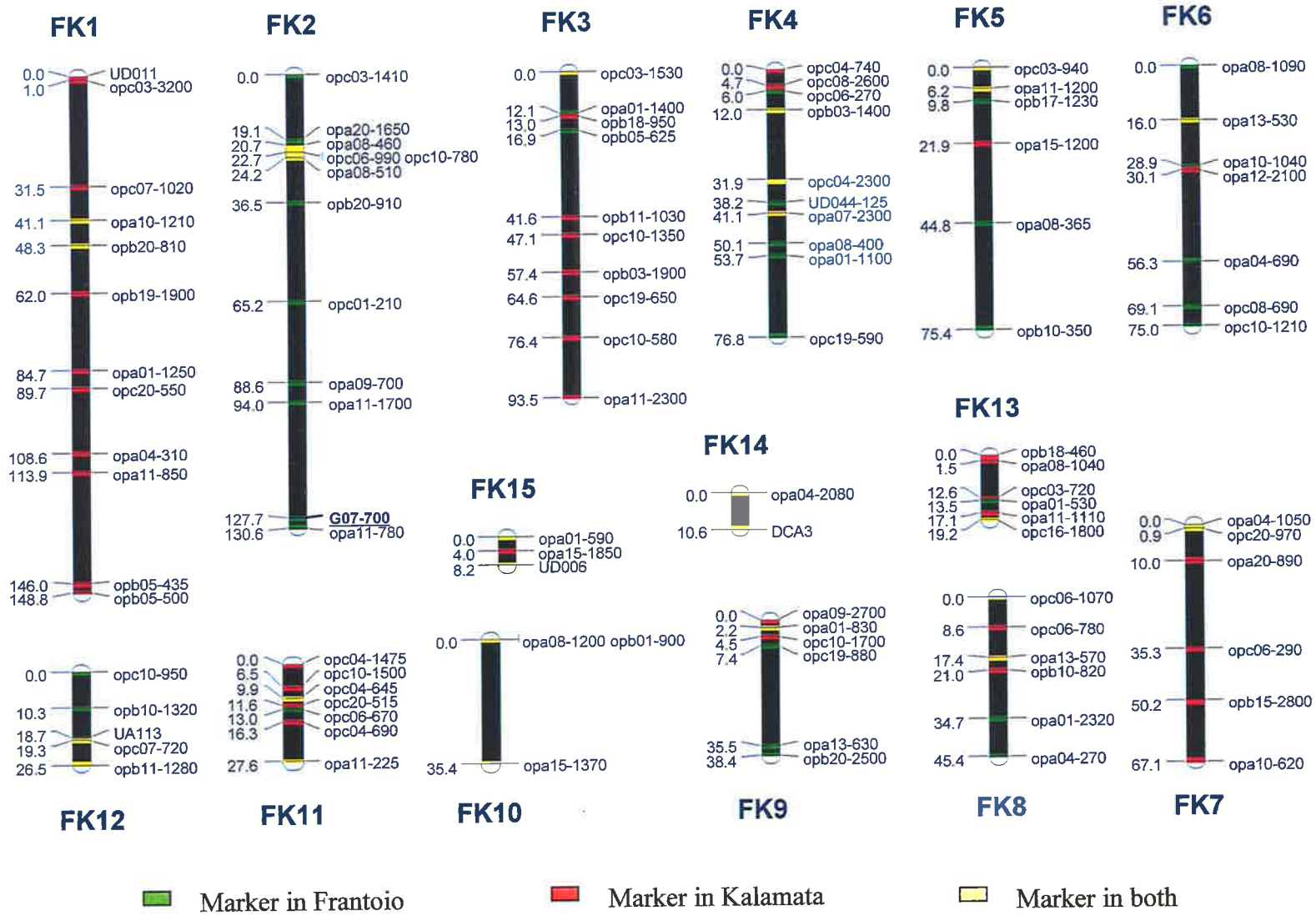

Fig. 7. 15 Integrated linkage map from Frantoio and Kalamata consisting of 15 linkage groups

7.3.9 Estimation of the genome size of olive

The method of Hulbert *et al.* (1988) was used to estimate the genomic sizes of Frantoio and Kalamata as 3427 cM and 2614 cM respectively, based on their partial linkage data. Therefore, for the olive species, the estimated genomic size should be around 3000 cM.

7. 4 Discussion

Baldoni *et al.* (1999) reported linkage maps for the olive cultivars Leccino and Dolce Agogia, but did not produce an integrated map. In this chapter, linkage maps were prepared for Frantoio and Kalamata, based on a combination of RAPD, SCAR, and SSR markers. These two maps were combined to produce the first integrated map for the genus *Olea* and the species *O. europaea* L.

7.4.1 Mapping population development

The olive is a species that shows both self- and cross-incompatibility, with the result that fruit set is often low (Griggs *et al.*, 1975; Rallo *et al.*, 1990). Therefore, to produce the 100 or so F_1 offspring necessary to develop a linkage map, a large number of pollinations was required. Four different crosses were made, involving the pollination of about 3,000 flowers for each. One of these crosses, Frantoio x Kalamata, produced 104 seedlings, whereas the other three produced between 0 and 23 seedlings.

The progeny of the cross between Frantoio and Kalamata is planted at the Roseworthy Campus of the University of Adelaide, 25 km north of Adelaide, South Australia, and will be used as a permanent population for studies into molecular markers for quality traits in olive. At present, the plants are expressing juvenile characters, but eventually important adult agronomic features will be expressed, and these will be added to the linkage map.

7.4.2 Hybridity of the F_1 individuals

It is important to determine that the progeny are hybrids of the parents, and that no stray pollen has been introduced, which may lead to a significant bias in the segregation of the alleles. This was demonstrated in an initial analysis of the segregation data in a mapping program based on *Camellia sinensis* (Hackett *et al.*, 2000), where two extra male parents were identified and the mapping data were reorganised as three separate sets.

In this study, the introduction of stray pollen was controlled by several factors. Firstly, Frantoio is a self-incompatible cultivar, as demonstrated in Chapters three and four, and therefore self pollen does not contribute to false hybrids, secondly, Kalamata pollen had the highest viability of the other pollen used, and thirdly, pollinations were conducted immediately after emasculation, as the highest stigma receptivity was on the day of flower opening (Chapter four), and the flowers were then bagged to keep the pistils from contamination by other pollen.

In the present study, all 104 progeny were shown to be hybrids of Frantoio and Kalamata based on 1. a similarity dendrogram using 300 RAPD markers, 2. nonmetric multidimensional scaling using 300 RAPD markers, and 3. genotype matching based on nine SSR markers. Therefore the results from all progeny were used as a data set.

7.4.3 Characterisation of microsatellites

Microsatellite sequences have been identified in olive, mainly based on the dinucleotides AC, GT, AG, and AT, including 78 that have been reported recently by four different sources (Carriero *et al.*, 2002; Cipriani *et al.*, 2002; Rallo *et al.*, 2000; Sefc *et al.*, 2000). The repeat motifs included perfect, imperfect, and compound, and the motif lengths varied from 8 to 114 bp.

In the present study, a genomic library highly enriched for $(AC)_n$ repeats was prepared from Kalamata, one of the parents of the mapping population, and 30 microsatellite sequences were identified. The motif types included perfect (17%), imperfect (55%), and compound (28%), and the motif lengths varied from 22 to 228 bp. This result diverges from previous reports for olives (Carriero *et al.*, 2002; Cipriani *et al.*, 2002; Rallo *et al.*, 2000; Sefc *et al.*, 2000), where the values for perfect repeats varied from 40 to 60%, for imperfect repeats from 13 to 46%, and for compound repeats from 8 to 27%. In addition, the configuration of the imperfect repeat motifs was more complicated than those reported, as shown in Table 7.5. For example, sub-sections of the repeat sequences were sometimes found to form secondary repeat units within the primary repeat motif, as shown for the sequences F003 and R132. This type of repeat sequence has not been reported previously in olive. Furthermore, the lengths of the repeat motifs were usually more than 60 bp (Tables 7.4, 7.5), whereas in other reports, the lengths were usually less than 60 bp. The complexity of these microsatellite motifs indicates that substitution, insertion, and duplication of sequences (Zhu *et al.*, 2000), leading to the development of intricate tandem repeat sequences, is probably a frequent occurrence in olive genomic DNA.

The efficiency of the enrichment process was 26%, which is similar to that achieved for other tree crops, such as apple (Guilford *et al.*, 1997), *Cocos* (Rivera *et al.*, 1999), *Prunus* (Cipriani *et al.*, 1999), and *Eucalyptus* (Brondani *et al.*, 1998). However, it is relatively high compared to other reports for olive (Cipriani *et al.*, 2002; Rallo *et al.*, 2000), where the enrichment efficiencies were 5% and 6.6%, respectively. Cipriani *et al.* (2002) stated that the low efficiency was due to technical problems that occurred during the enrichment procedure rather than to a low absolute number of repeat motifs in the genome.

Some of the inserts were found to contain the same sequence, which is a common occurrence in the development of microsatellites (Cipriani *et al.*, 2002). The reasons for this include 1. multiple copies of the same DNA fragments were captured during library enrichment and 2. the existence of more than one copy of the microsatellite, with its flanking sequences, in the olive genome. In addition, two groups of inserts shared the same, or very similar, sequences on one side of the microsatellite, whereas the sequences on the other side were heterologous. For example, R025 and F137 showed 100% homology along 378 bp of one flanking sequence, and F120 and F122, showed 98.8% (242 out of 245 bp). One possible explanation for this is that mis-alignment of chromosome segments may occur, although infrequently, at microsatellite sites during mitosis leading to the exchange of the flanking sequences from heterologous chromosomes. Another possible explanation could be the occurrence of allopolyploidy in olive, which has been suggested by Breviglieri and Battaglia (1954) and supported by chromosome C-banding analyses (Falistocco and Tosti 1996).

Four of the sequences flanking the microsatellite regions showed homology with GenBank accessions over lengths that varied from 76 to 230 bp. Three of the these were homologous with the olive accessions, "pOLEU-900", "OLEU-828", and "clone212f", indicating the presence of conserved sequences in the olive genome. The fourth, F120, contained a 177 bp fragment that was homologous to "F20B17" from *Arabidopsis thaliana* (putative kinase predicted mRNA), which may be related to a kinase gene in the olive.

Primer pairs were designed for 23 out of the 30 microsatellite sequences identified, using the rules suggested by Innis and Gelfand (1990). However, only four primer pairs produced bands of the expected size, which is a low efficiency of amplification by designed primers. One of the reasons for this is that the sequences flanking the microsatellite regions often had a high content of A and T, and these sometimes occurred as runs of either A or T. Consequently, the regions for primer design were limited, and often the melting temperatures of the primers were

less than desirable (Table 7.5), leading to the production of non-specific amplification products. Another factor in the low efficiency of amplification could be the relatively large size of the olive genome. Garner (2002) suggested that, as the size of the genomic DNA increases, there is a concomitant decrease in the ability of the target DNA to be amplified, firstly because of the relatively large amount of non-target DNA, and secondly because of dilution of the available primer pool by non-specific binding. The olive has a haploid number of 23 chromosomes (Taylor, 1945) with about 2.2 pg of DNA per haploid nucleus (Rugini *et al.*, 1996), or about 3000 cM. This is a comparatively large genome, compared to the length of 1000 cM for many cultivated plants (Beckman and Soller, 1983).

7.4.4 Trials of some SCAR markers

SCAR markers are considered to be highly specific and transferable, and therefore several of these were tested. The SCAR marker linked to olive peacock disease resistance (Mekuria *et al.*, 2002b) segregated in the mapping population, as did some other unidentified bands that amplified with the same primers. The primers, ConF and ConR, developed by (Channuntapipat *et al.*, 2001) to target self-incompatibility alleles in almond were also tested, because self-incompatibility is reported to be present in olives (Bartolini and Guerriero, 1995; Griggs *et al.*, 1975) These primers were not successful, most likely because of the distance between the two taxa, and the possibility that the self-incompatibility mechanisms in almond and olive are different. The third SCAR marker used was ScoeMS-2, which was reported by (Hernandez *et al.*, 2001a) to indicate a flesh:pit ratio of = 6.2:1. The flesh:pit ratios for Frantoio and Kalamata are 5.1:1 and 8:1 respectively (Dymiotis, 2002). The sequences of the primers were obtained from the authors (Hernandez *et al.*, 2001a), but failed to amplify with either Frantoio or Kalamata. In addition, the primers were tested on several other cultivars, including Manzanillo, which has a flesh:stone ratio of 8.2:1 (Garrido-Fernandez *et al.*, 1997), and was reported to produce a positive response by Hernandez *et al.* (2001a). However, the results were still negative, despite modifying the PCR protocol, purchasing the primers from different companies, and trialling different brands of *Taq* polymerase.

7.4.5 Morphological traits of the population

Morphological characters are important for cultivar selection and for the production of olive products. However, in this study, most of the economically important characters were not available for assessment because the plants in the mapping population were still in their juvenile phase, and only a few leaf characters could be measured. Four of these showed polymorphism,

and two segregated with a Mendelian ratio of 3:1, but because of the possibility of introducing variance into the segregation, they were not included in the linkage groups of Frantoio and Kalamata at this stage.

7.4.6 Distorted segregation of the markers

The dynamics of gene frequencies in diploids depends on two factors, the fitness of the genotypes, and the relative dominance of the various alleles. Fitness is a measure the ability of individuals to survive, reproduce, and transmit their genes to the next generation, and the genetic load is defined as the reduction in fitness from the maximum possible in a population (Excoffier, 2002). With regard to gene flow within a population, the null hypothesis is that heterozygous individuals will transmit their alleles in equal proportions. However, this does not always occur, and there are many examples in plants of unequal transmission probabilities of alleles within a locus, which is referred to as segregation distortion (Baldoni *et al.*, 1999; Levi *et al.*, 2001). Segregation distortion can occur for several reasons, including statistical bias, errors in genotyping and scoring (Plomion *et al.*, 1995), and biological effects such as loss of chromosomes, the presence of lethal genes, non-random union of gametes, self-incompatibility, female choice, zygotic survival, and changes in genetic load (Bradshaw and Stettler, 1994; Excoffier, 2002). In the present study, thirty seven of the 196 markers analysed, or 19.1%, showed segregation patterns skewed from the expected ratio at $\alpha = 0.05$. This level of distorted markers is consistent with the 19.6% reported in the mapping population investigated by Baldoni *et al.* (1999), and is comparable to those reported in other crops, e.g., apple (17.0 and 24.0%) (Conner *et al.*, 1997), chestnut (10.0%) (Casasoli *et al.*, 2001), lentil (10.3%) (Eujayl *et al.*, 1998), and watermelon (25.7%) (Levi *et al.*, 2001). Conner *et al.* (1997) suggested that both pre- and post-zygotic events may be responsible for distorted ratios in apple. Both of the parent cultivars used in the present study, Frantoio and Kalamata, are self-incompatible (Chapters 3, 4; Wu *et al.*, 2002; Wu *et al.*, 2000), and in addition, the fruit set in olives is generally low (Chapter 5; Griggs *et al.*, 1975; Rallo *et al.*, 1990). It is possible that the self-incompatibility mechanism created a stress during the pre-zygotic phase, and that this resulted in an imbalance in the gametes at fertilisation. In addition, the low fruit yield may have resulted in stress during the post-zygotic phase, which would affect the ability of some genotypes to form viable seeds.

In apple and watermelon, most of the distorted markers have a tendency to cluster into specific linkage groups (Conner *et al.*, 1998; Levi *et al.*, 2001). This implies that selection

may be acting against the expression of certain genes on these linkage groups. In the present study, while four groups of distorted markers clustered in linkage group Kal2, and three in Kal10, most of the other distorted markers were distributed more or less evenly across the map. Therefore, the selection of gametes in olive may be more complex than in other plants, and may be related, as previously mentioned, to the features of self-incompatibility (prezygotic selection), and low fruit set (postzygotic selection).

7.4.7 Linkage map construction

RAPD markers have been used extensively in studies of genetic diversity and genotyping of olives (Belaj *et al.*, 2001; Fabbri *et al.*, 1995; Mekuria *et al.*, 1999; Mekuria *et al.*, 2002a; Sanz-Cortes *et al.*, 2001; Vergari *et al.*, 1996; Wiesman *et al.*, 1998), and Grattapaglia and Sederoff (1994) and Nelson *et al.* (1993) showed that the application of RAPD markers is an efficient first step towards establishing a genomic map for plants where little or nothing is known about the sequence of the genomic DNA, such as olive. The RAPD markers used in the present study were chosen on the basis of their reproducibility and the production of good patterns of segregation. They provided a rapid and efficient way to construct the map, and were followed by the addition of SCAR and SSR markers.

SCAR markers have been developed in the olive in recent years (Hernandez *et al.*, 2001a; Hernandez *et al.*, 2001b; Mekuria *et al.*, 2002b), and three were mapped into the linkage groups obtained, although two of them showed distorted segregation ratios. The SCAR marker, G07, which is associated with resistance to peacock disease of olives (Mekuria *et al.*, 2002b), was assigned to linkage group 1 of Frantoio (Fra1), and integrated linkage group 2 (FK2). This provided preliminary information about the location of the gene that confers resistance to peacock disease.

SSR markers are considered to be reliable for developing linkage maps due to their reproducibility and the fact that they are codominant (Rafalski and Tingey, 1993; Staub *et al.*, 1996). Several recent studies on the identification of SSR markers in olives (Carriero *et al.*, 2002; Cipriani *et al.*, 2002; Rallo *et al.*, 2000; Sefc *et al.*, 2000) have included the sequences of the primers and some of these were used in this study to develop the linkage maps. In addition, microsatellite regions in Kalamata were identified and sequenced, and primers were developed to target these for mapping. In all, six SSR loci were mapped, and all showed good segregation at the level of $\alpha = 0.05$. More SSR markers will be added in the future, and they will be useful

to establish reliable correspondence between genetic maps derived from different mapping populations, and as bridge markers for comparative mapping studies.

One other report of a linkage map for olive is available (Baldoni *et al.*, 1999), and this provides limited information about the linkage groups for the cultivars, Leccino and Dolce Agogia, but does not provide an integrated map. In the present study, two comprehensive maps were developed for Frantoio and Kalamata, and one integrated map was produced by combining the data from both parents. This is the first reported integrated map for olive of the author, and covers 879 cM of the genome with an average distance between loci of 10.2 cM. When the markers that could not be integrated are included in the calculation, the total coverage of the genome is 1333 cM .

The number of linkage groups obtained for Kalamata was 23, which is equal to the haploid chromosome of olive, n = 23, while the number of linkage groups obtained for Frantoio was 27. The size of the genomic DNA was estimated (Hulbert and Michelmore, 1988) to be 2614 cM for Kalamata, and 3427 cM for Frantoio. Possible reasons for the difference between the two estimates include 1. the distribution of markers along the chromosomes differs between Frantoio and Kalamata, 2. different recombination frequencies may have occurred for the male and female gametes, 3. statistical and experimental errors within the data. In spite of the difference, a reasonable estimate of the genome size for olive is approximately 3000 cM. By comparison, the haploid chromosome number for humans is also 23, and the genome sizes are 2800 cM for males and 4800 cM for females (Liu, 1998).

The size of the olive genome is greater than that found for many other tree plants. For example, apple n = 17 (Conner *et al.*, 1997), almond n = 8 (Viruel *et al.*, 1995), chestnut n = 12 (Casasoli *et al.*, 2001), cocoa n = 10 (Crouzillat *et al.*, 1996), eucalypt n = 11 (Thamarus *et al.*, 2002), and tea n = 15 (Hackett *et al.*, 2000). This means that considerably more molecular markers are required to obtain a saturated linkage map for olive. Using the equation of Beckman and Soller (1983), and allowing for adjustments to the ends of the chromosomes (Lange and Boehnke, 1982), about 80% of a genome would be mapped to an average distance of 10 cM between markers, given 360 markers and a map length of 3000 cM. For the olive mapping data, 152 markers covered 1333 cM , which would account for about 45% of the genome. Therefore, an additional 200 markers would be needed in order that 80% of the genome would lie within 10 cM of a marker, and 500 markers would be needed to reach 95%.

The mapping population produced by this research program has been planted at the Roseworthy Campus of the University of Adelaide. When the trees pass through their juvenile stage, agronomically important traits, such as flowering time, disease resistance, oil quality, and fruit and oil yields, will be added in the present linkage groups. Once the linkage relationships are established for those traits with molecular markers, the linkage map would be applied to the selection of superior trees in the feral population that is widespread throughout southern Australia, and in future breeding programs. The ultimate benefit will be to increase the efficiency of olive improvement in both Australian and international research programs.

CHAPTER EIGHT

General Discussion

Olea europaea is an ancient cultivated tree crop, which is thought to have been cultivated and traded for its oil by Semitic people in Syria as early as 3000 BC (Zohary 1994). In Australia, however, the olive industry is still relatively undeveloped, despite numerous attempts since the mid 1800s (Collins 1998). After World War II, and the concomitant increase in European migrants, the demand for olive products, particularly oil, greatly increased. Imports now amount to more than A$100 m *per annum*, and the potential profits to be made from olives has stimulated a current investment boom in Australia, as indicated by new plantings of more than 100,000 trees each year across the country (Australian Bureau of Statistics 2001).

However, while investment is increasing, the Australian olive industry is still under pressure from traditional olive growing countries, particularly those around the Mediterranean coast. To be competitive, the Australian industry will need to concentrate on improving and maintaining yield and quality of both fruit and oil by planting combinations of cultivars that are cross-compatible, and by developing cultivars that are well adapted to local environmental conditions. This will be achieved in part by the determining the compatibility relationships between and among the common commercial cultivars, and by the use of molecular markers for quality traits that can be used in plant improvement. The construction of a genetic linkage map would be an important approach to aid marker-assisted selection.

This program aimed at clarifying the self- and cross-compatibility of several commercial cultivars widely grown in Australia, evaluating fruit set, optimising seed germination methods, and constructing a molecular genetic map for olives. These aspects are critical for both olive breeding and the olive industry, and therefore the outcomes of the present project will benefit olive growers as well as researchers, and ultimately contribute to the renaissance of the Australian olive industry.

The olive has been reported to be partially self-incompatible, with some cultivars being self-incompatible, and others self-compatible. Therefore, appropriate pollinators are needed for the cultivars that show self-incompatibility. Although there have been reports on the compatibility status of some olive cultivars (Ateyyeh *et al.* 2000; Bartolini and Guerriero 1995; Griggs *et al.* 1975; Lavee *et al.* 2002), the information is still minimal considering that more than 2000 cultivars are planted world-wide. In Australia, about 200 olive cultivars are

grown commercially (Burr, 1999), and because many of these have been imported from different countries, their cross-compatibility status is unknown. Moreover, in the olive growing regions of Europe, olive groves tend to be relatively small, so that pollen drift between plantings is common, in contrast to the isolated large-scale plantings that are more characteristic of Australian groves. Hence it is likely that pollination problems will arise in Australia, and in other new olive producing countries. Chapters 3, 4, and 5 investigated the self- and cross-compatibility within and between several olive cultivars commonly grown in Australia, and identified the pattern of fruit set.

Because there was no prior knowledge about either pollen germination, or pollen tube growth in the pistil, a preliminary test was undertaken to determine the minimum number of flowers to be assessed for statistical analysis, and also the best time to collect pistils after pollination for pollen tube observation. This preliminary test was carried out in 1999 using three cultivars (Chapter 3). The results showed that 20 flowers were required for each cross, with three trees as replicates, and the best time for the collection of pistils is at day 7 after pollination.

These results were applied in the following year (Chapter 4) with a 5 x 5 diallel matrix using the cultivars 'Frantoio', 'Manzanillo', 'Kalamata', 'Pendolino', and 'Picual'. Since environmental condition, such as weather, are reported to affect pollination in tree crops (Lavee and Datt, 1978; Martin, 1990; Sedgley, 1994), maximum and minimum temperatures and relative humidity, were compared between the year under study and the long-term (73 yr) averages. Temperatures from July to early November, which encompass the period from floral initiation to anthesis, did not deviate appreciably in 2000 from the long-term averages. The relative humidity was higher than the average for September, and although this may have affected some aspects of floral development, anthesis occurred later than this and at the same time in all five cultivars. Therefore it is likely that the results obtained for sex ratio and self- and cross-compatibility presented in this study are representative for the Adelaide area.

With the exception of Manzanillo, the other four cultivars were self-incompatible, indicating that groves consisting of a single cultivar should be avoided by growers when setting up a new olive orchard. However, the results also showed that cross-pollination does not always enhance pollen tube growth in the pistil and penetration to the ovule, because four of the five cultivars showed poor cross-compatibility. These were Kalamata x Manzanillo, Kalamata x Pendolino, Kalamata x Picual, Manzanillo x Pendolino, Manzanillo x Picual, Pendolino x Picual, and the reciprocals. Therefore, appropriate combinations of cultivars must be chosen

for interplanting. Of the five cultivars tested, Frantoio was the best polliniser for the other four, and consequently, it is highly recommended as a polliniser if Kalamata, Manzanillo, Pendolino, and Picual are planted in an orchard.

The assessment of self- and cross-compatibility by measuring the growth of pollen tubes is an important and refined approach in plant species (Sedgley 1994). The method can be applied to estimate fruit set prior to the fruit becoming mature, which is an advantage for olives, since only 1- 4 % of the flowers are able to develop into mature fruits. The use of fruit set in a diallel matrix to investigate compatibility, instead of pollen tubes, would be very tedious. For example, if 20 fruits were required to estimate compatibility, the total pollination of flowers would be 75,000 rather than the 1,500 that were measured using the pollen tube method.

In all, six cultivars were investigated in this study for their self- and cross-compatibility, and other aspects of their reproductive biology. Although they are amongst the most popular cultivars grown in Australian groves, the compatibility of many other cultivars is still to be determined. The results reported in other countries are available as a reference for Australian growers, but differences in environmental conditions can affect compatibility. For example, Frantoio has been reported to be self-compatible in India by Sharma *et al.* (1976), whereas the results presented here for two year's data consistently showed that it is not self-compatible.

Knowledge about the mechanism of self-incompatibility in olive is limited, although the gametophytic type has been proposed (Ateyyeh *et al.*, 2000; Bini and Lensi, 1981; Cuevas and Polito, 1997). However, there is no information about the genetic control of self-incompatibility in olives, and therefore the system of self-incompatibility in olive is still conjecture. In *Prunus*, however, at least 30 gametophytic self-incompatible alleles have been identified genetically and some of the partial genomic sequences have been identified (Channuntapipat *et al.* 2001; 2002). When olive genomic DNA was amplified using primers specific for the self-incompatibility alleles in almond, the result was not successful, possibly because the two self-incompatibility systems are different. Further genetic, reproductive, and molecular biological studies need to be carried out with olive to reveal the underlying mechanism for self-incompatibility.

Apart from sexual compatibility, other aspects of the reproductive biology of olives are important for olive production. Pollen viability, sex ratio, and male sterility are several factors that may affect pollination and therefore fruit set. As demonstrated in Chapter 4, both pollen viability and sex ratio varied between the cultivars that were examined. These characters may

be under genetic control, but are affected also by seasonal and local environmental conditions (Lavee and Datt, 1978; Martin, 1990; Sedgley, 1994), and therefore further investigations are needed to cover a range of seasons.

Male sterility has been reported in olives (Besnard *et al.* 2000; Villemur *et al.* 1984), and is a feature that would lead to lack of pollination for the receptors in the orchards. In this study, SA Verdale was found to be male fertile in 1999 but showed high sterility in 2000, which indicates that seasonal factors probably play an important role in male sterility. Male sterility has not been considered as a problem for the Australian industry, and the precise environmental conditions that induce it need to be determined. Taken in combination with the presence of self-incompatibility, this finding emphasises the need for growers to plant appropriate combinations of cultivars to ensure adequate pollination.

Olives are considered to be wind-pollinated (Griggs *et al.*, 1975; Sibbett *et al.*, 1992), although little is known about the distance that pollen is carried in this way. Further studies on olive pollination should be directed towards acquiring information about this character, and the importance or otherwise of taking into account the direction of prevailing winds in orchard design. This information would help growers to determine the maximum distance between pollinator trees and receptor trees to optimise yields.

All of the cultivars used in the Australian olive industry have been introduced from overseas. While olive improvement and production in other countries is expanding, the Australian industry needs to compete against foreign counterparts by planting high quality olive cultivars, in particular those best suited to the Australian environment. This is a focus of the olive improvement and breeding program at the University of Adelaide (Guerin *et al.* 2000; Sedgley and Wirthenshon 2000).

The germination of olive seeds has been a problem due to the mechanical resistance of the endocarp (Crisosto and Sutter 1985; Sotomayor-Leon and Caballero 1990), seed dormancy (Lagarda and Martin 1983), and the collection of either premature or over-mature seeds (Lagarda *et al.* 1983; Linan *et al.* 1999). The optimisation of seed germination was reported in Chapter 6.

In most cases, olives are multiplied vegetatively, so seed germination is not important for propagation. However, seed germination is an integral part of a breeding program, or to establish the progeny for a mapping population, and three important parameters were

determined for seed germination. Firstly, the seeds were freshly collected, secondly, the endocarp was removed, and thirdly, the seeds were exposed to a chilling period at 10°C. This combination resulted in the germination of 84% of the seeds within 4 weeks.

Huge numbers of wild or "feral" olives cover large areas of southern Australia. Because these are derived from crosses between the world's best varieties, they form a natural gene pool, and the Australian olive improvement program concentrates on selecting the best of these ferals (Sedgley and Wirthenshon 1999). However, because the selection is made on mature trees, and olive has a long juvenile stage up to ten years, it is necessary to develop a quick method to select for desirable quality traits. The determination of gene flow by counting pollen tubes can be made more efficient by using molecular markers to detect paternity under open pollination (Buteler *et al.* 1997). With the increasing availability of these markers in olives, the efficiency of assessing compatibility will be greatly improved. Marker-assisted selection would be the method of choice, and the best approach would be to construct a genetic map that included both molecular and morphological characters. Microsatellite markers seem to be one of the choices for this purpose because of their codominant manner and ease of detection, as demonstrated in Chapter 7.

There is only one report of a genetic linkage map of olive, and that was constructed from the progeny of a cross between Dolce Agogia and Leccino (Baldoni *et al.* 1999). Individual maps were developed for the two parents, but there was no integrated map. In Chapter 7, linkage maps were prepared for Frantoio and Kalamata, based on a combination of RAPD, SCAR, and SSR markers. These two maps were combined to produce the first integrated map for the genus *Olea* and the species *O. europaea* L. The progeny from the cross are planted at the Roseworthy Campus of the University of Adelaide, 25 km north of Adelaide, South Australia, and will be used as a permanent population for studies into molecular markers for quality traits in olive. The hybridity of the mapping population was confirmed by genetic similarity using 300 RAPD markers, nonmetric multidimensional scaling using 300 RAPD markers, and genotype matching based on nine SSR markers. For the RAPD markers, which are dominant, and after accounting for those that were polymorphic between the parents but not between the progeny, and vice versa, 178 were used for linkage analysis. 117 loci that were heterozygous in one parent and homozygous null in the other segregated with a ratio of 1 : 1, and 61 loci that were heterozygous in both parents segregated with a ratio of 3 : 1.

For the microsatellite data, which are codominant, there were two different sources. Six sets of primers were derived from published sequences (Cipriani *et al.* 2002; Sefc *et al.* 2000), and four sets were prepared from the genomic DNA from Kalamata (Chapter 7). In addition, three different SCAR markers were tested, but only the peacock spot disease-resistance primers reported by Mekuria *et al.* (2002) were successful, and this marker was successfully mapped on linkage group 2 of the integrated map. In all, 194 molecular markers were used to construct the linkage maps.

Twenty-three linkage groups were obtained for Kalamata and 27 for Frantoio, which is in accordance with the haploid chromosome of olive of 23 (Taylor 1945), and the sizes of the genomic DNAs were estimated to be 2614 cM and 3427 cM respectively. It is estimated that the 152 markers used would cover 1333 cM, and this would account for about 45% of the genome. Therefore, an additional 200 markers would be needed in order that 80% of the genome would lie within 10 cM of a marker, and 500 markers would be needed to reach 95%. When the progeny pass through their juvenile phase and adopt their adult characters, the morphological markers will be added. If the self-incompatibility system in olive could be determined, and the locus placed on the linkage map, identification and isolation of SI gene(s) in the olive would be possible.

This program has contributed to four areas of significance for the improvement of olives that will benefit both the industry in Australia and other olive growing countries. The areas are firstly, sexual compatibility within and between olive cultivars, secondly, fruit set, thirdly, seed germination, and fourthly, the molecular linkage mapping. The information provided by this research program has filled some deficiencies in the knowledge of the breeding, floral biology, and genetics of pollination of olives, and has enabled certain areas to be highlighted where research should be directed in the future.

Bibliography

Acebedo, M. M., S. Lavee, J. Linan, A. Troncoso, 1997 *In vitro* germination of embryos for speeding up seedling development in olive breeding programmes. *Scientia Horticulturae* **69:** 207-215.

Adamblondon, A. F., M. Sevignac, H. Bannerot, M. Dron, 1994 Scar, RAPD and RFLP markers linked to a dominant gene (are) conferring resistance to anthracnose in common bean. *Theoretical & Applied Genetics* **88**: 865-870

Androulakis, I. I., M. H. Loupassaki, 1990 Studies on the self-fertility of some olive cultivars in the area of Crete. *Acta Horticulturae* **286:** 159-162.

Angiolillo, A., M. Mencuccini, L. Baldoni, 1999 Olive genetic diversity assessed using amplified fragment length polymorphisms. *Theoretical and Applied Genetics* **98:** 411-421.

Antognozzi, E., A. Standardi, 1978 A study of flowering biology in the olive cvs Gentile di Chieti and Dritta di Moscufo. *Rivista della OrtofloroFrutticoltura Italiana* **62:** 461-469.

Araki, S., I. Washitani, 2000 Seed dormancy/germination traits of seven *Persicaria* species and their implication in soil seed-bank strategy. *Ecological Research* **15:** 33-46.

Ateyyeh, A. F., Stosser, R., Qrunfleh, M. 2000 Reproductive biology of the olive (*Olea europaea* L.) cultivar 'Nabali Baladi'. *Journal of Applied Botany Angewandte Botanik* **74,** 255-270.

Australian Bureau of Statistics, 2001 *Agriculture Australia 1999-2000.* Australian Bureau of Statistics, Canberra.

Baldoni, L., A. Angiolillo, M. Pellegrini, M. Mencuccini, I. T. Metzidakis, *et al.*, 1999 A linkage genome map for olive as an important tool for marker-assisted selection. *Acta Horticulturae* **474:** 111-115.

Baratta, B., T. Caruso, P. Inglese, 1985 Three years of study on the floral biology of the olive cultivar Cerasuola. *Rivista della OrtofloroFrutticoltura Italiana.* **69:** 375-384.

Barreneche, T., C. Bodenes, C. Lexer *et al.*, 1998 A genetic linkage map of *Quercus robur* L. (pedunculate oak) based on RAPD, SCAR, microsatellite, inisatellite, isozyme and 5S rDNA markers. *Theoretical and Applied Genetics* **97:** 1090-1103.

Bartolini, S., R. Guerriero, 1995 Self-compatibility in several clones of oil olive cv. Leccino. *Advances in Horticultural Science* **9:** 71-74.

Bartolini, G., C. Messeri, G. Prevost, S. Lavee, I. Klein, 1994 Olive tree germplasm: descriptor lists of cultivated varieties in the world. *Acta Horticulturae* **356:** 116-118.

Bartolini, G., G. Prevost, C. Messeri, G. Carignani, 1999 Olive cultivar names and synonyms and collections detected in a literature review. *Acta Horticulturae* **474:** 159 - 162.

Bartolini, S., R. Viti, R. Guerriero, 2000 Observations on fertilization process in self-pollinated flowers of cv Leccino. *Proceedings of Fourth International Symposium on Olive Growing*, **1:** 374.

Bassam, B., G. Caetano-Anolles, P. Gresshoff, 1991 Fast and sensitive silver staining of DNA in polyacrylamide gels. *Analytical Biochemistry* **196:** 80-83.

Beckman, J., M. Soller, 1986 Restriction fragment length polymorphism and genetic improvement of agricultural species. *Euphytica* **35:** 111-124.

Beckmann, J., M. Soller, 1983 Restriction fragment length polymorphism in genetic improvement: methodologies, mapping and costs. *Theoretical and Applied Genetics* **67:** 35-43.

Bell, P. R., 1995 Incompatibility in flowering plants - Adaptation of an ancient response. *Plant Cell* **7:** 5-16.

Beloj, A., I. Trujillo, R. D. L. Rosa, L. Rallo, 1999 Selection of RAPD markers for olive cultivar identification. *Australian Olive Grower* **10:** 27-28.

Besnard, G., A. Berville, 2002 On chloroplast DNA variations in the olive (*Olea europaea* L.) complex: comparison of RFLP and PCR polymorphisms. *Theoretical & Applied Genetics* **104:** 1157-1163.

Besnard, G., B. Khadari, P. Villemur, A. Berville, 2000 Cytoplasmic male sterility in the olive (*Olea europaea* L.). *Theoretical and Applied Genetics* **100:** 1018-1024.

Bini, G., 1984 Flowering and pollination in olive. Studies on the pollination period, stigma receptivity, and development of the female gametophyte. *Rivista della Ortofloro Frutticoltura Italiana* **68:** 57-69.

Bini, G., M. Lensi, 1981 Observations on various aspects of floral ontogeny in the olive. *Rivista della OrtofloroFrutticoltura Italiana* **65:** 371-380.

Black, J. M., E. L. Robertson, 1980 *Flora of South Australia. Part IV. Oleaceae - Compositae*, 2nd Ed. Government Printer, South Australia, Adelaide.

Bogani, P., D. Cavalieri, R. Petruccelli, L. Polsinelli, G. Roselli *et al.*, 1994 Identification of olive tree cultivars by using random amplified polymorphic DNA. *Acta Horticulturae* **356:** 98-101.

Booth, N., G. Davies, 1996 *Olive Growth in South Australia.* Primary Industries South Australia, Adelaide, South Australia.

Bostein, D., R. White, M. Skolnick, R. Davies, 1980 Construction of a genetic map in man using restriction fragment length polymorphism. *American Journal of Human* **32:** 314-331.

Bouranis, D. L., C. K. Kitsaki, S. N. Chorianopoulou, G. Aivalakis, J. B. Drossopoulos, 1999 Nutritional dynamics of olive tree flowers. *Journal of Plant Nutrition* **22:** 245-257.

Bradshaw, H. D., R. F. Stettler, 1994 Molecular genetics of growth and development in populus .2. segregation distortion due to genetic load. *Theoretical & Applied Genetics* **89:** 551-558.

Brhadda, N., D. El Macane, W. Loudyi, A. Abousalim, D. Benali, 2000 Effect of temperature and endosperm on the dormancy and germination of olive embryos, *Olea europaea* L. Moroccan Picholine Variety. *Agronomie* **20:** 643-653.

Breviglieri N., E. Battaglia 1954 Ricerche cariologiche in *Olea europea* L. *Caryologia* **6**: 271–283.

Brondani, R. P. V., C. Brondani, R. Tarchini, D. Grattapaglia, 1998 Development, characterization and mapping of microsatellite markers in *Eucalyptus grandis* and *E. urophylla*. *Theoretical & Applied Genetics* **97:** 816-827.

Brooks, R. M., 1948 Seasonal incidence of perfect and staminate olive flowers. *Proceedings of American Society of Horticultural Science* **52:** 213-218.

Burr, M. 1999 *Australian olives: A guide for growers and producers of virgin olive oils.* M. Burr, Beetaloo Valley, South Australia.

Buteler, M. I., D. R. Labonte, R. E. Macchiavelli, 1997 Determining paternity in polyploids - hexaploid simulation studies. *Euphytica* **96:** 353-361.

Cai, Q., C. L. Guy, G. A. Moore, 1994 Extension of the linkage map in *Citrus* using random amplified polymorphic DNA (RAPD) markers and RFLP mapping of cold-acclimation-responsive loci. *Theoretical and Applied Genetics* **89:** 606-614.

Carriero, F., G. Fontanazza, F. Cellini, G. Giorio, 2002 Identification of simple sequence repeats (SSRs) in olive (*Olea europaea* L.). *Theoretical & Applied Genetics* **104:** 301-307.

Casasoli, M., C. Mattioni, M. Cherubini, F. Villani, 2001 A genetic linkage map of European chestnut (*Castanea sativa* Mill.) based on RAPD, ISSR and isozyme markers. *Theoretical & Applied Genetics* **102:** 1190-1199.

Carles, L., 1983 Intensive olive culture. *L'Arbor Fruit* **30:** 38-42.

Caruso, T., D. Giovannini, F. P. Marra, 1993 Reproductive and vegetative behavior of four table olive cultivars. *Fruit Varieties Journal* **47:** 109-114.

Channuntapipat, C., M. Sedgley, G. Collins, 2001 Sequences of the cDNAs and genomic DNAs encoding the S1, S7, S8, and Sf alleles from almond, *Prunus dulcis*. *Theoretical & Applied Genetics* **103:** 1115-1122.

Chareonboonsit, S.W. E. Splittstoesser, 1984 Pollination, fruit-set and parthenocarpy in 'Severianin' tomato. *Plant Physiology* **75 (Supplement):** 136

Ciampolini, F., M. Cresti, R. N. Kapil, 1983 Fine structural and cytochemical characteristics of style and stigma in olive. *Caryologia* **36:** 211-230.

Cipriani, G., G. Lot, W. G. Huang *et al.*, 1999 AC/GT and AG/CT microsatellite repeats in peach. *Theoretical and Applied Genetics* **99:** 65-72.

Cipriani, G., M. T. Marrazzo, R. Marconi, A. Cimato, R. Testolin, 2002 Microsatellite markers isolated in olive (*Olea europaea* L.) are suitable for individual fingerprinting and reveal polymorphism within ancient cultivars. *Theoretical & Applied Genetics* **104:** 223-228.

Collins, G., 1998 The South Australian olive breeding program, pp.117-119 in *Breeding and Biotechnology for Fruit Trees*, edited by M. Omura, T. Hayashi and N. S. Scott. NIFTS / CSIRO, Tatura.

Collins, G. G., R. H. Symons, 1993 Polymorphisms in grapevine DNA detected by the RAPD PCR technique. *Plant Molecular Biology Reporter* **11:** 105-112.

Conner, P. J., S. K. Brown, N. F. Weeden, 1997 Randomly amplified polymorphic DNA-based genetic linkage maps of three apple cultivars. *Journal of American Society for Horticultural Science* **122:** 350-359.

Conner, P. J., S. K. Brown, N. F. Weeden, 1998 Molecular-marker analysis of quantitative traits for growth and development in juvenile apple trees. *Theoretical & Applied Genetics* **96:** 1027-1035.

Cresti, M., H. F. Linskens, D. L. Mulcahy, S. Bush, V. d. Stilio *et al.*, 1997 Preliminary communication about the identification of DNA in leaves and in olive oil of *Olea europaea*. *Olivæ* **69:** 36-37.

Crisosto, C., E. Sutter, 1985a Improving 'Manzanillo' olive seed germination. *HortScience* **20:** 100-102.

Crisosto, C., E. G. Sutter, 1985b Role of the endocarp in Manzanillo olive seed germination. *Journal of American Society for Horticultural Science* **110:** 50-52.

Cristofani, M., M. A. Machado, D. Grattapaglia, 1999 Genetic linkage maps of *Citrus sunki* Hort. ex. Tan. and *Poncirus trifoliata* (L.) Raf. and mapping of citrus tristeza virus resistance gene. *Euphytica*. **109:** 25-32.

Cronquist, A., 1981 *An Integrated System of Classification of Flowering Plants*. Columbia University Press, New York.

Crouzillat, D., E. Lerceteau, V. Petiard, J. Morera, H. Rodriguez, *et al.*, 1996 *Theobroma cacao* L - A genetic linkage map and quantitative trait loci analysis. *Theoretical & Applied Genetics* **93:** 205-214.

Cruzan, M. B. 1989 Pollen tube attrition in *Erythronium grandiflorum*. *American Journal of Botany* **76:** 562-570.

Cuevas, J., A. J. Diaz-Hermoso, D. Galian, J. J. Hueso, V. Pinillos *et al.*, 2001 Response to cross pollination and choice of pollinisers for the olive cultivars (*Olea europaea* L.) 'Manzanilla de Sevilla', 'Hojiblanca' and 'Picual'. *Olivæ* **85:** 26 - 32.

Cuevas, J., V. S. Polito, 1997 Compatibility relationships in 'Manzanillo' olive. *HortScience* **32:** 1056-1058.

Cuevas, J., L. Rallo, 1990 Response to cross-pollination in olive trees with different levels of flowering. *Acta Horticulturae*, **286:**179-182.

Cuevas, J., L. Rallo, H. F. Rapoport, 1994a Crop load effects on floral quality in olive. *Scientia Horticulturae* **59:** 123-130.

Cuevas, J., L. Rallo, H.F. Rapoport, 1994b Initial fruit set at high temperature in olive, *Olea europaea* L. *Journal of Horticultural Science* **69**:665-672.

Cuevas, J., L. Rallo, H. F. Rapoport, S. Lavee, I. Klein, 1994 Staining procedure for the observation of olive pollen tube behaviour. *Acta Horticulturae* **356:** 264-267.

Cuevas, J., H. F. RapoportL. Rallo, 1995 Relationships among reproductive processes and fruitlet abscission in 'Arbequina' olive. *Advances in Horticultural Science* **9:** 92-96.

de Caraffa, V. B., J. Maury, C. Gambotti, C. Breton, A. Berville, *et al.*, 2002 Mitochondrial DNA variation and RAPD mark oleasters, olive and feral olive from Western and Eastern Mediterranean. *Theoretical & Applied Genetics* **104:** 1209-1216.

de Fossard, R. A., 1976 *Tissue Culture for Plant Propogation*. Univerisity of New England, Armidale, Australia.

de Nettancourt, D., 1977 *Incompatibility in Angiosperms*. Springer-Verlag, Berlin.

de Nettancourt, D., 1997 Incompatibility in Angiosperms. *Sexual Plant Reproduction* **10:** 185-199.

Del-Tredici, P., 1987 Lost and found: *Elliottia racemosa*. *Arnoldia, USA* **47:** 2-8.

Deng, Z., N., S. Huang, S. Y. Xiao, F. G. Gmitter (1997) Development and characterization of scab markers linked to the citrus tristeza virus resistance gene from *Poncirus trifoliata*. *Genome* **40**:697-704.

Dimassi, K., Therios, I., Balatsos, A., Metzidakis, I. T., Voyiatzis, D. G. (1999). The blooming period and self-fruitfulness in twelve Greek and three foreign olive cultivars. *Acta Horticulturae* **474:** 275-278.

Dionigi, A., 1973 The biology and *Genetics* of the olive. *Frutticoltura* **35:** 3-7.

Dirlewanger, E., V. Pronier, C. Parvery *et al.*, 1998 Genetic linkage map of peach. *Theoretical and Applied Genetics* **97:** 888-895.

Dirlewanger, E., C. Bodo, 1994 Molecular genetic mapping of peach. *Euphytica* **77:** 101-103.

Don, R., P. Cox, B. Wainwright, K. Baker, J. Mattick, 1991 Touchdown PCR to circumvent spurious priming during gene amplification. *Nucleic Acids Research* **19:** 4008.

Donini, B., P. Mannino, G. Roselli, 1982 A compact mutant variety of olive induced by mutation. *Mutation Breeding Newsletter* **19:** 3.

Dubcovsky, J., M. Luo, G. Zhong, R. Bransteitter, A. Desai *et al.*, 1996 Genetic map of diploid wheat, *Triticum monococcum* L., and its comparison with maps of *Hordeum vulgare* L. *Genetics* **143:** 983-999.

Dymiotis, M., 2002 Table olive processing.http://www.olivebusiness.com/OBGuest/MDym/Table%20Olive%20Processing.pdf

Eujayl, I., M. Baum, W. Powell, W. Erskine, E. Pehu, 1998 A genetic linkage map of lentil (*Lens sp.*) based on RAPD and AFLP markers using recombinant inbred lines. *Theoretical and Applied Genetics* **97:** 83-89.

Excoffier, L. 2002 Introduction to Population Genetics (5). Selection models. Computation and Molecular Population Genetics Lab (CMPG), Zoological Institute, Baltzerstrasse 6, 3012 Bern. http://www.cmpg.unibe.ch.

Fabbri, A., J. I. Hormaza, V. S. Polito, 1995 Random amplified polymorphic DNA analysis of olive (*Olea europaea* L.) cultivars. *Journal of American Society for Horticultural Science***120:** 538-542.

Fall A., L., P. F. Byrne, G. Jung , D. P. Coyne , M. A. Brick , H. F. Schwartz, 2001 Detection and mapping of a major locus for *Fusarium* Wilt resistance in common bean. *Crop Science* **41**:1494-1498.

Falistocco E., N.Tosti 1996 Cytogenetic investigation in *Olea europaea* L. *Journal of Genetics and Breeding* 50:235–238.

Fang, D. Q., C. T. Federici, M. L. Roose, 1997 Development of molecular markers linked to a gene controlling fruit acidity in citrus. *Genome* **40:** 841-849.

Fernandez, E. R., G. G Valledor, 1985 Cross-pollination in 'Gordal Sevillana' olives. *HortScience* **20:** 191-192.

Finneseth, C. H., D. R. Layne, R. L. Geneve, 1998 Requirements for seed germination in North American pawpaw (*Asimina triloba* (L.) Dunal). *Seed Science & Technology* **26:** 471-480.

Fontanazza, G., L. Baldoni, 1990 Proposed programme for the genetic improvement of the olive. *Olivæ* **34:** 32-40.

Fuss, A. M., M. Sedgely, 1991 Pollen tube growth and seed set of *Banksia coccinea* R.Br. (Proteaceae). *Annals of Botany* **68:** 377-384.

Gardiner, S. E., J. M. Zhu, H. C. M. Whitehead, 1994 The New Zealand apple genome mapping project - a progress report. *Euphytica* **77:** 77-81.

Garner, T. W. J., 2002 Genome size and microsatellites: the effect of nuclear size on amplification potential. *Genome* **45:** 212-215.

Garrido-Fernandez, A., M. Fernandez-Diez, M. Adams, 1997 *Table olives : production and processing*. Chapman & Hall, LondonM.R.

Ghrisi, N., B. Boulouha, M. Benichou, S. Hilali, 1999 Agro-physiological evaluation of the phenomenon of pollen compatibility in olive. Case of the Mediterranean collection at the Menara Station, Marrakech. *Olivae* **79:** 51-59.

Gianfranceschi, L., N. Seglias, R. Tarchini, M. Komjanc, C. Gessler, 1998 Simple sequence repeats for the genetic analysis of apple. *Theoretical and Applied Genetics* **96:** 1069-1076.

Gibbs, P. E., M. Bianchi, 1993 Post-pollination events in species of *Chorisia* (Bombacaceae) and *Tabebuia* (Bignoniaceae) with late-acting self-incompatibility. Bot Acta **106:** 64-71.

Grattapaglia, D., R. Sederoff, 1994 Genetic linkage maps of *Eucalyptus grandis* and *Eucalyptus urophylla* using a pseudo-testcross - mapping strategy and RAPD markers. *Genetics* **137:** 1121-1137.

Griffing, B., 1956 Concept of general and specific combining ability in relation to diallel crossing systems. *Australian Journal of Biological Sciences* **9:** 463-493.

Griggs, W. H., H. T. Hartmann, M. V. Bradley, B. T. Iwakiri, J. E. Whisler, 1975 Olive pollination in California. *California Agricultural Experiment Station Bulletin* **869:** 1-49.

Guilford, P., S. Prakash, J. M. Zhu, E. Rikkerink, S. Gardiner, *et al.*, 1997 Microsatellites in *Malus* x *Domestica* (Apple) - Abundance, polymorphism and cultivar identification. *Theoretical & Applied Genetics* **94:** 249-254.

Guerin, J., G. Collins, M. Sedgley, 2000 Selection and breeding of olive cultivars. *Outlook on Agriculture* **29:** 269-274.

Gupta, P. K., R. K. Varshney, P. C. Sharma, B. Ramesh, 1999 Molecular markers and their applications in wheat breeding. *Plant Breeding. Nov.* **118:** 369-390.

Hackett, C. A., F. N. Wachira, S. Paul, W. Powell, R. Waugh, 2000 Construction of a genetic linkage map for *Camellia sinensis* (tea). *Heredity* **85:** 346-355.

Hartmann, H. T., R. M. Hoffman, 1953 Olive fruit behavior. *California Agriculture* **7:** 9-10.

Hartmann, H. T., C. Panetsos, 1962 Effect of soil moisture deficiency during floral development on fruitfulness in the olive. *Proceedings of American Society for Horticultural Science* **78:** 209-217.

Helentjaris, T., G. King, M. Slocum, C. Sidenstrang, S. Wegmen, 1985 Restriction fragment polymorphisms as probes for plant diversity and their development as tool for applied plant breeding. *Plant Molecular Biolology* **5:** 109-118.

Hemmat, M., N. F. Weeden, P. J. Conner, S. K. Brown, 1997 A DNA marker for columnar growth habit in apple contains a simple sequence repeat. *Journal of American Society for Horticultural Science* **122:** 347-349.

Hemmat, M., N. F. Weeden, A. G. Manganaris, D. M. Lawson, 1994 Molecular Marker Linkage Map For Apple. *Journal of Heredity* **85:** 4-11.

Hernandez, P., R. de la Rosa, L. Rallo, G. Dorado, A. Martin, 2001a Development of SCAR markers in olive (*Olea europaea*) by direct sequencing of RAPD products: applications in olive germplasm evaluation and mapping. *Theoretical & Applied Genetics* **103:** 788-791.

Hernandez, P., R. de la Rosa, L. Rallo, A. Martin, G. Dorado, 2001b First evidence of a retrotransposon-like element in olive (*Olea europaea*): implications in plant variety identification by SCAR-marker development. *Theoretical & Applied Genetics* **102:** 1082-1087.

Herrero, M., J. I. Hormaza, 1996 Pistil strategies controlling pollen tube growth. *Sexual Plant Reproduction* **9:** 343-347.

Heslop-Harrison, Y., K. R. Shivanna, 1977 The receptive surface of the angiosperm stigma. *Annals of Botany* **41:** 1233-1258.

Hulbert, S. H., R. W. Michelmore, 1988 DNA restriction fragment length polymorphism and somatic variation in the lettuce downy mildew fungus, *Bremia lactucae*. *Molecular Plant Microbe Interactions* **1:** 17-24.

Iannotta, N., C. B. Bati, L. Perri, C. Tocci, I. T. Metzidakis *et al.*, 1999 Interfertility tests using different pollinizers for the Carolea cultivar (*Olea europaea* L.). *Acta Horticulturae* **474:** 237-239.

Iannotta, N., L. Perri, F. Zaffina, 1996 Studies on the floral biology of Carolea (*Olea europaea* L.) in different areas of Calabria. pp 399-407 in *Atti del Convegno*, edited by N. Lombardo, N. Iannotta and C. B. Bati. Istituto Sperimentale per l'Olivicoltura, Cosenza, Italy.

Inglese, P., E. Barone, G. Gullo, 1996 The effect of complementary irrigation on fruit growth, ripening pattern and oil characteristics of olive (*Olea europaea* L) cv Carolea. *Journal of Horticultural Science* **71:** 257-263.

Innis, M., D. Gelfand, 1990 Optimization of PCRs, 3-12 in *PCR protocols: a guide to methods and applications*, edited by M. A. Innis, D. H. Gelfand, J. J. Sninsky, *et al.* Academic Press, New York.

Istanbouli, A., P. Neville, 1977 A study of the dormancy of olive (*Olea europea*) seeds. The favourable influence of light in the presence of obstacles to germination. *Comtes-Rendus-Hebdomadaires-des-Seances-de-l'Academie-des-Sciences,-D* **285:** 41-44.

Jermstad, K. D., D. L. Bassoni, N. C. Wheeler, D. B. Neale, 1998 A sex-averaged genetic linkage map in coastal douglas-fir (*Pseudotsuga menziesii*). *Theoretical & Applied Genetics* **97:** 762-770.

Jones, A. G., C. A. Stockwell, D. Walker, J. C. Avise, 1998 The molecular basis of a microsatellite null allele from the white sands pupfish. *Journal of Heredity* **89:** 339-342.

Kar, P. L., R. P. Singh, 1984 Pollination and compatibility studies in some olive cultivars. *Himachal Journal of Agricultural Research* **10:** 55-57.

Kosambi, D., 1944 The estimation of map distances from recombination values. *Annals of Eugenics* **12:** 172-175.

Lagarda, A., G. C. Martin, 1983 'Manzanillo' olive seed dormancy as influenced by exogenous hormone application and endogenous abscisic acid concentration. *HortScience* **18:** 869-871.

Lagarda, A., G. C. Martin, D. E. Kester, 1983 Influence of environment, seed tissue, and seed maturity on 'Manzanillo' olive seed germination. *HortScience* **18:** 868-869.

Lagercrantz, U., H. Ellegren, L. Andersson, 1993 The abundance of various polymorphic microsatelitte motifs differ betweeen plants and vertebrate. *Nuclear Acids Research* **21:** 1111-1115.

Lambardi, M., L. M. R. Rinaldi, D. Menabeni, A. Cimato, S. Lavee, *et al.*, 1994 Ethylene effect on "in vitro" olive seed germination (*Olea europaea* L.). *Acta Horticulturae* **356:** 54-57.

Lande, R., 1991 Marker-assisted selection in relation to traditional methods of plant breeding, 437-451 in *Plant breeding in the 1990s*, edited by H. Stalker and J. Murphy. CAB Intl., Wallingford, U.K.

Lange, K., M. Boehnke, 1982 How many polumorhpic genes will it take to span the human genome? *American Journal of Human Genetics* **34:** 842-845.

Lavee, S., 1990 Aims, methods, and advances in breeding of new olive (*Olea europaea* L.) cultivars. *Acta Horticulturae* **286:** 23-36.

Lavee, S., Z. Datt, 1978 The necessity of cross-pollination for fruit set of Manzanillo olives. *Journal of Horticultural Science* **53:** 261-266.

Lavee, S., L. Rallo, H. F. Rapoport , A. Troncoso, 1996 The floral biology of the olive: effect of flower number, type and distribution on fruitset. *Scientia Horticulturae* **66:** 149-158.

Lavee, S., L. Rallo, H. F. Rapoport, A. Troncoso, 1999 The floral biology of the olive. II. The effect of inflorescence load and distribution per shoot on fruit set and load. *Scientia Horticulturae* **82:** 181-192.

Lavigne, C., A. Mignot, J. Stocklin, 1999 Genetic variation in the response of pollen germination to nutrient availability and elevated atmospheric CO_2 concentrations in *Epilobium angustifolium*. *International Journal of Plant Sciences* **160:** 109-115.

Lefort, F., S. Brachet, N. Frascaria-Lacoste, K. J. Edwards, G. C. Douglas, 1999 Identification and characterization of microsatellite loci in ash (*Fraxinus excelsior* L.) and their conservation in the olive family (Oleaceae). *Molecular Ecology* **8:** 1088-1090.

Lefort, P., N. Legisle, 1977 Quantitative stock-scion relationships in vine. Preliminary investigations by the analysis of reciprocal graftings. *Vitis* **16:** 149-61.

Legesse, N., 1993 Investigations on the germination behavior of wild olive seeds and the nursery establishment of the germinants. *Sinet, an Ethiopian Journal of Science* **16:** 71-81.

Levi, A., C. E. Thomas, X. P. Zhang, T. Joobeur, R. A. Dean, *et al.*, 2001 A genetic linkage map for watermelon based on randomly amplified polymorphic DNA markers. *Journal of the American Society for Horticultural Science* **126:** 730-737.

Linan, J., A. Troncoso, H. F. Rapoport, I. T. Metzidakis, D. G. Voyiatzis, 1999 Olive embryo development stage and the possibility of obtaining viable seedlings. *Acta Horticulturae* **474:** 75-78.

Litt M., J. A. Luty, 1989 A hypervariable microsatellite revealed by *in vitro* amplification of a dinucleotide repeat within the cardiac muscle actin gene. *American Journal of Human Genetics* **44:** 397-401.

Liu, B., 1998 *Statistical genomics : linkage, mapping, and QTL analysis*. CRC Press, Boca Raton.

Loukas, M., and C. A. Pontikis, 1981 Is the phenotypic variation observed within cultivars of *Olea europaea* L. reflected in isozyme banding patterns? *Journal of Horticultural Science* **56:** 377-379.

Maliepaard, C., F. H. Alston, G. v. Arkel *et al.*, 1998 Aligning male and female linkage maps of apple (*Malus pumila* Mill.) using multi-allelic markers. *Theoretical and Applied Genetics* **97:** 60-73.

Marco, L. d., D. Giovannini, F. P. Marra, G. Viglianisi, L. Di Marco, 1990 Reproductive and vegetative behaviour of four table-olive cultivars. *Acta Horticulturae* **286:** 187-190.

Martin, G. C., 1990 Olive flower and fruit population dynamics. *Acta Horticulturae* **No. 286:** 141-153.

Martin, F. W., 1959 Staining and observing pollen tubes in the style by means of fluorescence. *Stain Technology* **34:** 125-8.

Masuda, M., M. Maki, T. Yahara, 1999 Effects of salinity and temperature on seed germination in a Japanese endangered halophyte *Triglochin maritimum* (Juncaginaceae). *Journal of Plant Research* **112:** 457-461.

McCclean, P., 1998 Mapping quantitative trait loci. http://www.ndsu.nodak.edu/instruct/mcclean/plsc731/quant/index.htm.

McGregor, S. E., 1976 *Insect pollination of cultivated crop plants*. USDA, Washington.

Mekuria, G. T., G. Collins, M. Sedgley, 2002a Genetic diversity within an isolated olive (*Olea europaea* L.) population in relation to feral spread. *Scientia Horticulturae* **94:** 91-105.

Mekuria, G. T., M. Sedgley, G. Collins, S. Lavee, 2002b Development of a sequence-tagged site for the RAPD marker linked to leaf spot resistance in olive. *Journal of the American Society for Horticultural Science* **127:** 673-676.

Mekuria, G. T., G. Collins, M. Sedgley, S. Lavee, 2001 Identification of genetic markers in olive linked to olive leaf spot resistance and susceptibility. *Journal of the American Society for Horticultural Science* **126:** 305-308.

Mekuria, G. T., G. G. Collins, M. Sedgley, 1999 Genetic variability between different accessions of some common commercial olive cultivars. *The Journal of Horticultural Science & Biotechnology* **74:** 309-314.

Mitrakos, K., S. Diamantoglou, 1984 Endosperm dormancy breakage in olive seeds. *Physiologia Plantarum* **62:** 8-10.

Morettini, A., G. Bini, E. Bellini, 1972 The performance of several French and Spanish table olive cultivars in the Maremma district of Tuscany. *Rivista della OrtofloroFrutticoltura Italiana* **56:** 3-19.

Morgante, M., A. Olivieri, 1993 PCR-amplified microsatellites as markers in plant *Genetics. Plant Journal* **3:** 175-182.

Morgante, M., M. Vogel, 1999 Compound microsatellite primers for the detection of genetic polymorphisms. U.S. Patent and Trademarker Office PN: 5,955,276.

Murashige, T., F. Skoog, 1962 A revised medium for rapid growth and bioassays with tobacco tissue culture. *Physiologia Plantarum* **15:** 473-497.

Nagamura, Y., B. Antonio, T. Sasaki, 1997 Rice molecular genetic map using RFLPs and its applications. *Plant Molecular Biology* **35:** 79-87.

Nelson, C. D., W. L. Nance, R. L. Doudrick, 1993 A partial genetic linkage map of slash pine (*Pinus elliottii* Engelm *var elliottii*) based on random amplified polymorphic DNAs. *Theoretical & Applied Genetics* **87:** 145-151.

Newbigin, E., M. A. Anderson, A. E. Clarke, 1993 Gametophytic self-incompatibility systems. *Plant Cell* **5:** 1315-1324.

Newbury, H., B. Ford-Lloyd, 1993 The use of RAPD for assessing variation in plants. *Journal of Plant Growth Regulation* **12:** 43-51.

Ouazzani, N., R. Lumaret, P. Villemur, F. d. Guisto, F. Di Giusto, 1993 Leaf allozyme variation in cultivated and wild olive trees (*Olea europaea* L.). *Journal of Heredity* **84:** 34-42.

Paran, I., R. W. Michelmore (1993) Development of reliable PCR-based markers linked to downy mildew resistance genes in lettuce. *Theoretical and Applied Genetics* **85**:985-99.

Patumi, M., R. d'Andria, G. Fontanazza, G. Morelli, P. Giorio *et al.*, 1999 Yield and oil quality of intensively trained trees of three cultivars of olive (*Olea europaea* L.) under different irrigation regimes. *Journal of Horticultural Science & Biotechnology.* **74:** 729-737.

Pemberton, J. M., J. Slate, D. R. Bancroft, J. A. Barrett, 1995 Nonamplifying alleles at microsatellite loci - a caution for parentage and population studies. *Molecular Ecology* **4:** 249-252.

Pfahler, P. L., M. J. Pereira, R. D. Barnett, 1997 Genetic variation for *in vitro* sesame pollen germination and tube growth. *Theoretical & Applied Genetics* **95:** 1218-1222.

Pinney, K., V. S. Polito, 1990 Olive pollen storage and *in vitro* germination. *Acta Horticulturae* **286:** 207-210.

Plomion, C., D. M. Omalley, C. E. Durel, 1995 Genomic analysis in maritime pine (*Pinus pinaster*) - comparison of two RAPD maps using selfed and open-pollinated seeds of the same individual. *Theoretical & Applied Genetics* **90:** 1028-1034.

Poli, M., 1986 The olive's alternating production pattern. *Olivae* **3:** 7-27.

Pontikes, K., M. Loukas, C. A. Pontikis, 1981 Distinguishing olive cultivars through the use of genetic markers. Genetic variation within and between cultivars. *Georgike Ereuna* **5:** 15-24.

Proietti, P., 1990 Photosynthesis and respiration in olive fruit. *Acta Horticulturae* **286:** 211-214.

Rafalski, J. A., S. V. Tingey, 1993 Genetic diagnostics in plant breeding - RAPD, microsatellites and machines. *Trends in Genetics* **9:** 275-280.

Rajapakse, S., L. E. Belthoff, G. He *et al.*, 1995 Genetic linkage mapping in peach using morphological, RFLP and RAPD Markers. *Theoretical and Applied Genetics* **90:** 503-510.

Rallo, L., D. Barranco, 1986 Influence of the time of application on the response of olive to chemical thinning. *Acta Horticulturae* **179:** 709-710.

Rallo, L., J. Cuevas, H.F. Rapoport, 1990 Fruit set pattern in self- and open-pollinated olive cultivars. *Acta Horticulturae* **286:** 219-222.

Rallo, P., G. Dorado, A. Martin, 2000 Development of simple sequence repeats (SSRs) in olive tree (*Olea europaea* L.). *Theoretical & Applied Genetics* **101:** 984-989.

Rallo, L., E. R. Fernandez, 1985 Influence of cultivar and flower thinning within the inflorescence on competition among olive fruit. *Journal of American Society for Horticultural Science* **110:** 303-308.

Rallo, L., G. C. Martin, S. Lavee, 1981 Relationship between abnormal embryo sac development and fruitfulness in olive. *Journal of the American Society for Horticultural Science* **106:** 813-817.

Rallo, P., G. Dorado, A. Martin, 2000 Development of simple sequence repeats (SSRs) in olive tree (*Olea europaea* L.). *Theoretical & Applied Genetics* **101:** 984-989.

Rapoport, H. F., S. Lavee, I. Klein, 1994 The timing and developmental context of olive embryo growth. *Acta Horticulturae* **356:** 268-271.

Rapoport, H. F., L. Rallo, 1990 Ovule development in normal and parthenocarpic olive fruits. *Acta Horticulturae* **286:** 223-226.

Rapoport, H. F., L. Rallo, 1991a Fruit set and enlargement in fertilized and unfertilized olive ovaries. *HortScience* **26:** 896-898.

Rapoport, H. F., L. Rallo, 1991b Postanthesis flower and fruit abscission in 'Manzanillo' olive. *Journal of the American Society for Horticultural Science* **116:** 720-723.

Reddy, T. V., 1988 Presowing treatments to improve *Cassia* and *Calliandra* seed germination. *Acta Horticulturae* **226:** 537-540.

Rivera, R., K. J. Edwards, J. H. A. Barker, G. M. Arnold, G. Ayad, *et al.*, 1999 Isolation and characterization of polymorphic microsatellites in *Cocos nucifera* L. *Genome* **42:** 668-675.

Roche, P., F. H. Alston, C. Maliepaard, K. M. Evans, R. Vrielink *et al.*, 1997 RFLP and RAPD markers linked to the rosy leaf curling aphid resistance gene (Sd(1)) in apple. *Theoretical and Applied Genetics* **94:** 528-533.

Rolf, F., 1998 NTSYS-pc: Numerical taxonomy and multivariate analysis system, version 2.01, user guide. Exeter Software: Setauket, N.Y.

Rotundo, A., F. Fiume, 1978 Floral and fruiting biology of the olive cultivar Itrana. *Annali della Facolta di Scienze Agrarie della Universita Degli Studi di Napoli Portici* **12:** 91-99.

Rotundo, A., S. Rotundo, 1988 "Olivo da Olio", the most widespread olive cultivar in the Sorrento Peninsula. *L'Informatore Agrario* **44:** 68-72.

Rugini, E., G. Caricato, 1995 Somatic embryogenesis and plant recovery from mature tissues of olive cultivars (*Olea europaea* L.) "Canino" and "Moraiolo". *Plant Cell Reports* **14:** 257-260.

Rugini, E., E. Fedli, 1990 Olive (*Olea europaea* L.) as an oilseed crop, pp. 593-641 in *Biotechnology in agriculture and forestry*, edited by Y. Bajaj. Springer-Verlag, Berlin, Heidelberg.

Rugini, E., S. Lavee, 1992 Olive, pp. 371-382 in *Biotechnology of Perennial Fruit Crops* edited by F. A. Hammerschlag and R. E. Litz. CAB International, Wallingford, UK.

Rugini, E., G. Pannelli, M. Ceccarelli, M. Muganu, 1996 Isolation of triploid and tetraploid olive (*Olea europaea* L.) plants from mixoploid cv. 'Frantoio' and 'Leccino' mutants by in vivo and *in vitro* selection. *Plant Breeding* **115:** 23-27.

Sage, T. L., R. I. Bertin, E. G. Williams, 1994 Ovarian and other late-acting self-incompatibility systems, pp. 116-140 in *Genetic Control of Self-Incompatibility and Reproductive Development in Flowering Plants*, edited by E. G. Williams, A. E. Clarke and R. B. Knox. Kluwer Academic Publishers, Dordrecht

Sambrook, J., E.F. Fritsch, T. Maniatis, (1989) *Molecular cloning: a laboratory manual.* Cold Spring Harbor Laboratory Press, N.Y.

Sanz-Cortes, F., M. L. Badenes, S. Paz, A. Iniguez, G. Llacer, 2001 Molecular characterization of olive cultivars using RAPD markers. *Journal of the American Society for Horticultural Science* **126:** 7-12.

Sarker, R. H., S. K. Paul, A. Haque, M. I. Hoque, 1997 Pollen tube growth and variation in pollen tube callose plugs in some *Corchorus* species. *Phytomorphology* **47:** 311-317.

Seavey, S. R., K. S. Bawa, 1986 Late-acting self-incompatibility in angiosperms. *Botanical Review* **52:** 195-219.

Sedgley, M., 1994 Self-incompatibility in woody horticultural species, pp. 141-163 in *Genetic Control of Self-Incompatibility and Reproductive Development in Flowering Plants*, edited by E. G. Williams, A. E. Clarke and R. B. Knox. Kluwer Academic Publishers, Dordrecht; Boston; London.

Sedgley, M., F. D. H. Bell, D. Bell, C. W. Winks, S. J. Pattison *et al.*, 1990 Self- and cross-compatibility of macadamia cultivars. *Journal of Horticultural Science* **65,** 205-214.

Sedgley, M., M. Wirthensohn, 1999 Better olive cultivars for Australia. *Olive Press* **Winter:**12-14.

Sedgley, M., M. Wirthensohn, 2000 The Australian olive improvement programme. *Olivæ* **83:** 27-30.

Sefc, K. M., S. Lopes, D. Mendonca, M. R. Dos Santos, M. L. D. Machado, *et al.*, 2000 Identification of microsatellite loci in olive (*Olea europaea*) and their characterization in Italian and Iberian olive trees. *Molecular Ecology* **9:** 1171-1173.

Sharma, P. C., D. R. Thakur, M. R. Sharma, 1976 Pollination and fruit development studies in olive, *Olea europea. Food and Farming Agriculture*. **8:** 24-26.

Shimada, T., T. Yamamoto, H. Yaegaki *et al.*, 1999 Application of AFLP to molecular genetic analysis in peach. *Journal of Japanese Society for Horticultural Science* **68:** 67-69.

Sibbett, G. S., M. Freeman, L. Ferguson, V. S. Polito, 1992 Effect of topically applied 'Sevillano' pollen on normal-seeded and parthenocarpic "shotberry" fruit set of 'Manzanillo' olive. *HortTechnology* **2:** 228-230.

Singh, R. P., P. L. Kar, 1980 Compatibility studies in some olive cultivars. *Progressive Horticulture* **12:** 9-15.

Sotomayor-Leon, E. M., J. M. Caballero, 1990 An easy method of breaking olive stones to remove mechanical dormancy. *Acta Horticulturae* **286:** 113-116.

Sotomayor-Leon, E. M., J. M. Duran Altisent, S. Lavee, I. Klein, 1994 Breaking of dormancy in olive *(Olea europaea* L.) seeds. *Acta Horticulturae* **356:** 137-142.

Stam, P., 1993 Construction of integrated genetic linkage maps by means of a new computer package: JOINMAP. *Plant Journal* **3:** 739-744.

Staub, J. E., F. C. Serquen, M. Gupta, 1996 Genetic markers, map construction, and their application in plant breeding. *Hortscience* **31:** 729-741.

Stead, A. D., I. N. Roberts, H. G. Dickinson, 1979 Pollen-pistil interaction in *Brassica oleracea*. Events prior to pollen germination. *Planta* **146:** 211-216.

Steenkamp, J., I. Wiid, A. Lourens, P. v. Helden, P. Van Helden, 1994 Improved method for DNA extraction from Vitis vinifera. *American Journal of Enology and Viticulture* **45:** 102-106.

Sutherland, S., 1986 Patterns of fruit-set: What controls fruit-flower ratios in plants? *Evolution* **40:** 117-128.

Tanksley, S. D., 1993 Mapping polygenes. *Annual Review of Genetics* **27:** 205-233.

Tansley, S., N. Young, A. Paterson, M. Bonierbale, 1989 RFLP mapping in plant breeding: new tools for an old science. *Biotechnology* **7**: 257-264.

Taylor, H., 1945 Cyto-taxonomy and phylogeny of the Oleaceae. *Brittonia* **5:** 337-367.

Thamarus, K. A., K. Groom, J. Murrell, M. Byrne, G. F. Moran, 2002 A genetic linkage map for *Eucalyptus globulas* with candidate loci for wood, fibre, and floral traits. *Theoretical & Applied Genetics* **104:** 379-387.

Thompson,J.D., T.J. Gibson, F. Plewniak, F. Jeanmougin, D.G. Higgins, 1997 The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research* **24:** 4876-4882.

Tingy, S., J. D. Tufo, 1993 Genetic analysis with random amplified polymorphic DNA markers. *Plant Physiology* **101:** 349-352.

Tombesi, A., S. Lavee, I. Klein, 1994 Olive fruit growth and metabolism. *Acta Horticulturae* **356:** 225-232.

Trujillo, I., L. Rallo, E. A. Carbonell, M. J. Asins, 1990 Isoenzymatic variability of olive cultivars according to their origin. *Acta Horticulturae* **286:** 137-139.

Ugrinovic, K., F. Stampar, 1996 Fertilization of olive (*Olea europaea* L.) cultivars 'Istrska Belica', 'Pendolino' and 'Leccino' by different pollinators. *Zbornik Biotehniske Fakultete Univerze v Ljubljani, Kmetijstvo.* **67:** 183-188.

Ukai, Y. 1989 A microcomputer program DIALL for diallel analysis of quantitative characters. *Japanese Journal of Breeding* **39:** 107-109.

Vachkoo, A. M., M. S. Mughal, O. P. Gupta, K. C. Sharma, 1993 Effect of some chemical and mechanical treatments on seed germination in olive (*Olea cuspidata* Wall.). *Advances in Horticulture and Forestry* **3:** 105-108.

Vassiliadis, C., J. Lepart, P. Saumitou-Laprade, Vernet, P. (2000). Self-incompatibility and male fertilization success in *Phillyrea angustifolia* (Oleaceae). *International Journal of Plant Sciences.* **161:** 393-402.

Vergari, G., M. Patumi, G. Fontanazza, 1996 Use of RAPD markers in the characterisation of olive germplasm. *Olivæ* **60:** 19-22.

Verhaegen, D., C. Plomion, 1996 Genetic mapping in *Eucalyptus urophylla* and *Eucalyptus grandis* using RAPD markers. *Genome* **39:** 1051-1061.

Villemur, P., U. S. Musho, J. M. Delmas, M. Maamar, A. Ouksili, 1984 Contribution to the study of the floral biology of the olive (*Olea europaea* L.): male sterility, pollen flow and effective pollination period. *Fruits* **39:** 467-473.

Viruel, M. A., R. Messeguer, M. C. Devicente, J. Garciamas, P. Puigdomenech, *et al.*, 1995 A linkage map with RFLP and isozyme markers for almond. *Theoretical & Applied Genetics* **91:** 964-971.

Vivian-Smith, A., A. M. Koltunow, 1999 Genetic analysis of growth-regulator-induced parthenocarpy in *Arabidopsis*. *Plant Physiology* **121:** 437-451.

Voorrips, R., 2002 MapChart: Software for the graphical presentation of linkage maps and QTLs. *The Journal of Heredity* **93:** 77-78.

Vos, P., R. Hogers, M. Bleeker, M. Reijans, T. V. D Lee *et al.*, 1995 AFLP: a new technique for DNA fingerprinting. *Nucleic Acids Research* **23:** 4407-4414.

Voyiatzis, D. G., 1993 Overcoming self-incompatibility and increasing fruit set in olive trees with benzyladenine. *Acta Horticulturae* **329:**194-196.

Voyiatzis, D. G., 1995 Dormancy and germination of olive embryos as affected by temperature. *Physiologia Plantarum* **95:** 444-448.

Voyiatzis, D. G., I. C. Porlingis, 1987 Temperature requirements for the germination of olive seeds (*Olea europaea* L.). *Journal of Horticultural Science* **62:** 405-411.

Wang, Z., J. Zhong, S. Tanksley, 1994 Survey of plant short tandom DNA repeats. *Theoretical and Applied Genetics* **88:** 1-6.

Warburton, M. L., V. L. Becerravelasquez, J. C. Goffreda, F. A. Bliss, 1996 Utility of RAPD markers in identifying genetic linkages to genes of economic interest in peach. *Theoretical and Applied Genetics* **93:** 920-925.

Watson, J. C., W. F. Thompson, 1986 Purification and restriction endonuclease analysis of plant nuclear DNA. *Methods in Enzymology* **118:** 57-75.

Weeden, N. F., 1994 Approaches to mapping in horticultural crops, pp. 57-68 in *Plant Genome Analysis*, edited by P. Gresshoff. CRC Press, Boca Raton.

Welsh, J., and M. McClelland, 1990 Fingerprinting genomes using PCR with arbitrary primers. *Nuclear Acids Research* **18:** 7213-7218.

Wiesman, Z., N. Avidan, S. Lavee, B. Quebedeaux, 1998 Molecular characterization of common olive varieties in Israel and West Bank using randomly amplified polymorphic DNA (RAPD) markers. *Journal of American Society for Horticultural Science* **123:** 837-841.

Wiliams, J., A. Kubelik, K. Livak, J. Rafalski, S. Tingey, 1990 DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nuclear Acids Research* **18:** 6531-6535.

Wu, S., G. Collins, M. Sedgley, 2002 Sexual compatibility of olive varieties cultivated in Australia. *Journal of Horticultural Science and Biotechnology* **77: (in press):**

Wu, S., M. Wirthenshon, G. Collins, M. Sedgley, 2000 Olive trees need the right pollinator. *The Olive Press* **Winter:** 13-15.

Wunsch, A., J. I. Hormaza, 2002 Cultivar identification and genetic fingerprinting of temperate fruit tree species using DNA markers. *Euphytica* **125:** 59-67.

Young, N., 1994 Constructing a plant genetic linkage map with DNA markers, pp. 39-57 in *DNA-Based Markers in Plants*, edited by R. Phillips and I. Vasil. kluwer Academic Publishers, Dordrecht.

Yuce, B., 1979 Studies on the germination of olive seed. *Tarimsal Arastirma Dergisi* **1:** 189-203.

Zhu, Y., J. E. Strassmann, D. C. Queller, 2000 Insertions, substitutions, and the origin of microsatellites. *Genetical Research* **76:** 227-236.

Zietkiewicz, E., A. Rafalski, D. Labuda, 1994 Genome fingerprinting by simple sequence repeat (SSR)- anchored polymorphism chain reaction amplification. *Genomics* **20:** 176-183.

Zohary, D., 1994 The wild genetic resources of the cultivated olive. *Acta Horticulturae* **356:** 62-65.

Appendix

Papers published based on the thesis research

Wu, S., M. Wirthenshon, G. Collins, M. Sedgley, 2000 Olive trees need the right pollinator. *The Olive Press* **Winter:** 13-15.

Wu, S., Collins, G., M., Sedgley, 2000 Optimising olive seed germination. *The Olive Press* **Autumn,** 26-27.

Wu, S., G. Collins, M. Sedgley 2002 Sexual compatibility of olive varieties cultivated in Australia. *Journal of Horticultural Science and Biotechnology* **77:** (in press).

Wu, S., Mekuria, G., Collins, G., M. Sedgley 2002 Construction of a genetic linkage map for olive (*Olea europaea*). In *Australasian Plant Breeding Conference*, Perth, Australia.

Guerin, J, Mekuria, G., Wu, S., G., Collins, G., M. Sedgley. 2002 Selection of superior olive cultivars. *Symposia at the XXVIth International Horticultural Congress.* p343.

Wu, S., Wirthensohn, M., Collins, G. & Sedgley, M. (2000). Olive trees need the right pollinator. *The Olive Press*, *winter*, 13-15.

Wu, S., Collins, G. & Sedgley, M. (2000). Optimising olive seed germination. *The Olive Press, autumn*, 26-27.

Journal of Horticultural Science & Biotechnology (2002) 77 (4) 000–000

Sexual compatibility within and between olive cultivars

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(Accepted 23 July 2002)

SUMMARY

Self- and cross-incompatibility of the olive cultivars Frantoio, Manzanillo, Kalamata, Pendolino, and Picual were investigated using a 5 × 5 diallel matrix. Pistils were collected seven days after controlled pollinations on the day of flower opening, and pollen tubes were detected by fluorescence microscopy. Diallel analysis showed significant specific combining ability, general combining ability and reciprocal effects between cultivars for pollen tube growth in the pistil. 'Frantoio' was cross-compatible, as either a male or female parent, with each of the other cultivars, but showed a high degree of self-incompatibility. 'Manzanillo', 'Kalamata', 'Pendolino', and 'Picual' were cross-incompatible, and all except for 'Manzanillo', were self-incompatible. It is concluded that 'Frantoio' is a good general polleniser for the other cultivars investigated. Pollen tube growth decreased in discrete steps from stigma to upper style, and from upper style to lower style, with the result that only one, and rarely more, pollen tube penetrated ovules. The sex ratio of flowers, and pollen viability using fluroescein diacetate staining and *in vitro* germination, were examined. 'Frantoio', 'Manzanillo' and 'Pendolino' had more than 80% perfect flowers, while 'Kalamata' and 'Picual' had less than 30%. 'Frantoio' had the highest pollen viability, 'Kalamata' and 'Picual' were intermediate, and 'Manzanillo' and 'Pendolino' the lowest. Pollen staining and both *in vitro* and *in vivo* germination provided the same male fertility rankings of cultivars.

Olive (*Olea europaea* L.) is a wind-pollination species, and self-incompatibility has been widely reported in recent decades (Griggs *et al.*, 1975; Cuevas *et al.*, 2001). Different cultivars may be totally self-incompatible or show some level of self-compatibility. In olive, self-incompatibility can become a problem when a single cultivar is planned in an isolated area, where other olive pollen is not available (Cuevas and Polito, 1997; Ateyyeh *et al.*, 2000). Cross-pollination can enhance fertilization as shown by reports of higher fruit set (Cuevas and Polito, 1997; Cuevas *et al.*, 2001), and lower production of shot berries, which are small and usually seedless olive fruits (Griggs *et al.*, 1975; Sibbett *et al.*, 1992). Fruit set after cross-pollination relies on compatibility between recipient and polleniser trees, and in some cases, polenisers may fail to improve olive fruit set because of cross-incompatibility (Lavee and Datt, 1978; Cuevas and Polito, 1997).

Inconsistent results are reported about the classification of some cultivars as self-compatible or self-incompatible. For example, 'Manzanillo' was considered to be highly self-compatible by Androulakis and Loupassaki (1990) and Sibbett *et al.* (1992), but mainly self-incompatible by Griggs *et al.* (1975), Lavee and Datt (1978), and Cuevas and Polito (1997). 'Leccino' was reported to be self-incompatible by Ugrinovic and Stampar (1996) and other authors (cited by Bartolini and Guerriero, 1995), but both pollen tube penetration of the ovule, and satisfactory fruit set following self-pollination were reported in several accessions by Bartolini and Guerriero (1995). The reasons for this include the effect of local environmental conditions on self-compatibility (Griggs *et al.*, 1975), and on the genetic background of the trees used, as there is confusion about the names of cultivars grown around the world (Bartolini *et al.*, 1994; Mekuria *et al.*, 1999). The situation is further complicated as olives produce both perfect (complete) and staminate flowers (Brooks, 1948; Griggs *et al.*, 1975). The ratio appears to differ in different cultivars, as well as under varying environmental conditions, such as insufficient winter chilling (Martin, 1990).

In Australia, about 200 olive cultivars are recorded as being grown commercially (Burr, 1999); these have been sourced from around the world. Thus cultivar combinations are available in Australia that would not occur overseas, but limited knowledge is available on their self- and cross-fertility. 'Pendolino' has been regarded as being compatible with a number of other cultivars and it has therefore been planted as a common polleniser (Booth and Davies, 1996). 'Frantoio' is considered to be self-compatible, and so has been planted as a single cultivar without the concern of possible low yields (Sharma *et al.*, 1976).

'Frantoio', 'Kalamata', 'Manzanillo', 'Pendolino', and 'Picual' are five popular cultivars that are widely cultivated in olive plantings, some for table olives ('Kalamata' and 'Manzanillo') and others for oil ('Frantoio', 'Manzanillo', 'Pendolino', and 'Picual') (Booth and Davies, 1996). This research aimed to determine the self- and cross-compatibility of these cultivars. Preliminary stigma receptivity investigations were also conducted on the cultivar 'SA (South Australian) Verdale'.

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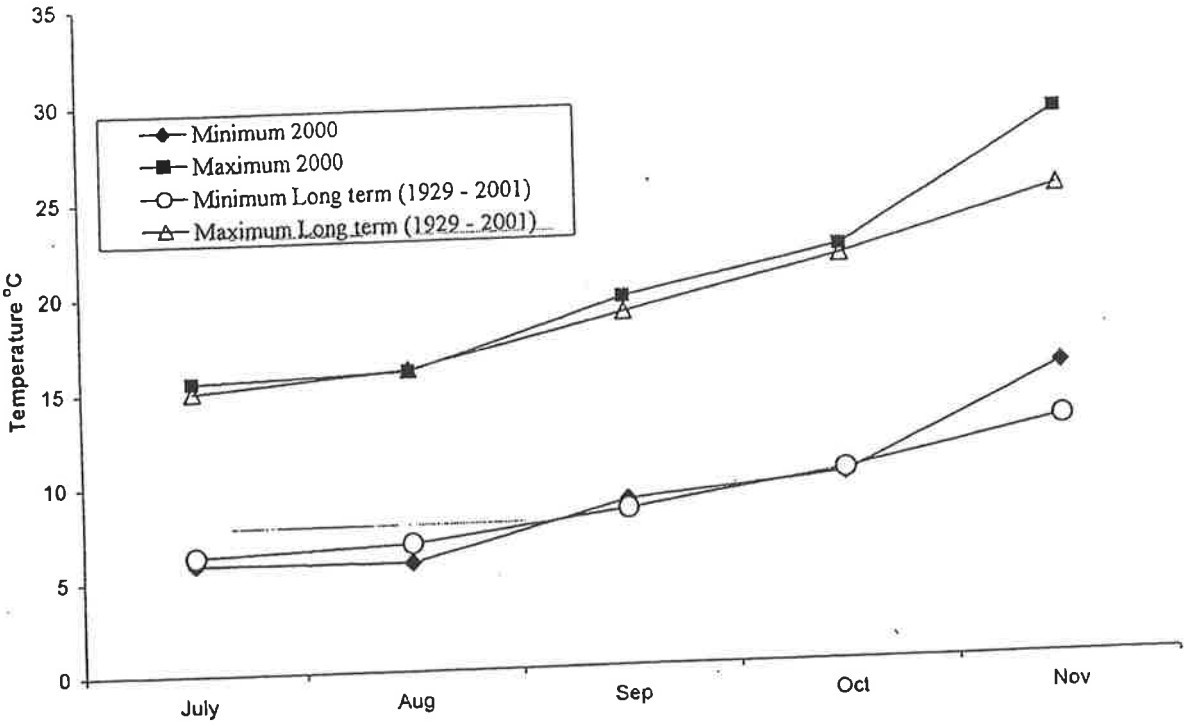

FIG. 1
Monthly means of daily maximum and minimum temperatures in the Adelaide area of South Australia from July to December for the year 2000 compared with the long term averages. Data were collected from Parafield Airport (34°48'S, 138°38'E) (Australian Bureau of Meteorology).

MATERIALS AND METHODS

Climatic conditions

The experiments were conducted in the Adelaide area of South Australia (Latitude: 34°32'–34°40'S, Longitude: 138°30'–138°43'E) during the 2000 flowering season. The climatic data before and during flower opening, and the long-term averages (1929–2001) for the corresponding period, were collected (Figures 1, 2). Although flowering lasted from late October to early November, the climatic conditions for winter (July and August) are also included because winter chilling has important effects on floral initiation and the production of staminate flowers (Martin, 1990).

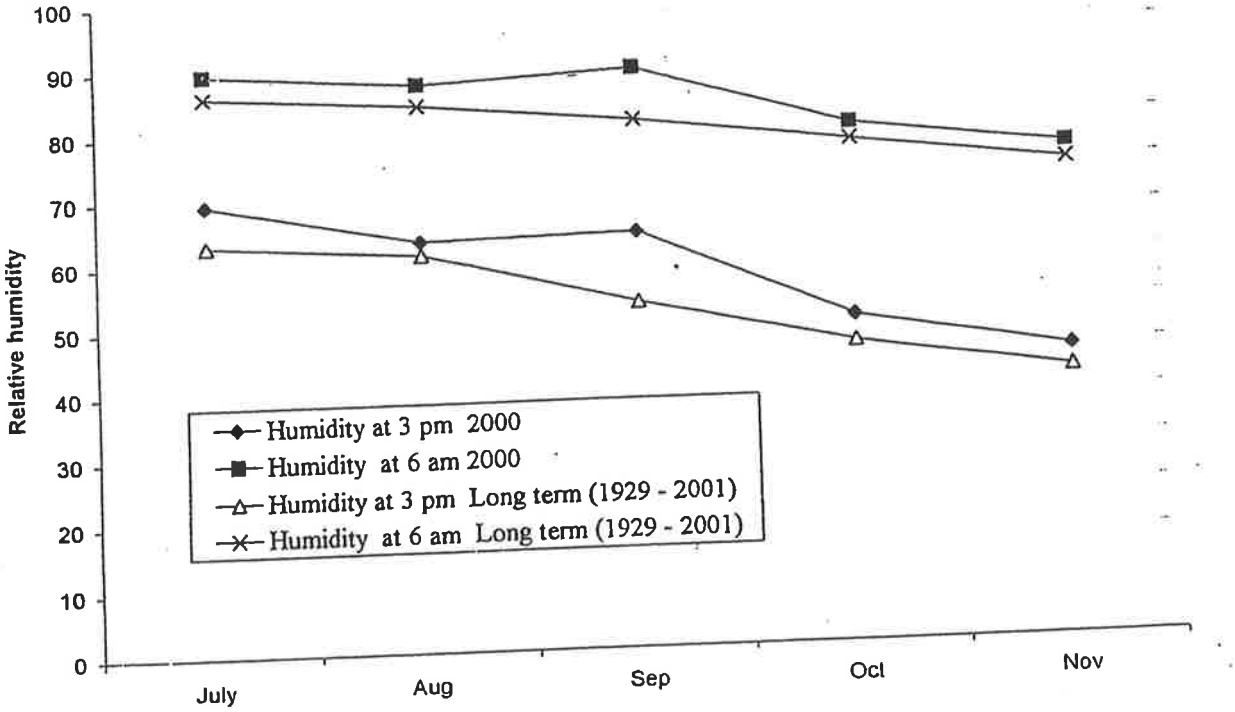

FIG. 2
Comparison of Adelaide monthly means of relative humidity at 0600 hours and 1500 hours from July to December for the year 2000 compared with the long term averages. Data were collected from Parafield Airport (34°48'S, 138°38'E) (Australian Bureau of Meteorology).

Trees

Three trees each of 'Frantoio', 'Kalamata', 'Manzanillo', 'Pendolino', and 'Picual' were selected on commercial properties. Each tree was considered as a replicate within a diallel matrix (Sedgley, 1994) in an investigation of pollen/pistil compatibility. A preliminary investigation of stigma receptivity was carried out with 'SA Verdale'.

Prior to pollination, the identities of the trees used were confirmed by DNA fingerprinting as described by Mekuria *et al.* (1999). The standards used for identification were 'Frantoio', 'Manzanillo de Sevilla', 'Pendolino' and 'Picual' from the Olive World Collection, Centro de Investigacion y Desarollo Agrario, Cordoba, Spain, 'Kalamata' from Consiglio Nationale delle Ricerche, Instituto di Ricerca Sulla Olivicultura, Perugia, Italy, and 'Verdale' from Foundation Plant Material Service, University of California, Davis, California, USA. Genetic similarities among all pairs of individuals were estimated using the hierarchical distance method of simple matching coefficient with NTSYS-pc (Exeter software, NY, USA). Cluster analysis was performed on the estimated similaritiy using the unweighted pair group method with arithmetic average (UPGMA) and the SAHN algorithm.

Inflorescence structure and sex ratio

The number of flowers per inflorescence, and the sex ratio of flowers were determined for 'Frantoio', 'Kalamata', 'Manzanillo', 'Pendolino', and 'Picual' during flowering in October. For each of the 15 trees, two inflorescences were randomly collected from the south, north, west, and east of the tree.

Complete flowers were those with both well-developed anthers and a pistil, whereas staminate flowers had either no, or only remnants of, pistils. There were no pistillate flowers that lacked anthers. The sex ratio was calculated from the ratio of complete flower number to total flower number, where the number of complete flowers represented the number of females and number of total flowers represented the number of males.

Pollen viability

Inflorescences were enclosed in white paper bags before the flowers opened, and the branch was shaken on the day the flowers were in bloom. Pollen grains were collected, placed on a glass slide, immersed in a drop of fluorescein diacetate (FDA, 2 mg ml^{-1} in acetone), and incubated for 10 min. Fluorescence was detected at 520 nm after excitation at 450–490 nm (Pinney and Polito, 1990). Viability percentages were determined using approximately 100 grains over five fields of views.

Pollen germination *in vitro* was assessed on a medium composed of 0.8% Bacto-agar, 15% sucrose, 100 ppm boric acid, and 60 ppm tetracycline (Pinney and Polito, 1990). Pollen was considered to have germinated if the pollen tubes extended to a length equal to at least twice the diameter of the grain after 24 h. Germination percentages were determined using five randomly chosen fields of approximately 100 grains.

Controlled pollination

Pistil receptivity was tested on 'SA Verdale', an early flowering cultivar in South Australia. The DNA fingerprint of this cultivar matches that of 'Verdale' from the Foundation Plant Material Service, University of California, Davis, California, USA (Figure 3). Forty flowers of 'SA Verdale' were emasculated immediately after the flowers opened, and pollen from 'Kalamata' was applied to ten pistils at 0, 3, 7, and 14 d. Seven days after the pollen was applied, the pistils were collected, and pollen tube germination on the stigma, growth in the stigma and style, and penetration of the ovules were observed.

Pollen/pistil compatibility was examined using a 5 × 5 diallel matrix (Sedgley, 1994). Twenty flowers were used per cross with three matrices as replicates. Flowers were emasculated just before anthesis, and the appropriate pollen was applied to the stigma at day 0. Unused flowers were removed, and the inflorescence was securely closed to guard against contamination.

After seven days, the pistils were harvested, fixed in Carnoys fluid (absolute ethanol:chloroform: acetic acid, 6:3:1) for 24 h, and stored in 70% ethanol at 4°C. Fixed pistils were hydrated through 50% and 30% ethanol to distilled water, 30 min for each, softened with 0.8 M NaOH for 6 h at room temperature, and washed in running water overnight. Styles and ovules were prepared separately, stained with 0.1% aniline blue (Martin, 1959) in alkaline phosphate buffer (pH 11.5) for 1 h, mounted in 80% glycerol, and observed under UV light using a Zeiss photomicroscope (Axiophot) equipped with a filter set of exciter filter 395–440, interference beam splitter FT 460, and barrier filter LP 470. The number of pollen grains and pollen tubes on the stigma, pollen tubes in the upper and lower style, and pollen tubes penetrating the ovules were recorded. Pollen was considered to have germinated if the pollen tube extended to a length equal to at least twice the diameter of the grain. In total, 1500 pistils were collected and 3000 slides were prepared.

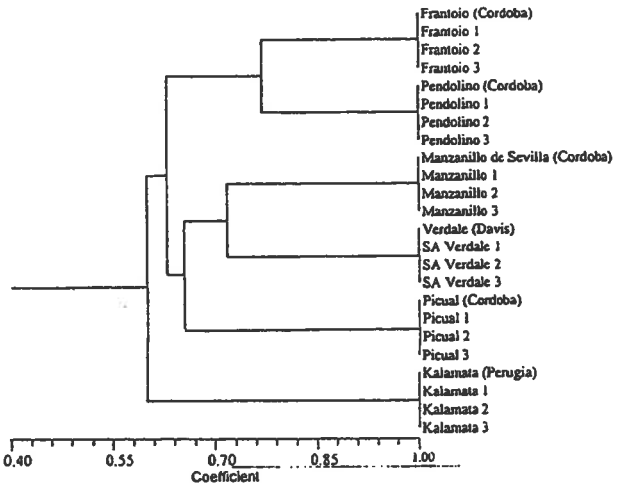

FIG. 3

Dendrogram of the trees used for pollinations compared with standard cultivar samples. Genetic similarities among all pairs of individuals were estimated using the hierarchical distance method of simple matching coefficient with NTSYS-pc (Exeter software, NY, USA). Cluster analysis was performed on the estimated similarity using the unweighted pair group method with arithmetic average (UPGMA), and the SAHN algorithm.

Statistical analysis

Flower number per inflorescence and pollen viability were analysed as one-way ANOVAs using MINITAB (Version 13.20, Minitab Inc., PA, USA). Confidence intervals of the means were applied to determine the significance of differences between cultivars.

Pollen tube growth was assessed on the basis of the number of pollen tubes in the lower style, because the probability of a pollen tube in the lower style reaching the ovule was considerably higher than one in the upper style. Since the number of pollen tubes in this region was rarely more than 1, the presence of one or more pollen tubes was allocated 1, and the absence of pollen tubes was allocated 0. A binary matrix was developed for the diallel cross data, and this was analysed with Genstat 5 for Windows Release 4.1, 4th Edition (Lawes Agricultural Trust) as a logistic regression using a generalized linear model (GLM) assuming a binomial distribution and a logit link (Lefort and Legisle, 1977). The probability of a flower having a pollen tube in the lower style was calculated using:

$$\text{Logit}\,(p_{ij}) = \log\left(\frac{p_{ij}}{1-p_{ij}}\right) = \mu + (Female)_i + (Male)_j + (Female \cdot Male)_{ij}$$

where

$i = 1, \ldots, 5$ corresponding to each cultivar as a female parent.

$j = 1, \ldots, 5$ corresponding to each cultivar as a male parent.

p_{ij} is the probability that a flower has a pollen tube in the lower style ($0 < p_{ij} < 1$).

$1 - p_{ij}$ is the probability that a flower does not have a pollen tube in the lower style.

μ is the grand mean.

The probability that a pollen tube will reach the lower style in a particular flower (p_{ij}) for the various combinations of female and male trees was determined using a likelihood ratio test, which is distributed approximately according to the chi-squared analysis.

In addition, pollen tube numbers in various parts of the pistil were analysed by the method described by Griffing (1956). The variability in the means was partitioned into general combining ability (gca), specific combining ability (sca), reciprocal effect (r), and experimental error, using DIAL (Ukai, 1989). Since the numbers of pollen tubes in the lower style and the ovule were fewer than 10, and zeros were present in the data, which were not normally distributed, a transformation was conducted using $\sqrt{x + 0.5}$ (Steel and Torrie, 1960). The procedure of analysis and the presentation of results followed that used by Sedgley *et al.* (1990), and Fuss and Sedgley (1991). Significant differences between various combinations were determined after one-way ANOVA was applied using MINITAB (Version 13.20, Minitab Inc., PA, USA).

RESULTS

Comparison of climatic conditions

The monthly means of daily maximum and minimum temperatures during the flowering period for the area under study are shown in Figure 1 together with the long-term averages. Temperatures from July to October matched those of the long-term averages, whereas the maximum and minimum temperatures in November were 4.0°C and 2.8°C higher than the long-term averages, respectively.

The monthly means of daily relative humidity at 0600 hours and 1500 hours during the flowering period are shown in Figure 2 together with the long-term average. Relative humidity for July, August, October, and November matched those of the long-term averages, whereas in September the means were 7.9% and 10.7% higher than those of the long-term average for 0600 hours and 1500 hours, respectively.

Identification of the cultivars

The DNA fingerprints of the trees used were identical to those for 'Frantoio', 'Manzanillo de Sevilla', 'Pendolino' and 'Picual' from the Olive World Collection, Centro de Investigacion y Desarollo Agrario, Cordoba, Spain, 'Kalamata' from Consiglio Nationale delle Ricerche, Instituto di Ricerca Sulla Olivicultura, Perugia, Italy, and Verdale from Foundation Plant material Service, University of California, Davis, California, USA, respectively (Figure 3).

Inflorescence structure and sex ratio

Flower number per inflorescence varied from 12 to 45 for the cultivars investigated. 'Kalamata' and 'Pendolino' had the highest mean numbers of flowers per inflorescence, whereas 'Picual' had the lowest, and 'Frantoio' and 'Manzanillo' were intermediate (Table I).

Complete flowers were predominant in 'Frantoio', 'Manzanillo', and 'Pendolino'. In contrast, staminate flowers were more frequent in 'Kalamata' and 'Picual'. The highest percentage of complete flowers was 86.6% for 'Frantoio', and the lowest was 22.9% for 'Kalamata'. There were no significant differences between the three different trees examined for each cultivar (data not shown).

Pollen viability

Pollen viability was assessed using both FDA staining and *in vitro* germination (Figure 4). The results from FDA staining showed that 'Frantoio' had the highest pollen viability of 78.7%, and this was significantly

TABLE I

Flower number per inflorescence, and percentage of complete flowers of five olive cultivars

Cultivar	Flowers/inflorescence*	Complete flowers (%)	Sex ratio (Female : Male)
Frantoio	21.1 b	86.6 a	0.87 : 1
Kalamata	28.3 a	22.9 b	0.23 : 1
Manzanillo	18.4 b	80.6 a	0.81 : 1
Pendolino	25.9 a	85.4 a	0.85 : 1
Picual	14.6 c	29.3 b	0.29 : 1

*Within a column, mean values followed by different letters are significantly different at $P = 0.05$.

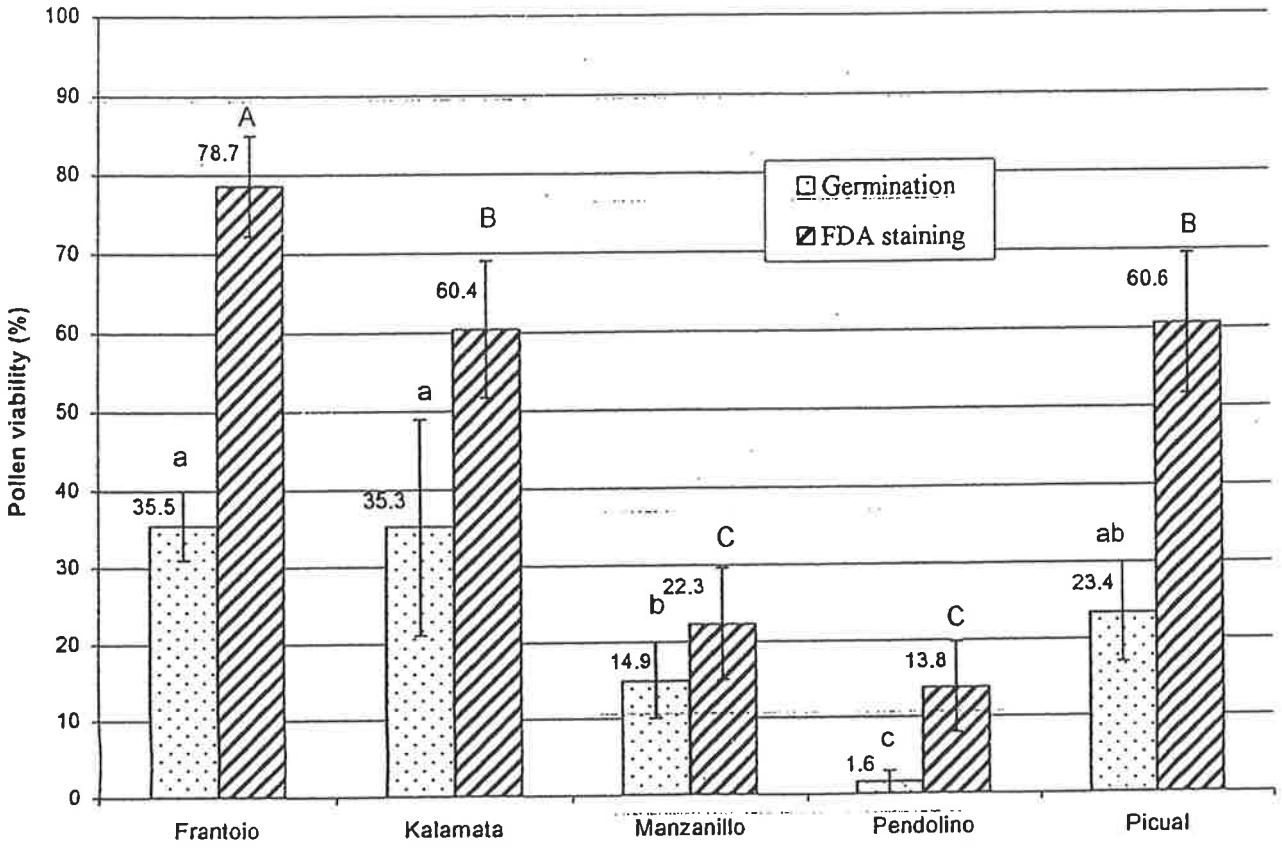

Fig. 4'
Pollen viability in olive cultivars compared by FDA staining and *in vitro* germination. Columns with the same letter, upper case for FDA staining, and lower case for *in vitro* germination, are not significantly different. Bars represent 95% confidence interval. Values are the means of pollen viability percentage.

higher than for the other four cultivars at $P<0.05$. 'Kalamata' and 'Picual' were intermediate with about 60%, and 'Manzanillo' and 'Pendolino' were the lowest at 22.3% and 13.8% respectively. Viability assessed by *in vitro* germination was always lower than for FDA staining, but the order between the cultivars was unchanged. The coefficient of determination (r^2) for the two methods was 0.86 ($P<0.05$).

Stigma receptivity

Germination of 'Kalamata' pollen on the stigma of 'SA Verdale' was 12.9% on the day of flower opening, 10.5% on day 3, 9.3% on day 7, and, due to shrivelling of the stigmas, absent on day 14. All subsequent pollinations were conducted on the day of flower opening.

Pollen germination in vivo

The highest germinations (Figure 5) were recorded for pollen of 'Frantoio' on stigmas of 'Manzanillo' (27.7%), and pollen of 'Kalamata' and 'Picual' on stigmas of 'Frantoio' (23.4% for each). Germination rates of less than 5% were recorded for pollen of 'Pendolino' on the other four cultivars, and for 'Manzanillo' on 'Frantoio' and 'Picual'. Self-pollinations for 'Frantoio', 'Kalamata', 'Manzanillo', 'Pendolino', and 'Picual' were 2.8, 18.3, 19.2, 3.1 and 12.4% respectively.

The average germination of pollen across all cultivars was highest for 'Frantoio', and lowest for 'Pendolino' (Table II) and the ranking was the same as that found for pollen viability (Table III). Stigma receptivity across all cultivars was highest for 'Manzanillo' and lowest for 'Pendolino' and 'Picual'.

Pollen tube growth

Pollen tube growth decreased in discrete steps from stigma to upper style, and from upper style to lower style. Usually, about 70% of the pollen tubes arriving at the upper style were blocked at a point just below the junction between stigma and style. The highest numbers of pollen tubes occurring in the upper style were recorded for the pollen of 'Frantoio' on 'Picual' and 'Kalamata'. The average varied from 2.4 to 13.4 per pistil. There were significant general combining ability (gca), specific combining activity (sca) and reciprocal effects (Table IV).

Regardless of the number of pollen tubes in the upper style, the maximum found in the lower style was rarely more than two, and most commonly zero or one. The highest number was recorded for 'Frantoio' pollen on 'Picual' where up to four pollen tubes were observed.

Ovule penetration by a pollen tube always occurred via the micropyle and generally in only one of the four ovules. The highest percentage of pistils that showed pollen tube penetration of the ovules was for 'Pendolino' and 'Picual' on 'Frantoio' (Table V).

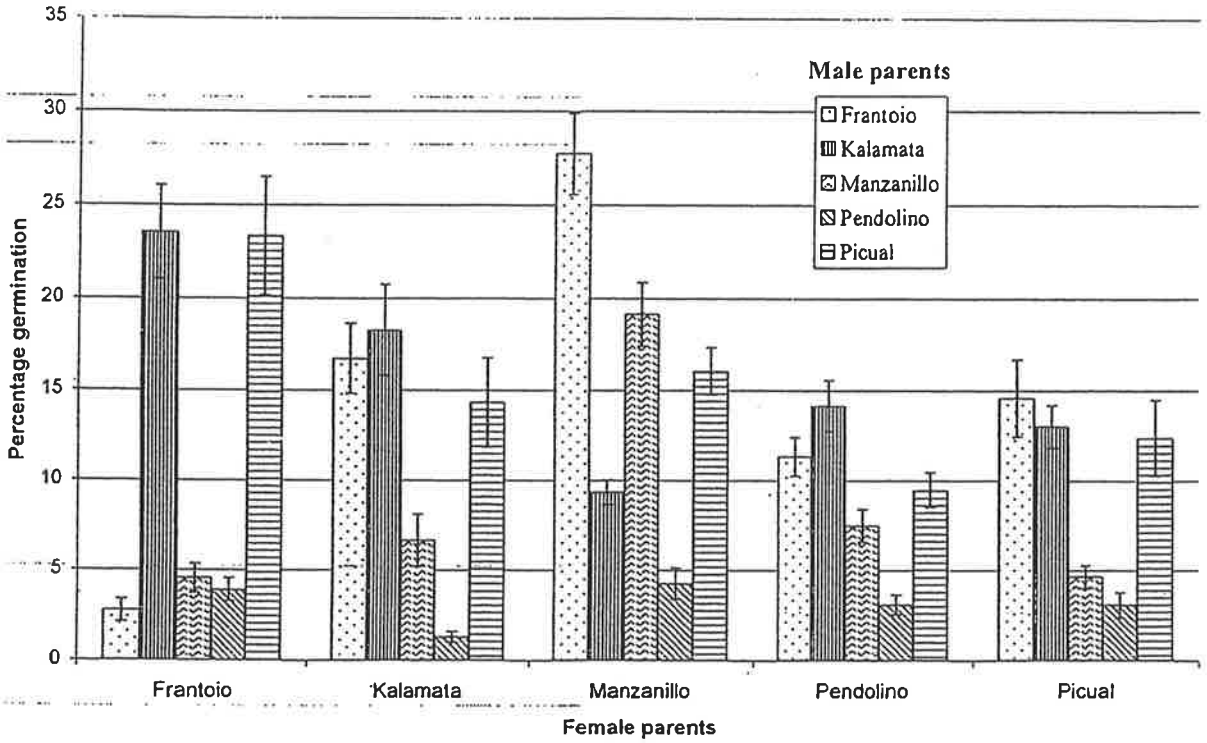

FIG. 5
Results of diallel cross between five olive cultivars showing pollen germination on the stigma. Bars represent 95% confidence intervals.

Self- and cross-compatibility

The ANOVA shown in Table IV indicates that sca, gca, and reciprocal effects were significant for pollen tube growth in both upper and lower styles, and penetration of ovules.

The predicted probabilities of flowers having pollen tubes in the lower style were calculated from the logistic regression (Table VI). With the exception of 'Manzanillo', the other four cultivars showed a high degree of self-incompatibility. 'Frantoio' was successful as either a male or female parent, and all combinations that did not involve 'Frantoio' appeared to have a low probability of successful fertilization.

Table VII shows the order of fertility for each cross, based on pollen tubes in the upper and lower style. The most fertile combination was 'Picual' pollen on the stigmas of 'Frantoio'.

DISCUSSION

'Frantoio', 'Kalamata', 'Picual' and 'Pendolino' showed a high degree of self-incompatibility, thus confirming the presence of self-incompatibility in olives, as reported for other cultivars (Lavee and Datt, 1978; Ateyyeh *et al.*, 2000). Therefore, large orchards containing only these cultivars should be avoided. 'Frantoio' has been reported to be self-incompatible (Sharma *et al.*, 1976), but in the present study, pollen germination was only 2.8% on its own stigmas, and pollen tube growth was not observed in the style. The difference in self-pollination found in this study from others may be due to differences in climatic conditions in Australia compared with the countries where the previous research was carried out (Booth and Davies, 1996). There are also conflicting reports about the self-incompatibility of 'Manzanillo', ranging from self-incompatible (Lavee and Datt, 1978; Cuevas and Polito, 1997), through partially self-incompatible (Dimassi *et al.*, 1999) to self-compatible (Androulakis and Loupassaki, 1990; Sibbett *et al.*, 1992). Under the environmental conditions that pertained during this study, 'Manzanillo' performed as a partially self-incompatible cultivar.

The olive cultivars investigated in this experiment showed significant variation caused by general and specific combining ability and significant reciprocal effects with regard to pollen tube growth in both upper and lower styles. A similar effect has been reported for macadamia cultivars (Sedgley *et al.*, 1990). Cross-pollination generally enhances fruit set of olives (Griggs *et al.*, 1975; Ghrisi *et al.*, 1999; Cuevas *et al.*, 2001), although not all cultivars are cross-compatible (Griggs

TABLE II
Comparison of means of in vivo *pollen germination percentages on the stigma between cultivars both as male and female parents*

Cultivars	As male parent*		As female parent	
	Pollen germination (%)	Significance	Pollen germination (%)	Significance
Frantoio	14.6 ± 1.2	a	11.6 ± 1.4	b
Kalamata	11.9 ± 0.8	b	11.5 ± 1.1	b
Manzanillo	11.0 ± 1.1	b	15.5 ± 1.1	a
Pendolino	7.7 ± 0.8	c	9.1 ± 0.6	c
Picual	12.0 ± 1.2	b	9.5 ± 0.9	bc

*Within a column, mean values followed by different letters are significantly different at $P = 0.0.5$

Table III
The rank of cultivars for in vitro *and* in vivo *pollen germination (pooled for all cultivars)*

Rank	*in vitro**		*in vivo*	
1	Frantoio	a	1 Frantoio	a
2	Kalamata	b	2 Kalamata	b
2	Picual	b	2 Picual	b
3	Manzanillo	c	2 Manzanillo	b
3	Pendolino	c	3 Pendolino	c

*Within the column, mean values followed by different letters are significantly different at $P = 0.05$.

et al., 1975; Cuevas and Polito, 1997). Among the five cultivars investigated, 'Frantoio' showed the best combining ability with the others as both male and female parent, indicating that it is a general polleniser as well as a good recipient. The other four cultivars showed low cross-compatibility in both directions, and would need appropriate pollenisers to obtain reasonable pollination in orchards. This is consistent with the findings of Cuevas *et al.* (2001) who found that the three cultivars Gordal Sevillana, Picual, and Arbequina were most appropriate pollenisers for 'Manzanilla de Sevilla', 'Hojiblanca', and 'Picual', respectively. Of the five cultivars examined in this study 'Kalamata' is Greek, 'Pendolino' and 'Frantoio' are Italian and 'Manzanillo' and 'Picual' are Spanish. While it is unusual to find these cultivars growing together in Europe, new world oilve production is currently experimenting with many varieties. Moreover, European olive groves tend to be relatively small, and are generally located in olive production regions where pollen drift between plantings is common. In contrast, isolated large-scale plantings are more characteristic of Australian groves. Hence pollination problems may arise in Australia and in other new olive producing countries, which may not be manifest in traditional areas.

For the olive cultivars examined, the number of pollen tubes declined markedly in the pistil with distance from the stigma. Pollen tubes converged towards the central part of the style and only one, or occasionally more, pollen tubes were able to grow down to the ovary. This explains why olive fruit usually develop only one or rarely two seeds, even though four ovules are produced in the ovary. Similar pollen tube attrition has also been reported in *Phillyrea angustifolia* (Vassiliadis *et al.*, 2000) which belongs to the same family as olive. In spite of this strong pollen tube attrition, the cross 'Frantoio' × 'Picual' was found to have up to four pollen tubes in the lower style in comparison with one for other combinations. However, only one of the four ovules was penetrated. In addition to pollen tube attrition, there is early fruit shed in olive. Maximum fruit shed occurs during one month after flowering, and fruit set reaches a level of ca 1% approximately seven weeks after full bloom (Grigg *et al.*, 1975; Rallo and Fernández-Escobar, 1985). Pollen tube attrition may be one of the reasons for early fruit shed due to lack of fertilization of some pistils, as well as competition between developing fruits for resources.

The gametophytic system of self-incompatibility has been proposed for olive (Cuevas and Polito, 1997; Ateyyeh *et al.*, 2000). Other plants that exhibit gametophytic self-incompatibility are usually characterized by wet stigma and binucleate pollen (Heslop-Harrison and Shivanna, 1977), as is the case in olive. Incompatible pollen germinates successfully on the surface of the stigma, but growth of the pollen tube is arrested in the style (Newbigin *et al.*, 1993). Further work is required to determine whether the apparent cross-incompatibility between 'Manzanillo', 'Picual', 'Pendolino', and 'Kalamata' is attributable to shared incompatibility alleles.

Table IV
Analysis of variance of diallel cross of five olive cultivars based on pollen tubes within upper style, lower style, and ovule. The data were transformed using $(X+0.5)^{1/2}$ (Steel and Torrie, 1960) for the lower style and ovule

Source of variation		d.f.	Mean square	Significance
Replicates	(upper style)	2	796.25	$P > 0.1$
	(lower style)	2	0.00	$P > 0.1$
	(ovules)	2	0.00	$P > 0.1$
General combining ability	(upper style)	4	61515.42	$P < 0.01$
	(lower style)	4	0.55	$P < 0.01$
	(ovules)	4	0.03	$P < 0.01$
Specific combining ability	(upper style)	5	5793.07	$P < 0.01$
	(lower style)	5	0.01	$P < 0.01$
	(ovules)	5	0.00	$P < 0.01$
Reciprocal	(upper style)	10	7909.85	$P < 0.01$
	(lower style)	10	0.01	$P < 0.01$
	(ovules)	10	0.09	$P < 0.01$
Error	(upper style)	38	438.44	
	(lower style)	38	0.00	
	(ovules)	38	0.00	
Total	(upper style)	59		
	(lower style)	59		
	(ovules)	59		

Table V
Mean of pollen tubes penetrating the ovule per pistil

	Male parent									
Female parent	Frantoio		Kalamata		Manzanillo		Pendolino		Picual	
Frantoio	0.00	c*	0.15	ab	0.05	bc	0.28	ab	0.33	a
Kalamata	0.08	bc	0.02	bc	0.02	bc	0.00	c	0.02	bc
Manzanillo	0.15	ab	0.00	c	0.03	bc	0.00	c	0.00	c
Pendolino	0.12	b	0.00	c	0.00	c	0.00	c	0.00	c
Picual	0.20	ab	0.02	bc	0.02	bc	0.02	bc	0.00	c

*Means followed by the same letter are not significantly different.

TABLE VI
Predicted proportion of pollen tubes in the lower style

Female parent	Male parent				
	Frantoio	Kalamata	Manzanillo	Pendolino	Picual
Frantoio	0.00	0.85	0.60	0.67	0.90
Kalamata	0.62	0.07	0.02	0.00	0.03
Manzanillo	0.63	0.00	0.22	0.00	0.00
Pendolino	0.57	0.00	0.00	0.00	0.00
Picual	0.63	0.02	0.05	0.02	0.05

TABLE VII
Olive crosses in order of decreasing fertility based on pollen tube number in the upper and lower style (ANOVA) results by MINITAB 13.20)

Pollen tube in upper style				Pollen tube in lower style			
Female parent		Male parent	Significance*	Female parent		Male parent	Significance
Frantoio	×	Picual	a	Frantoio	×	Picual	a
Frantoio	×	Kalamata	b	Frantoio	×	Kalamata	b
Manzanillo	×	Frantoio	c	Frantoio	×	Manzanillo	b
Kalamata	×	Frantoio	d	Kalamata	×	Frantoio	b
Picual	×	Frantoio	e	Picual	×	Frantoio	b
Pendolino	×	Frantoio	e	Manzanillo	×	Frantoio	b
Frantoio	×	Manzanillo	ef	Frantoio	×	Pendolino	b
Frantoio	×	Pendolino	f	Pendolino	×	Frantoio	c
Manzanillo	×	Manzanillo	g	Manzanillo	×	Manzanillo	d
Kalamata	×	Kalamata	g	Kalamata	×	Kalamata	e
Kalamata	×	Manzanillo	g	Picual	×	Picual	e
Picual	×	Pendolino	g	Picual	×	Manzanillo	e
Manzanillo	×	Picual	g	Kalamata	×	Picual	e
Picual	×	Kalamata	g	Picual	×	Kalamata	e
Picual	×	Picual	g	Kalamata	×	Manzanillo	e
Picual	×	Manzanillo	g	Picual	×	Pendolino	e
Kalamata	×	Picual	g	Manzanillo	×	Picual	e
Pendolino	×	Kalamata	g	Pendolino	×	Kalamata	e
Manzanillo	×	Kalamata	g	Manzanillo	×	Kalamata	e
Pendolino	×	Picual	g	Pendolino	×	Picual	e
Pendolino	×	Manzanillo	g	Pendolino	×	Manzanillo	e
Frantoio	×	Frantoio	g	Frantoio	×	Frantoio	e
Manzanillo	×	Pendolino	g	Manzanillo	×	Pendolino	e
Pendolino	×	Pendolino	g	Pendolino	×	Pendolino	e
Kalamata	×	Pendolino	g	Kalamata	×	Pendolino	e

*Within the column, mean values followed by different letters are significantly different at $P = 0.05$.

The reliability of *in vitro* germination tests has been questioned by Lavee and Datt (1978). In the present study, the results from FDA staining were consistently higher than for *in vitro* germination, and a regression analysis ($r^2 = 0.86$, $P<0.05$) showed that either technique could be used with an appropriate correction. Differences in the germination of pollen from different cultivars on the same stigma can be attributed partly to pollen viability as evidenced by the similarity in the rankings between germination on the stigma and pollen viability. However, the interaction between pollen and stigma is also important in pollen germination (Pfahler *et al.*, 1997), for example, the differences in the germination of pollen of 'Frantoio' on the stigmas of each of the cultivars investigated.

The results reported here follow controlled hand pollination of perfect flowers, and do not take into account the fact that large variation was observed in the sex ratio and pollen fertility of the cultivars. These factors would also be expected to impact on grove yield. There is evidence of environmental effects on pollination in olives and other woody crops (Lavee and Datt, 1978; Martin, 1990; Sedgley, 1994), and therefore the results may include genotype-environment interactions on sex ratios, pollen viability, and self-incompatibility. Maximum and minimum temperatures and relative humidity, were compared between the year under study with the long-term average, and temperatures from July to early November, which encompass the period from floral initiation to anthesis, did not deviate appreciably from the long-term (73 years) averages. The relative humidity was higher than average for September, and although this may have affected some aspects of floral development, anthesis occurred later than this and at the same time in all five cultivars. Therefore it is likely that the results obtained for sex ratio and self- and cross-compatibility presented in this study are representative all of the cultivars for most seasons in the Adelaide area. The relatively short period of receptivity of the stigma also requires that pollinator cultivars overlap precisely with the flowering period of the main cultivar for optimum fertility and yield. The research highlights the importance of selecting cultivars for a mixed orchard that are compatible to maximise pollination, and the implications for losses in yield by planting single cultivar orchards.

The authors are grateful to Dr Meredith Wallwork for technical advice, Xuemei Han for technical assistance, and Michelle Lorimer of Biometrics SA for statistical advice. Thanks also to the following growers for access to their properties: Chas Chamberlain, Peter Grilli, Nick Kyriakou, Sam Hristofis, and Milan Palvic. S.B.W. was supported by an International Postgraduate Research Scholarship of the Australian Government and a University of Adelaide Scholarship.

REFERENCES

Androulakis, I. I. and Loupassaki, M. H. (1990). Studies on the self-fertility of some olive cultivars in the area of Crete. *Acta Horticulturae*, **286**, 159–62.

Ateyyeh, A. F., Stosser, R. and Qrunfleh, M. (2000). Reproductive biology of the olive (*Olea europaea* L.) cultivar 'Nabali Baladi'. *Journal of Applied Botany*, **74**, 255–70.

Bartolini, G., Messeri, C. and Prevost, G. (1994). Olive tree germplasm: Desriptor list of cultivated varieties in the world. *Acta Horticulturae*, **356**, 116–8.

Bartolini, S. and Guerriero, R. (1995). Self-compatibility in several clones of oil olive cv. Leccino. *Advances in Horticultural Science*, **9**, 71–4.

Booth, N. and Davies, G. (1996). *An introduction to olive growing in southern Australia*. Primary Industries South Australia, Adelaide, Australia.

Brooks, R. M. (1948). Seasonal incidence of perfect and staminate olive flowers. *Proceedings of the American Society for Horticultural Science*, **52**, 213–8.

Burr, M. (1999). *Australian olives: A guide for growers and producers of virgin olive oils*. M. Burr, Beetaloo Valley, South Australia.

Cuevas, J., Diaz-Hermoso, A. J., Galian, D., Hueso, J. J., Pinillos, V., Prieto, M., Sola, D. and Polito, V. S. (2001). Response to cross pollination and choice of pollenisers for the olive cultivars (*Olea europaea* L.) 'Manzanilla de Sevilla', 'Hojiblanca' and 'Picual'. *Olivae*, **85**, 26–32.

Cuevas, J. and Polito, V. S. (1997). Compatibility relationships in 'Manzanillo' olive. *HortScience*, **32**, 1056–8.

Dimassi, K., Therios, I., Balatsos, A., Metzidakis, I. T. and Voyiatzis, D. G. (1999). The blooming period and self-fruitfulness in twelve Greek and three foreign olive cultivars. *Acta Horticulturae*, **474**, 275–8.

Fuss, A. M. and Sedgley, M. (1991). Pollen tube growth and seed set of *Banksia coccinea* R. Br. (Proteaceae). *Annals of Botany*, **68**, 377–84.

Ghrisi, N., Boulouha, B., Benichou, M. and Hilali, S. (1999). Agro-physiological evaluation of the phenomenon of pollen compatibility in olive. Case of the Meditterranean collection at the Menara Station, Marrakech. *Olivae*, **79**, 51–9.

Griffing, B. (1956). Concept of general and specific combining ability in relation to diallel crossing systems. *Australian Journal of Biological Sciences*, **9**, 463–93.

Griggs, W. H., Hartmann, H. T., Bradley, M. V., Iwakiri, B. T. and Whisler, J. E. (1975). Olive pollination in California. *California Agricultural Experiment Station*, **869**, 1–49.

Heslop-Harrison, Y. and Shivanna, K. R. (1977). The receptive surface of the angiosperm stigma. *Annals of Botany*, **41**, 1233–58.

Lavee, S. and Datt, Z. (1978). The necessity of cross-pollination for fruit set of 'Manzanillo' olives. *Journal of Horticultural Science*, **53**, 261–6.

Lefort, P. and Legisle, N. (1977). Quantitative stock-scion relationships in vine. Preliminary investigations by the analysis of reciprocal graftings. *Vitis*, **16**, 149–61.

Martin, G. C. (1990). Olive flower and fruit population dynamics. *Acta Horticulturae*, **286**, 141–53.

Martin, F. W. (1959). Staining and observing pollen tubes in the style by means of fluorescence. *Stain Technology*, **34**, 125–8.

Mekuria, G. T., Collins, G. G. and Sedgley, M. (1999). Genetic variability between different accessions of some common commercial olive cultivars. *Journal of Horticutural Science & Biotechnology*, **74**, 309–14.

Newbigin, E., Anderson, M. A. and Clarke, A. E. (1993). Gametophytic self-incompatibility systems. *Plant Cell*, **5**, 1315–24.

Pfahler, P. L., Pereira, M. J. and Barnett, R. D. (1997). Genetic variation for *in vitro* sesame pollen germination and tube growth. *Theoretical and Applied Genetics*, **95**, 1218–22.

Pinney, K. and Polito, V. S. (1990). Olive pollen storage and *in vitro* germination. *Acta Horticulturae*, **286**, 207–10.

Rallo, L. and Fernandez-Escobar, R. (1985). Influence of cultivar and flower thinning within the inflorescence on competition among olive fruit. *Journal of the American Society for Horticultural Science*, **110**, 303–8.

Sedgley, M. (1994). Self-incompatibility in woody horticultural species. In: *Genetic control of self-incompatibility and reproductive development in flowering plants* (Williams, E. G., Clarke, A. E. and Knox, R. B., Eds). Kluwer Academic Publishers, Dordrecht, Boston, and London, 141–63.

Sedgley, M., Bell, F. D. H., Bell, D., Winks, C. W., Pattison, S. J. and Hancock, T. W. (1990). Self- and cross-compatibility of macadamia cultivars. *Journal of Horticultural Science*, **65**, 205–14.

Sharma, P. C., Thakur, D. R. and Sharma, M. R. (1976). Pollination and fruit development studies in olive. *Olea europea*. *Food and Farming Agriculture*, **8**, 24–6.

Sibbett, G. S., Freeman, M., Ferguson, L. and Polito, V. S. (1992). Effect of topically applied 'Sevillano' pollen on normal-seeded and parthenocarpic 'shotberry' fruit set of 'Manzanillo' olive. *HortTechnology*, **2**, 228–30.

Steel, R. G. D. and Torrie, J. H. (1960). *Principles and procedures of statistics, with special reference to the biological sciences*. McGraw-Hill Book Company, Inc., New York, Toronto, and London.

Ugrinovic, K. and Stampar, F. (1996). Fertilization of olive (*Olea europaea* L.) cultivars 'Istrska Belica', 'Pendolino' and 'Leccino' by different pollinators. *Zbornik Biotehniske Fakultete Univerze*, **67**, 183–8.

Ukai, Y. (1989). A microcomputer program DIAL for diallel analysis of quantitative characters. *Japanese Journal of Breeding*, **39**, 107–9.

Vassiliadis, C., Lepart, J., Saumitou, L. P. and Vernet, P. (2000). Self-incompatibility and male fertilization success in *Phillyrea angustifolia* (Oleaceae). *International Journal of Plant Sciences*, **161**, 393–402.

Wu, S.-B., Mekuria, G., Collins, G. & Sedgley, M. (2002, September). Construction of a genetic linkage map for olive (Olea europaea). *Proceedings of the 12th Australasian Plant Breeding Conference.* Perth, Western Australia, 258-260