Genetic Identification of Cultivars and Marker Analysis in Olives (*Olea europaea* L.)

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Olive, *Olea europaea sativa*

Table of Contents

Abstract

Olive cultivation started in Australia in the early 1800s, with the first introduction to South Australia in 1836. Despite numerous attempts, and much government support, the industry has never been commercially successful, although the climate in South Australia is ideal for growing olives that produce high quality oil. Over the years, many olive groves were abandoned, and records about the cultivars, including many original names, have been lost. Natural outcrossing has led to the development of feral or escape olive trees that now extend over much of southern Australia.

Since 1950, many migrants from regions bordering the Mediterranean have emigrated to Australia creating a demand for olive products that now amounts to over $A100 million per annum. To cater for this increased demand, numerous olive groves have been planted in Australia, many financed by large investors. However, the future of the olive industry depends on a number of factors, including the correct identification of cultivars. Other approaches include the selection of trees from the feral population that are adapted to the local environment and have high quality oil, and the development of molecular markers to improve the efficiency of breeding programs. These topics were the focus of the research described in this thesis.

The results from cultivar identification showed that while some cultivars, such as Kalamata, Picual, and Manzanillo, exhibited little genetic variation, others such as Verdale and Mission showed such a high level of genetic variation that some accessions could be considered as separate cultivars. It appears likely that some of the accessions within the Verdale group were either misnamed or that errors were made during propagation. The misnaming of cultivars was clearly demonstrated for the accession of Calamata from Davis. The DNA fingerprint obtained for this accession could not be distinguished from that obtained for Manzanillo. In another instance, an accession of Mission also produced the same DNA fingerprint as Manzanillo. The DNA fingerprints developed in this study have formed the basis for a database that is now used for a commercial identification service to growers.

For any crop species, the gains that can be made from selection depend to a large extent on the genetic variability of the population. The genetic variability of feral olive trees was estimated by selecting an isolated population that was about 70 - 80 years old, consisting of an original

grove, with a few planted progeny, and numerous feral trees. Forty five individual trees were selected and their DNA fingerprints compared. The genetic similarities found between the 45 individuals varied from 67% to 99%, confirming the existence of high genetic diversity in the gene pool. Three molecular clusters were identified that were significantly different indicating that random gene flow was not a feature of this population, and that the spread of olives is mainly by fruit drop, with occasional transport over longer distances by birds or animals.

The genetic improvement of fruit trees is limited by a long juvenile phase and a relatively long life cycle. These problems can be overcome by using marker assisted selection. Olive leaf spot is caused by the fungal pathogen *Spilocea oleaginea*. By using trees from a breeding program in Israel, RAPD markers linked to both leaf spot resistance and susceptibilty were identified by bulked segregant analysis. The resistance marker was more closely linked to the leaf spot trait than the marker for susceptibility.

To increase the utility of the RAPD marker for resistance, it was converted to a sequence tagged site by developing primers specific for the sequence of the RAPD band. These primers were used to screen olives for leaf spot resistance. The STS marker produced results that were consistent with observations that were based on 20 years of field observation. However, the results were less consistent for trees that had been observed for only eight years. Because of the difficulty of ensuring infection under field conditions, the STS marker will enhance the accuracy of detecting leaf spot resistance in the progeny of breeding programs.

This research program has contributed significantly to the information available for olive improvement in Australia and therefore to the future of the olive industry. Some of the results, particularly for the identification of leaf spot resistance, will also be of benefit to other olive producing countries.

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List of Tables

List of Figures

Chapter One

General introduction

1.1 Introduction

The earliest source of information about olives is the Bible, including the religious and domestic uses of olive oil. There is a reference in Exodus where the Lord teaches Moses to prepare the oil for holy anointment and the Lord said: "Take the finest spices: of liquid myrrh and of sweet smelling cinnamon and of olive oil". It is also says: "Send your children to bring you the most virgin oil from the olive tree, so that it is always burning in the lamp".

The olive has become a part of the folklore and tradition of many countries. The olive branch has long been a symbol of peace, security and fruitfulness. The oil was particularly useful to the ancient Greeks who used it for massage to keep themselves in condition for athletics, dancing, games and war to keep their bodies supple and prevent strain and injury (Greer and Blau, 1995).

Olive cultivation is thought to have started around 3000 B.C. in Syria, and from there it spread to Turkey and Egypt, and then to other Mediterranean countries. Although the olive is said to be a species of the Mediterranean, it has been grown in different parts throughout the world where the climate is termed Mediterranean. Olives were introduced to Australia around the beginning of the 19th century. The tree is a major source of revenue for most of the producing countries, but it has been commercially successful in Australia on a relatively small scale.

1.2 Botanical classification of olives

The olive belongs to the *Oleaceae* family that includes approximately 30 genera and 600 species (Fontanazza and Baldoni, 1990). The cultivated olive belongs to the genus *Olea*, species *europaea*, sub-species *sativa*, one of the 20 species of *Olea* found in tropical and subtropical regions of the world. The wild form of olive is referred to as *europaea oleaster*. Of the genus *Olea*, only *europaea* L. produces edible fruit (Martin, 1994). Both the cultivated and the wild oleaster olives have the same chromosome number ($2n = 2x = 46$), and are fully interfertile (Zohary, 1994). Wild olives reproduce from seeds while cultivated

varieties are maintained by vegetative propagation and are clones (Zohary and Spiegel-Roy, 1975).

There is a divergence in opinion as to whether the wild olive tree originated by naturalisation from the cultivated olive or whether the wild forms are indigenous to the Mediterranean area (Ouazzani *et al.*, 1993). According to Chevalier (1948) and Ciferri (1950), the wild tree, sub-species *oleaster*, reverted from cultivated *europaea sativa.* However, Zohary (1994) argued that it is the cultivated olive, *europaea sativa*, derived from a variable aggregate of wild olives, which is widely distributed over the Mediterranean basin. Lavee (1990) argued that most olive cultivars are phenotypic selections from natural seedlings and are only one or two generations away from the wild types.

1.2.1 Cultivated olives

The mature domesticated olive tree is medium-sized, in some cases it can reach a height of 10 m, and the natural crown is rounded. The mature leaf of the olive is usually elongated and spindle-shaped, but leaf shape, size, and characteristics may differ from one cultivar to another. The tree can adapt to a range of environmental conditions, such as drought and high temperature, but the size of the tree and its fruiting potential are closely related to the environmental and management conditions (Lavee, 1996). The olive is a predominantly allogamous species showing a high degree of outcrossing, and is known to exhibit alternate bearing (Lavee, 1996).

In the Mediterranean basin, where olives have been cultivated for thousands of years, hundreds of cultivars have been selected over the centuries for their adaptation to various microclimates and soil types. They are generally characterised by specific forms which have been selected for local conditions. Some varieties produce a high percentage of oil and are best suited to oil production, while others are more suited to the table olive market, due to their large size and small pit. Cultivars with an average oil yield of less than 20 percent are usually used for table olives. In Spain, the major cultivars used for oil production include Picual, Cornicabra, Hojiblanco, and Lechin de Sevilla. Leccino and Frantoio are the major oil cultivars in Italy, Kalamata is a popular table olive cultivar in Greece, and Mission and Manzanillo are the principal varieties in California (Ogawa and English, 1991; Anon, 1992). The more common oil varieties in Australia include Frantoio, Leccino, Nevadillo, and

Pendolino. Among the table olives are Ascolano, Azaba, Barouni, and Kalamata; and dual purpose olives include Manzanillo, Verdale, and Mission (Booth and Davies, 1995).

Cultivars are often known by different names in the areas where they are grown and this creates considerable confusion (Bartolini *et al.*, 1994): the same name may be given to similar but clearly different selections. Manzanilla Cacerena for example, has two other names: Aluellania and Cacerena in Spain, and Azeiteria and Negrihna in Portugal. Similarly, Verdial de Huevar is known by two other names: Verdial Real and Verdial in Spain, and Verdial de Serpa and Verdial Alenteijana in Portugal (Barranco, 1995). In Australia, cultivars are also known by different names. Frantoio for example is known by five other names: Frantoiano, Correggiola, Correggiolo, Razzo, and Gentile, and Verdale is also known as South Australian Verdale and Wagga Verdale. Bartolini *et al.* (1994) ascertained the existence throughout the world of roughly 1,200 cultivars with over 3,000 synonyms. It is therefore important to develop an identification system to clarify these synonyms.

1.2.2 Wild olives

The wild olive, *O. europaea oleaster*, is a small shrubby tree, often with several trunks. It differs from cultivated olives by having smaller fruits with a relatively large stone, thinner mesocarp, lower oil content, and a relatively long juvenile stage (Zohary, 1994; Lavee, 1996). Studying the archaeological evidence, Loukas and Krimbas (1983) concluded that the size of both the olive stone and fruit have increased with time in cultivation, and a fruit size, similar to that of the large sized modern cultivars, appeared at least 2000 years ago. This suggests that selection has taken place over time.

1.2.3 Ferals or escapes

Between the wild and the cultivated olives, secondary forms called ferals can be identified. These originate from seeds derived from crosses either between varieties (cultivars) as well as between cultivars and both feral (escaped) and wild (oleaster) olives (Zohary and Spiegel-Roy, 1975).

As the olive is not native to Australia, the feral olive trees in Australia are believed to be derived from cultivated olives through seed dispersal. Cross-breeding from abandoned

groves over 150 years has resulted in populations of feral olives that grow in all regions of southern Australia (Sedgley and Wirthensohn, 1999). In South Australia, feral olives have spread through wide areas of the Adelaide Hills, Mid North, South East, Fleurieu Peninsula, and Kangaroo Island, and represent a large gene pool that may include economically or agronomically important traits that need to be considered for future olive breeding programs.

1.3 Olive cultivation

1.3.1 World

1.3.1.1 History

The olive tree has been part of Mediterranean civilisation since before recorded history. Depictions of the olive appeared on pottery, coins, and tomb decorations from the ancient world. The earliest records of olive stones, olive oil receptacles and pollen date back approximately 4000 years B.C. in the eastern Mediterranean regions, in the areas of Palestine, Lebanon, North West Syria, and Cyprus (Connell, 1994).

The cultivated olive is believed to have originated in Asia Minor, probably in Syria (Blázquez, 1996) and from there olive cultivation spread northward into Turkey, southward into Egypt, and from Egypt to the Arab world, through north Africa to southern Spain, and from there to other countries.

Three to four thousand years ago the Egyptians traded in olive oil and cured olives (Loukas and Krimbas, 1983). The inhabitants of ancient Crete were probably responsible for the spread of olive culture to Greece and Italy. In the fifth century B.C., Athens was a vast centre of Greek olive culture, and the Romans were becoming familiar with the uses of olive products and olive cultivation (Blázquez, 1996). Olive oil was a profitable export of the time in addition to the technology associated with olive culture and curing for table olive production.

There was little change in the geographical location of the olive until the advent of explorers and colonists. In 1560, olive cuttings were carried to Peru by Spanish explorers, and in the

early 1700s, Jesuits established missions in Mexico and Baja California and introduced olives to those areas (Connell, 1994). Over the past 200 years, olives have been introduced to many areas with Mediterranean climates although the Mediterranean region still dominates production and trade.

1.3.1.2 Major producing areas

The major olive producing countries are located between latitudes 30°N and 45°S with a long hot growing season and a relatively mild winter. The tree requires a mild climate with medium to high temperatures during the summer season. It cannot survive below about -12°C, and most cultivars are injured at -9°C. However, the olive tree must be subjected to a certain amount of chilling in order to initiate flowers for the following cropping season (Connell, 1994).

There is an estimated 8.9 m ha of olive plantings worldwide, of this, 8.4 m ha is in Mediterranean countries (Booth and Davies, 1995). About 10% of the olives produced are used for table olives. There is estimated to be an area of over 2 m ha in Spain and over 1 m ha in Italy, Greece, Tunisia, and Portugal planted to olives (Anon, 1992).

The majority of world olive production comes from four countries, Spain, Italy, Greece, and Tunisia which between them account for 85% of oil and 46% of fruit production (Anon, 2000). The most extensive olive cultivation is practiced in Spain, which produces nearly 29 percent of the world's olive oil and about 32 percent of the world's table olives. Table 1.1, extracted from Anon (2000) shows the major olive producing countries and their corresponding oil and table olive production.

Table 1.1 World production statistics for olive oil and table olives.

Country	Olive Oil Production		Table Olive Production	
	1999/2000 Prod. (,000 t)	World %	1999/2000 Prod. (,000 t)	World %
Spain	652.0	29.0	438.0	32.2
Italy	648.0	28.8	78.0	5.7
Greece	398.0	17.7	100.0	7.3
Tunisia	210.0	9.3	13.5	1.0
Syria	81.0	3.6	93.0	6.8
Turkey	54.0	2.4	150.0	11.0
Algeria	52.5	2.3	42.5	3.1
Portugal	42.9	1.9	8.0	0.6
Morocco	40.0	1.8	80.0	5.9
Argentina	11.0	0.5	58.0	4.3
USA	1.0	-	129.0	9.5
Others	57.2	2.5	171.5	12.6
Total	2248.0	100.0	1361.5	100.0

Source Anon (2000)

1.3.1.3 Economics

The major oil exporting countries are members of the European community (EC) (67%), and Tunisia (24%). The major exporters of table olives are the EC (57%), Morocco (15%), Argentina (12%), and Turkey (9%). The main importers of olive oil are the EC (25%), the USA (37%), and Libya (6%), and the main importers of table olives are the USA (30%), and the EC (17%)(Anon, 2000).

The top consumers of olive oil are Italy, Spain, Greece, USA., Syria, Tunisia, Turkey, and Morocco, which together account for 88.7% of consumption. The leading table olive consumers in the world are the EC (35%), the USA (16%), Turkey (8.5%), Syria (7.2%), Egypt (4.4%), Algeria (2.8%), and Morocco (2.6%) (Anon, 2000).

Importer only countries (which includes Australia) account for 3% of total consumption but have an annual growth of 13% (Booth and Davies, 1995; Anon, 2000). The figures indicate that olive oil consumption is spreading outside traditional consumer markets. This may well be on the basis of perceived health benefits of olive oil (Greer and Blau, 1995).

1.3.2 Australia

1.3.2.1 History

The olive was introduced to Australia in the early 19th century and there are reports of trees being planted at Parramatta, NSW in 1805 (Hobman, 1993). It was introduced to Western Australia in 1831, South Australia in 1836, Victoria in 1879, and later to the other States (Anon, 1983; Burr, 1997). Since its first introduction, there have been numerous attempts to establish the industry with cultivars imported from the Mediterranean and California. The earliest of these attempts was in 1844, when the South Australia Company imported five cultivars including Bouquettier, Verdale, Blanquette, Salouen, and Rubra, from France (Macdonald, 1915), some of which are still popular in the Australian industry. The trees were planted at Beaumont near Adelaide. In 1883, the cultivars Sevillano, Temprano, and Lohayme were imported from Italy, along with Picholine from Montpellier in France, and were established at Palmer, east of Adelaide (Burr, 1997).

By 1890, four olive orchards with a total area of 360 ha had been established in South Australia. They included parts of the Parklands near Adelaide and the suburbs of Stonyfell and Beaumont (Anon, 1899). In the early 1900s the Blackwood experimental orchard was established, with 27 olive cultivars and this was later expanded with another eight cultivars including Mission imported from California (Burr, 1997). By 1920, about 40 named cultivars had been introduced (Quinn, 1920), with the number planted at the Blackwood experimental orchard alone reaching 55 by the 1930s (Fowler, 1939). The results obtained from trials conducted at the different experimental orchards in the State at the time had verified the suitability of the South Australian climate for olive production and the quality of the products. By 1913, South Australia was producing an average of 66,000 L of oil per year, while imports into Australia amounted to 200,000 L (Perkins, 1917).

At the time, efforts were made by the government to encourage olive plantings. The government passed a Crown Law Amendment Act providing for special 21-year leases of land with low rental. This development continued into the early 1900s. However, problems such as the high cost of labour for olive picking, poor management practices,

and the relatively low cost of imported oil (Fowler, 1939) inhibited the expansion of the industry or even the proper maintenance of many established plantations.

Starting from 1939, a considerable number of olive trees was uprooted (Fowler, 1939), and by the 1950s, the Australian olive industry was in major decline, with many olive stocks, such as those at the Blackwood Experimental Orchard, being replaced by other fruits (Anon, 1964). For many of the remaining orchards, records about the names and derivations of the cultivars were often misplaced or confused, leading to a high degree of uncertainty about the identity of some cultivars now being grown. Feral plants, derived from abandoned plantings, thrived in the South Australian environment, and the olive eventually came to be considered as a weed.

The growing demand for olive products, and the profits that could accompany it, has led to the recent investment in, and expansion of, the industry in Australia. The Australian Olive Association was formed in 1995 with the aim of encouraging the sustained development of a national olive industry in Australia through research and dissemination of information (Burr, 1997). Currently, expansion of olive cultivation is occurring in many parts of the country.

1.3.2.2 Major producing areas

Australia has a climate similar to that of countries bordering the Mediterranean and is therefore ideally suited to olive growing and the production of high quality oil. Winter temperatures are relatively mild, yet able to satisfy the vernalisation requirement of olives, and summer temperatures are hot and provide a long ripening period necessary for the fruit to develop to a good size.

In Australia, the major olive plantings are located in the Riverland region of South Australia and the Murray/Mallee and Sunraysia regions of Victoria. Hobman (1993) suggested that certain areas in South Australia are similar to those found in Andulusia in Spain and have hot dry summers, wet winters, and moderately high pH (6.5-8.2) limestone soils, such as the Southern Vales near Adelaide and the irrigation areas along the Murray River. Suitable areas are also found in the Riverland region of SA, and large areas of the Murray Mallee and Sunraysia regions of Victoria and NSW. Olives are also grown in the temperate

regions of Umbria in Central Italy (Hobman, 1993) and similar areas to these are found in Tasmania and the southernmost areas of mainland Australia. These collectively suggest that the Australian climate and soil are favourable for the production of olives.

In 1955 there were 1500 ha of olive plantings recorded in Australia, rising to 2,000 ha by 1993 (Hobman, 1993). By 1998, it was estimated that there were about 5,000 ha across four states: South Australia, Victoria, NSW, and WA (Anon, 1998).

1.3.2.3 Economics

Australian olive production has been estimated at 2,500 t per year, of which 80% is harvested for table olives and is bottled or canned in brine for local consumption. Oil production is very limited, at 100 t, and is mainly for consumption by producer households (Booth and Davies, 1995). Considering the current olive plantings, it is estimated that by 2006, Australia will produce 28,000 t of olive oil (Sweeney, 2000). Australian imports of olive oil increased from about 11,000 t in 1989, to 17,000 t in 1992, and 22,549 in 1999. Imports of table olives have risen from over 4,000 t in 1985 up to 9,000 t in 2000 (Booth and Davies, 1995; Sweeney, 2000; Kailis, 2000). The majority of these imports were from Spain, Italy and Greece. In 1998 olive oil imports were worth about $90 million annually and have grown at annual average rate of about 15%. Table olives worth about 20 m are imported annually (Anon, 1998). Current domestic consumption is nearly equivalent to current imports (Sweeney, 2000). The current demand for olive products caused Australian producers to re-establish the industry, and the availability of large areas of land suitable for olive production should ensure its rapid expansion.

1.4 Olive diseases

1.4.1 Overview

There are a number of diseases that affect olive trees, such as olive leaf spot *(Spilocea oleaginea* Cast. Huges (syn. *Cycloconium oleaginum*), olive knot (*Pseudomonas syringae* pv. *savastanoi* Smith), sooty mould (a complex form of *Capnodium*, *Cladosporium*, and *Alternaria*), verticillium wilt (*Verticillium dahliae* Kleb), leaf and fruit spot of olive (*Mycocentrospora cladosporioids* Sacc.), and armillaria root rot (*Armillaria mellea* Vahl.).

Some cause severe infestation in certain regions while others are reported to have little economic impact (Ogawa and English, 1991; Anon, 1992). Amongst these, olive leaf spot, also known as peacock spot, olive knot, and verticillium wilt, are the major diseases of olives in most olive growing countries.

The symptom of olive knot is the appearance of tumours on twigs and small branches that develop from pruning cuts, or injuries that have occurred during mechanical harvest. The disease is restricted to the above ground parts of the tree and does not affect root tissues. Olive knot reduces production of olive crops by killing twigs and branches following defoliation (Ogawa and English, 1991). In addition, fruits from infected trees may have off flavours. All cultivars are susceptible and damage can be severe under favourable weather conditions (Teviotdale, 1994).

Verticillium wilt is found in many agricultural soils around the world (Teviotdale, 1994). It has a wide host range and is a pathogen of many crops including cotton, melons, peppers, stone fruits, and tomato. The symptoms include leaves on one or more branches suddenly wilting, with repeated attacks over several years causing the tree to die. Most verticillium infections occur in cool, moist soil during late winter and spring (Teviotdale, 1994).

1.4.2 Olive leaf spot disease

Olive leaf spot, peacock spot, peacock eye, and bird's eye spot are alternative names for the same disease (Ogawa and English, 1991). The causal fungus of olive leaf spot was first found in France and named *Cycloconium oleaginum* by Castagne in 1845 (Ogawa and English, 1991). Hughes (1953) suggested that this fungus properly belonged in the genus *Spilocaea*, and thus renamed it S. *oleaginea* (Cast.) Hughes. The disease has been recorded in all Mediterranean olive-growing countries since the mid 1800s, and it has been recognised as the most important leaf disease of olives in China, California, South Africa, South America, and Australia (Hughes, 1953; Ogawa and English, 1991).

The pathogen attacks mainly leaves, producing lesions and circular spots on the upper surface, but in rare cases fruits and fruit stems may also be attacked (Shabi *et al.*, 1994). The lesions first appear as small sooty blotches, and later develop into muddy green to black circular spots, which if numerous result in leaf yellowing and abscission (Ogawa and

English, 1991). The dark green to black spots on the yellow background of the infected leaf blades are said to resemble the spots on a peacock's tail, hence the name peacock spot (Teviotdale, 1994).

Infestation is associated with rainy periods, and is a particularly significant problem in damp, low lying areas during wet years. The disease is spread by rain drops to the lower part of the tree (where the disease is usually most severe), and lateral dissemination occurs to a small extent (Ogawa and English, 1991). Temperatures around 20°C are optimum in the development of the fungus, whereas temperatures higher than this appear to restrict spore germination and mycelial growth (Ogawa and English, 1991).

Because most infected leaves fall prematurely, small branches weaken and subsequently die resulting in a marked reduction in both flower bud differentiation, and fruit set in subsequent years (Shabi *et al.*, 1994). Hartmann (1957) reported that in California, some trees have been reduced in yield by as much as 20% due to partial defoliation of leaves caused by *S. oleaginum*. A complete loss of yield of some sensitive cultivars can occur under extreme inductive climatic conditions (Lavee *et al.*, 1999).

Susceptibility to infection varies amongst cultivars. For example, Leccino and Nevadillo show resistance for peacock disease while others, such as Mission, and Picual, are susceptible (Dal Pero Bertini, 1960). In California, Sutter (1994) found that the order of resistance amongst several cultivars from highest to lowest was Sevillano, Manzanillo, Ascolano, Barouni, and Mission, and, in Israel, Manzanillo is more resistant than Souri and Nabali (Lavee, 1990). In Spain Lechín de Sevilla is found to be tolerant to this disease (Rallo, 1995), and in Australia, most resistance is shown by Corregiola and Frantoio and least by Attica (J. Archer, pers. comm). As olive leaf spot has been a major disease of olives in many countries, the development of molecular markers will greatly assist in screening olive progeny for resistance to olive leaf spot in breeding programs.

1.5 Techniques for cultivar identification

Several authors have taken an interest in the identification of olives. Caballero *et al.* (1990) and Cantini *et al.* (1999) used morphological characters to identify certain known and unknown olive cultivars, while Pontikis *et al.* (1980), and Loukas and Krimbas (1983) used

isozyme analysis to distinguish between olive cultivars of Greek origin. Recently, random amplified polymorphic DNA (RAPD) analysis has been used to identify genetic similarity within and between olive cultivars (Fabbri *et al.*, 1995; Vergari *et al.*, 1996; Weisman *et al.*, 1998; Mekuria *et al.*, 1999; Gemas *et al.*, 2000; Belaj *et al.*, 2001). Other DNA based techniques, such as amplified fragment length polymorphism (AFLP) (Angiolillo *et al.*, 1999), and simple sequence repeats (Rallo *et al.*, 2000) have also been used.

1.5.1 Morphology

Traditionally, olive cultivars have been identified by various morphological characters such as fruit size and shape, pit shape, and pit furrow. Fruit size is correlated with criteria such as average fruit weight, and average flesh to pit ratio, which have been used to classify cultivars into groups based on their main use. While these methods are very effective for many purposes, morphological comparisons may have limitations. Varietal characterisation is complicated by the existence of numerous synonyms and homonyms, as well as by the fact that there are groups of cultivars that have very similar but not identical biomorphological characters (Fontanazza, 1988). In addition, environmental and cultivation factors can modify some morphological characters of individual cultivars.

1.5.2 Gene products

1.5.2.1 Isozyme

The different molecular forms of an enzyme arising from any cause are called isozymes (Markert and Moller, 1959). These form patterns, called zymograms, when plant extracts are separated by electrophoresis. Isozymes have proven useful for varietal identification of olive (Pontikis *et al.*, 1980), mandarin (Elisiário *et al.*, 1999), and in the characterisation of genetic resources in the genus *Chaenomeles* (Garkava *et al.*, 2000).

Pontikis *et al.* (1980) used 16 enzyme systems from pollen to identify 27 olive cultivars. By using five enzymes derived from olive pollen, Trujillo *et al.* (1990) distinguished 134 of 155 olive cultivars. Ouazzani *et al.* (1993) distinguished 33 of 44 olive cultivars using 9 enzymes from leaf tissue but found no clear relationships between isozyme polymorphism and the geographic origin of the cultivars. In examining the genetic variation in wild and

cultivated olive cultivars using isozyme analysis, Ouazzani *et al.* (1993) found no allele differences between wild trees and cultivated cultivars. Similarly, they found no significant differences in allele number and allele frequencies between wild and cultivated trees.

Isozymes are restricted in their ability to distinguish between cultivars because of the limited number of enzymes that can be visualised, and by the possibility that isozyme expression may be influenced by environmental conditions or management practices (Fabbri *et al.*, 1995). When pollen is used, only sexually mature individuals can be analysed, and pollen is only available during a short period in the year.

Alternative methods based on DNA fingerprinting, as presented in Table 1.2, may overcome the limitations of isozyme techniques and morphological traits in identifying varietal polymorphism and specific markers of interest.

Table 1.2 Characteristics of molecular systems for generating genetic markers.

Characteristics	RFLP	RAPD	Microsatellite	AFLP
Principles	Endonuclease restriction Southern blotting Hybridization	DNA amplification with random primers	PCR of simple sequence repeats	Endonuclease restriction DNA amplification with labelled primer
Type of polymorphism	Single base changes Insertion Deletion	Single base changes Insertion Deletion	Changes in length of repeats	Single base changes Insertion Deletion
Genomic abundance	High	Very high	Medium	Very high
Level of polymorphism	Medium	Medium	High	Very high
Dominance	Codominant	Dominant	Codominant	Dominant
Amount of DNA required	2-10 ug	10-25 ng	50-100 ng	0.5-2 ug
Sequence information required	No	No	Yes	No
Radioactive detection required	Yes/No	No	No	Yes/No
Development costs	Medium	Low	High	-
Start-up cost	Medium/High	Low	High	-

Source Rafalski and Tingey (1993) and Hill *et al.* (1996)

1.5.2.2 Restriction fragment length polymorphism (RFLP)

DNA-based molecular marker techniques have proven to be a powerful tool to estimate genetic diversity (Lu *et al.*, 1996; Sun *et al.*, 2001). Among them, RFLP was the first and still the most commonly used in the estimation of genetic diversity of eukaryotic species (Lu *et al.*, 1996). Detection of RFLP markers involves the preparation of radioactively or fluorescently labelled DNA probes, digestion of numerous genomic DNAs with restriction enzymes, gel electrophoresis, and southern blot hybridisation. The patterns of RFLP depend on variation in fragment length between individuals due to altered restriction sites resulting from the insertion or deletion of one or more bases in their genomic DNA. RFLPs have been used for mapping, and to tag useful genes in many agronomic crops, including tomato (Young *et al.*, 1988), and corn (Ahn and Tanksely, 1993), and to study genetic diversity of rice (Sun *et al.*, 2001), and pineapple (Duval *et al.*, 2001).

In some horticultural crops, such as citrus, the small number of probes available limits the use of RFLP methods (Morell *et al.* 1995). Many studies also showed that the number of RFLP polymorphisms identified per probe is low for the development of genetic linkage maps (Apuya *et al.*, 1988; Keim *et al.*, 1990; Lin *et al.*, 1996). The RFLP process is labour intensive, requires large amounts of genomic DNA (5-10 microgram per plant), and usually involves the use of radioactive isotopes (Lin *et al.*, 1996).

1.5.2.3 Microsatellite

Microsatellites are also known as simple sequence length polymorphisms (SSLP, Tautz 1989), sequence tagged microsatellites (STM, Beckman and Soller, 1990), or simple sequence repeats (SSR, Cregan, 1992). Microsatellites are highly polymorphic regions of DNA, containing simple repetitive motifs of di- tri-, and tetranucleotides. The most common motif in plants is AT_n (Lagercrantz *et al.*, 1993; Wang *et al.*, 1994). SSRs are analysed by PCR amplification of a short genomic region containing the repeated sequence, and size estimation of the repeat length by gel separation. SSRs are highly abundant in the plant genome and their loci are polymorphic (Rallo *et al.* 2000; Bertin *et al.*, 2001). In olives, Rallo *et al.* (2000) found 26 alleles among 46 olive cultivars analysed with 5 microsatellites. Similarly, Bertin *et al.* (2001) found 113 alleles among 30 spelt and nine common wheat accessions when analysing 17 microsatellites.

Microsatellites are expensive and laborious to produce since they require allele specific primers (Rallo *et al.*, 2000). A knowledge of suitable DNA sequences from the organism to be studied, or a related species is required, and the widespread application of STS-PCR in plants has been limited by the relatively few DNA sequences known (Goodfellow, 1993).

1.5.2.4 Amplified fragment length polymorphism (AFLP)

AFLP markers are among the most recent innovations in genetic marker technologies (Vos *et al.*, 1995). AFLP markers are based on restriction fragment polymorphisms that are detected by selective PCR amplification. This is accomplished by ligation of DNA adaptors to the ends of restriction fragments followed by amplification using adaptor-homologous primers. Selective restriction amplification is performed with a radioactive, chain luminescent, or fluorescent labelled primer for one end, and an unlabelled primer at the other end. Polymorphisms are usually detected as the presence or absence of amplified fragments and are therefore dominant (Meksem *et al.*, 1995).

Maheswaran *et al.* (1997) used the AFLP technique to study the polymorphism, distribution, and inheritance of AFLP markers in rice populations. Using 20 pairs of primer combinations, they detected 945 AFLP bands of which 208 were polymorphic. Maughan *et al.* (1996) evaluated the degree of AFLP variation both within and between wild and cultivated soybean. Using 15 primer pairs, they identified a total of 759 fragments and observed 274 polymorphic fragments, with an average of 18.3 polymorphic loci per AFLP analysis. By contrast RFLP probes and microsatellites detected only one polymorphic locus (Maughan *et al.*, 1995). Recently, Angiolillo *et al.* (1999) used the AFLP technique to study genetic diversity of cultivated and wild olives. Using five random primer combinations on 90 olive genotypes, they detected 419 fragments of which 288 were polymorphic. AFLP marker analysis is not as easy to perform as RAPD analysis, but more efficient than using RFLPs (Becker *et al.*, 1995) and microsatellites (which need sequencing).

1.5.2.5 Random amplified polymorphic DNA (RAPD)

RAPD analysis, first described by Williams *et al.* (1990) and Welsh and McClelland (1990), has proven to be a useful tool for genetic typing and mapping in both herbaceous and tree crops (Hu and Quiros, 1991; Woolley *et al.*, 2000). RAPD amplification is performed using PCR on genomic DNA with a single short oligonucleotide primer, usually a 10-mer. Primers are generally of random sequence with a G + C percentage of greater than 50% (Stiles *et al.*, 1993). The amplified fragments are separated by gel electrophoresis and visualised by UV transilluminator, after staining in ethidium bromide, or by silver staining.

Each amplification product is derived from a region of the genome that contains two DNA segments complementary to the primer on opposite DNA strands, and sufficiently close to each other to allow DNA amplification to occur. Using short primers and low annealing temperatures ensures that several sites, randomly distributed in the genome, give rise to amplification products. The polymorphisms between individuals result from sequence differences in one or both of the primer binding sites, and are visible as the presence or absence of a particular RAPD band. Such polymorphisms behave as dominant genetic markers (Rafalski and Tingey, 1993).

Tingey *et al.* (1992) compared the RAPD and RFLP techniques and reported that RAPDs are four to six times more efficient per assay and over ten times more efficient on time and labour requirements than RFLPs. This is comparing labour (6 units for RAPD and 23 units for RFLP) and time (14 hours versus 7 days respectively) involved in carrying out the process. With RAPDs, smaller amounts of DNA are required per data point (25-50 ng) compared to that required by RFLPs (2-10 μg). In addition, lack of a requirement for species-specific probe libraries makes it possible to work with a large variety of species for which such libraries are not available. RAPD polymorphisms are usually visualised without the need for labelled radioisotopes, which can be a significant handling and disposal concern. Reproducibility and band dominance are some of the limitations of RAPD techniques (Jones *et al.*, 1997; Hausner *et al.*, 1999).

Morell *et al.* (1995) identified the major cultivars of mandarin in Australia using 18 primers. A total of 91 bands was revealed of which 51 were polymorphic. In a similar study,

Woolley *et al.* (2000) used RAPD analyses to examine genetic similarity among 50 accessions of almond cultivars from five countries. Bradley *et al.* (1996) examined 20 Australian garlic cultivars and divided them into two major groups which co-incided with bolting and non-bolting/intermediate bolting characteristics, and four secondary groups based on early, mid and late season cultivars.

The applicability of the RAPD technique on olives has been verified in studies on the detection of polymorphisms between species belonging to the genus *Olea,* such as *O. cuspidata*, *O. oleaster*, and *O. europaea* (Vergari *et al.*, 1996) and among cultivars belonging to the species *O. europaea* (Bogani *et al.*, 1994; Fabbri *et al.*, 1995, Weisman *et al.*, 1998; Mekuria *et al.*, 1999; Gemas *et al.*, 2000). Fabbri *et al.* (1995) were able to distinguish 17 olive cultivars into small fruited oil cultivars and large fruited table varieties using RAPD analysis. They found, however, no apparent clustering of olives according to geographic location. A similar conclusion resulted from the isozyme analysis carried out by Ouazzani *et al.* (1993). Mekuria *et al.* (1999) assessed the genetic variability of different accessions of olive cultivars obtained from different sources and Weisman *et al.* (1998) identified some common olive varieties in Israel. In a similar study, Gemas *et al.* (2000) used the RAPD technique to identify genetic variability within and between olive cultivars.

1.6 Techniques for marker analysis

1.6.1 Overview

Due to the long juvenile periods, genetic improvement of tree crops has been difficult and time consuming. Thus, tree breeding will benefit from the use of molecular markers associated with genes for horticultural traits through marker aided selection (MAS). This technique would be especially useful where seedlings can be screened for economically important traits that are phenotypically expressed only in fully mature trees (Warburton *et al.*, 1996).

Besides differentiating between various cultivars, molecular methods are also used in the identification of markers linked to specific genes or genomic regions. Several approaches have been suggested to identify genomic regions of interest with molecular markers. These include preselection using nearly isogenic lines (NIL), bulked segregant analysis (BSA)

(Michelmore *et al.*, 1991), and the use of sequence-tagged sites (STS) (Olson *et al.* 1989), and/or sequence characterised regions SCARs (Paran and Michelmore, 1993).

NILs have been developed by introgression, which consists of repeatedly backcrossing a line carrying a gene of interest (Young *et al.*,1988). The basic objective of this breeding technique is the identification of markers located in the linkage block surrounding the introgressed gene (Haley *et al.*, 1993). Young *et al.* (1988) used NILs to identify a DNA marker linked to the *Tm-2a* gene in tomato using RFLP. Using RAPD analysis and NILs, Barua *et al.* (1993) identified markers linked to a *Rhynchosporium secalis* resistance locus in barley. This technique is suitable only for plants with a short generation time and so is of limited use for tree crops such as olive.

Michelmore *et al.* (1991) suggested the technique of bulked segregant analysis to identify genetic markers linked to specific genes of interest. The technique is performed by adding equal amounts of DNA from individuals from each population differing in the trait of interest, and the DNA fingerprints of the two resultant bulk DNA samples should differ significantly in the region linked to the trait.

BSA, when used with the RAPD-PCR technique (Williams *et al.*, 1990), requires no prior knowledge of the genomic DNA being studied. The only prerequisite is the existence of a population resulting from a cross that segregates for the genes of interest, and the success of the approach will depend on the genetic divergence between the parents in the target region (Michelmore *et al.*,1991). NILs require many backcrosses to develop and are therefore time consuming to generate; in contrast, bulked segregants can be made immediately for any locus or genomic region once the segregating population has been constructed. In addition, BSA is a relatively rapid and simple protocol to perform. (Michelmore *et al.*, 1991). The procedure has been used successfully in many studies involving the *Fom* 2 gene for resistance to fusarium wilt in musk melon (Wechter *et al.*, 1995), markers for freestone in peach (Warburton *et al.*, 1996), anthracnose resistance in sorghum (Boora *et al.*, 1998), glaucousness of leaves in cider gum (Wirthensohn *et al.*, 1999), and marker for seed coat colour in *brassica juncea* (Negi *et al.*, 2000). Michelmore *et al.* (1991) identified three RAPD markers linked to downy mildew resistance genes in lettuce using contrasting DNA-bulks composed of F_2 individuals of known genotypes. Using pooled DNA samples of genotyped individuals from two segregating populations of

common bean (*Phaseolus vulgaris*), Haley *et al.* (1993) identified two RAPD markers linked to a rust resistance gene in common bean. Warburton *et al.* (1996) identified linkages between RAPD markers and quantitative trait loci in *Prunus* (peach) using BSA. Using BSA and AFLP, Negi *et al.* (2000) identified a genetic marker linked to seed coat colour in *Brassica juncea.*

Some limitations of BSA are that polymorphic bands cannot always be confirmed as being linked to the trait of interest, and in a bulk, a band present at low frequency may not amplify since competition for primer sites may be too intense (Michelmore *et al.*, 1991). However, some bands present at very low frequencies (i.e present in only one individual in a bulk of ten) may successfully amplify in the bulk.

1.6.2 Sequence tagged site

Different approaches have been used to identify genetic markers linked to a trait of interest. In particular, the RAPD-PCR technique, together with BSA, has been used extensively to identify genetic markers linked to traits of interest in many studies (section 1.6.1). However, the dominant nature of RAPDs, the amplification of multiple loci, and their sensitivity to reaction conditions limit their further uses (Michelmore *et al.*, 1991; Brady *et al.*, 1996; Hausner *et al.*, 1999).

To overcome these problems associated with RAPDs, Paran and Michelmore (1993) developed sequence characterised regions (SCARs). They were able to separate RAPD fragments on an agarose gel, excise the bands from the gel, and reamplify individual bands using the original RAPD primer. The reamplified PCR product was then cloned and sequenced, and longer and more specific primers were developed from the sequence of the RAPD fragments. These longer primers generate a sequence-tagged site (STS) as described by Olson *et al.* (1989). An STS is a short, unique sequence, amplified by PCR, which identifies a known location on a chromosome (Olson *et al.*, 1989). In the development of STS markers, the most critical parameter is the design of primers (Prezioso, 2000). The primer sequence determines the length of the product, its melting temperature, and the yield, and thus several parameters must be taken into account when designing sequence specific primers. Such parameters are primer length (for efficient amplification of the PCR product, and for high specificity), melting temperature (to avoid mismatches),

complementary primer sequences (to prevent primer dimer or cross formation due to intera- and inter- primer homology), G/C content (to prevent non-specific annealing), and 3´ end sequences (for the control of mis-priming) (Prezioso, 2000).

STS and SCAR markers have been developed for many plant species, including markers linked to the downy mildew resistance gene in lettuce (Paran and Michelmore, 1993), the *Mi* gene for nematode resistance in tomato (Williamson *et al.*, 1994), seed coat colour in *Brassica juncea*, (Negi *et al.*, 2000), and the *Tm-2* locus of the genus *Lycopersicon* (Ohmori *et al.*, 2000).

1.7 Summary

The olive is one of the earliest cultivated plant species, and for millennia has been a part of the human diet, and a source of revenue in many countries. In Australia, as in most major producing countries in the world, the demand for olive products is continually increasing, and the industry has the potential to become a significant part of the agricultural sector, because of the cost of oil imports.

Cultivated olives include many cultivars some of unknown identity, and some with numerous synonyms. During the last 150 or so years of unsuccessful olive farming, many groves in Australia were abandoned and the records of the cultivars that were planted were lost. Thus many of the cultivar names that appear in early records are no longer evident. To add to the confusion, the same cultivar can have different names in different areas. In addition, the outcrossing nature of the olive has resulted in a prolific feral olive population that now covers many parts of southern Australia, and is probably the largest gene pool of olives in the world.

In common with many fruit trees, olives pass through a juvenile period and screening the progeny of a breeding program for traits of interest based on field observation is costly and time consuming. With the development of molecular techniques, it is now possible to identify molecular markers that are linked to a particular character, such as disease resistance.

1.8 Thesis aims

This research program will investigate three areas of interest in olive biology and production:

1. A comparison of genotypes of some common commercial olives grown in Australia with those obtained from collections in other countries.
2. An assessment of the genetic diversity and gene flow in an isolated feral population of olives.
3. The identification of a genetic marker linked to resistance or susceptibility to olive leaf spot, a common fungal disease of olives.

Chapter Two

General materials and methods

2.1 Leaf material

Young, fully-grown leaves that were apparently free of pests and diseases were used for genomic DNA isolation. The source and number of accessions used is described under each relevant chapter.

2.2 Leaf storage and handling

Since olive leaves were to be transported from overseas and interstate, it was necessary to develop a handling and storage technique for the leaves to ensure that DNA of high quality and adequate yield for subsequent analysis would be obtained. The method described by Field (1997) achieved this. Fresh leaves, still attached to the stem, were tightly wrapped in Cling Wrap to exclude as much air as possible. A label was attached setting out the variety, the date the leaves were collected, the name of the person who collected the leaves, and the location of the tree, and a further layer of Cling Wrap was applied. Upon arrival leaves were stored at 4°C. In this way it was possible to obtain DNA of high quality from leaves sent from various sources after more than 10 days in transit and if necessary, subsequent storage for a further 8-12 weeks at 4°C.

2.3 DNA isolation

Because olive fruits have been shown to have a relatively high content of phenolic compounds (Fontanazza *et al.*, 1994; Burr, 1997), it was assumed that the leaves would contain some polyphenols which could interfere with the recovery of DNA of high purity. For this reason, the method to extract DNA was based on those shown to be successful in other plants that have high levels of phenolics such as *Banksia* (Maguire *et al.*, 1994) and grapevines (Steenkamp *et al.*, 1994).

Approximately 2 g of fresh leaves were ground with liquid nitrogen into a fine powder in a pre-chilled mortar and pestle and transferred to 7.5 mL of cold (0°C) extraction buffer

containing 3% (w/v) cetyltrimethyl ammonium bromide (CTAB), 1.4 M NaCl, 0.1 M EDTA, pH 8.0, 1.0 M Tris HCl, pH 8.0. Just before use, 0.2% (v/v) of 2-mercaptoethanol, and 15 mg polyvinylpyrollidone (PVP-40) (SIGMA Chemical Co., MO, USA) was added.

The sample was incubated at 60°C for 30 min with occasional mixing and placed on ice to cool. An equal volume of chloroform/iso-amyl alcohol [24:1 (v/v)] was added and mixed gently for 10 min followed by centrifuging at 3000 rpm for 20 min at room temperature (RT). The upper aqueous layer was removed and mixed gently with 0.6 vol of cold (0°C) isopropanol and the DNA allowed to precipitate at -20°C for 30 min. The DNA was spooled onto a glass rod and transferred to a clean tube containing 20 mL wash buffer (76% ethanol (v/v), 10 mM NH_4Ac) (Steenkamp *et al.*, 1994). The DNA was washed until white and then dissolved in 1 mL TE buffer (10 mM Tris HCl, 0.1 mM EDTA, pH 8.0).

RNA was removed by digesting with 2 µL of 10 mg/mL DNase-free RNase A (AMRESCO®, Solon, OHIO, USA, prepared as described in Sambrook *et al.*, 1989) for 30 min at 37°C. The solution was diluted with 2 mL of TE buffer and 1 mL of 7.5 M NH_4Ac and placed on ice for 20 min to precipitate protein, including RNase A (Crouse and Amorese, 1997), followed by centrifuging at 10,000 rpm for 20 min at 4°C. The aqueous layer was transferred into a sterile centrifuge tube, mixed with 2 volumes of cold ethanol (-20°C), placed on ice for 20 min, and centrifuged at 8000 rpm for 10 min at 4°C. After draining the tube, the pellet was recovered in 1 mL of TE buffer (pH 8). The absorbance of the solution was determined at 230, 260, and 280 nm, and the quality of the DNA was estimated by calculating the ratios of the absorbances at 260 and 230 and at 260 and 280 (Johnson, 1994). DNA samples with absorbance ratios of 1.8 and greater were used for further analysis, and stored at -20°C.

2.4 DNA amplification

Random oligodeoxyribonucleotide primers, 10 bases in length (10-mers), were used for the polymerase chain reaction (PCR) amplification (Williams *et al.*, 1990). These were either purchased from Operon Technologies, Almeda, CA, USA, or made by the Nucleic Acid and Protein Chemistry Unit, Adelaide University.

PCR was performed in a volume of 20 µL containing 40 ng of genomic DNA, 1.5 mM $MgCl_2$, 0.25 µM 10-mer primer, 200 µM each of dGTP, dATP, dTTP, and dCTP, 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 (GIBCOBRL®, Life Technologies Inc., Gaithersburg, Md, USA), and overlaid with two drops of mineral oil. A control PCR mix containing all components but no genomic DNA was run with each primer to check for DNA contamination.

DNA amplification was performed in a DNA thermocycler (Programmable Thermal Controller, MJ Research Inc., Watertown, Mass., USA) according to the following programme: 2 min at 95°C for initial strand separation, then 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).

2.5 Separation of PCR products

DNA separation was accomplished using both agarose and polyacrylamide gel electrophoresis (PAGE).

2.5.1 Agarose gels (ethidium bromide stained)

For each sample, 8 µL of the amplification products were mixed with two µL of loading buffer (50% glycerol, 75 mM EDTA, 0.2% sodium dodecyl sulfate (SDS), 0.07% each of bromophenol blue and xylene cyanol FF) (Watson and Thompson, 1986), and separated on agarose gel. The concentration and types of agarose varied, based on the type of experiment performed. Gel electrophoresis was accomplished in 1 x TBE buffer (89 mM Tris HCl, pH 8.3, 89 mM boric acid, 5 mM EDTA) at constant amount of 80 mA. After electrophoresis, gels were stained with ethidium bromide (0.5 µg/mL), destained with distilled water, visualised under 254 nm UV light and photographed using Polaroid 667 film. Gels images were captured using the Tekcap computer program (version 1.0. Tekram Corporation, 1998), and saved in a bitmap format using PaintShop Pro (version 5.00, Jasc Software Inc, 1998).

2.5.2 Polyacrylamide gels (silver stained)

DNA amplification fragments were separated by polyacrylamide gel electrophoresis as described by Collins and Symons (1993). Denaturing gel electrophoresis was carried out using a vertical 16 x 13 cm gel running apparatus. One mm thick gel was prepared using 6% polyacrylamide (Accugel^{TM} 40% 19:1 acrylamide: bisacrylamide, Kimberly Research, Atlanta., Georgia) and 7 M urea in 1 x TBE. 10 µL/mL ammonium persulphate (APS) (10%) and 1.2 µL/ mL N,N,N',N',-tetramethylethylenediamine (TEMED) (both from Sigma Chemical Co. St. Louis, USA) were added as polymerisation catalysts.

Twelve µL of PCR reaction was mixed with an equal volume of formamide loading dye (95% 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 90°C and chilled on ice for 5 min just before loading. Gels were pre-heated for 15 min at 25 mA before the samples were loaded and run for 1 hr at 25 mA. For greater resolution of higher molecular weight products, 3 M unbuffered NaAc (0.1 vol) was added to the lower chamber one hour after loading and electrophoresis was continued for a further 2½ hrs at 25 mA.

After PAGE, the PCR products were visualised by silver staining following the protocol of Bassam *et al.* (1991). Gels were gently shaken in 10% acetic acid for 20 min. After 3 washes each of 2 min with sterilised water, the gels were stained with silver solution (0.2 gm $AgN0_3$ and 300 µL of 38% formaldehyde (HCOH), in 200 mL of water) for 30 min. After a rapid rinse with water (20 sec), the gel was developed in cold (4°C) alkaline solution (12 gm $NaCO_3$, 600 µL of 30% HCOH, and 800 µL NaS_2O_3 (1 mg/mL) in 400 mL of water) until bands were of the required intensity. The reaction was then stoped 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.

2.6 Data analysis

Each sample of DNA was subjected to three separate PCR reactions. Bands that were consistently amplified over the three reactions were scored, and the size of the DNA polymorphic fragments were estimated and scored either visually and/or by Scion Image digital gel analyser program (Rasband, 1997) using a 100 bp ladder (DMW-100M, GeneWorks, Adelaide, Australia). The bands in each lane were scored for their presence and absence compared to other lanes, where 1 was allocated for the presence of an amplified fragment and 0 for its absence.

Various numerical methods have been developed for expressing the genetic similarity within or between a population. The simple matching coefficient (Sokal and Michener, 1958), Jaccard's coefficient (Jaccard, 1908), and Nei and Li (Nei and Li, 1979) are the most commonly used methods for genetic similarity assessment.

The simple matching coefficient has the algorithm:

Sab = $[(x + y)/(n + z)] \times 100$ where Sab is the percentage of genetic similarity between two individuals a and b; x is the sum of the matched positive bands (11); y is the sum of the matched negative bands (00); z is the sum of unmatched bands (01, 10), and n is the sum of x + y.

The coefficient of Jaccard has the algorithm:

Sab = $x/[(n + z)-y] \times 100$. This is the equivalent of the simple matching coefficient but omits negative matches.

The coefficient of Nei and Li has the algorithm:

Sab = $2x/(2x + z) \times 100$. This emphasises matched pairs by giving them twice the weight of unmatched pairs, and, like the Jaccard coefficient, omits negative matches.

The incorporation of negative matches into a coefficient of association has been discussed by Sokal and Sneath (1963), and Stiles *et al.* (1993). Stiles *et al.* (1993) argued that when the cultivars under study are closely related, the same sequence variation may be responsible

for the lack of amplification products, and the absence of a band in two individuals may be the result of an identical ancestral mutation (Apostol *et al.*, 1993). This may indicate that the absence of a particular band in a DNA fingerprint is as important as its presence, and gives credibility to the simple matching coefficient. Lamboy (1994) found that the simple matching coefficient exhibits less percent bias than the Jaccard coefficient for closely related organisms. Thus, in the present study, the analysis of the genetic similarities of olives, which are assumed to be closely related, were calculated using the simple matching coefficient.

The Numerical Taxonomy Systems Software package NTSYS-pc (Applied Biostatistics Inc. v. 2.02k)(Rohlf, 1993), was used to analyse the data. A similarity matrix was calculated using the SIMQUAL (Similarity for qualitative data) component of this software using the simple matching coefficient (Sokal and Michener, 1958). Dendrograms showing genetic similarities were constructed using the Unweighed Pair Group Mathematical Average (UPGMA) with the SAHN (sequential, agglomerative, hierarchical, and nested clustering methods) algorithm (Rohlf, 1993).

Chapter Three

Genetic similarities between accessions of commercial cultivars

3.1 Abstract

Genetic similarities between accessions of olive cultivars, *Olea europaea*, obtained from overseas collections and various sites within Australia, were investigated using RAPD, simple matching coefficient, and UPGMA clustering. Twenty two accessions of olives representing three cultivars (Manzanillo, Kalamata, and Verdale), and 17 accessions representing two groups that are suspected to include synonyms (Corregiola, Corregiolo, Frantoio, Frantoja, and Frantojol; Nevadillo, Nevadillo Blanco, and Picual) were examined. The results showed that, for Manzanillo and Kalamata, there was a within-cultivar genetic similarity of at least 98% irrespective of source. In contrast, Verdale showed a high genetic variability, with some accessions having less than 80% similarity to others. Picual and Nevadillo (including Nevadillo Blanco) clustered at 69%, indicating that they are probably distinct cultivars. The identity of Corregiola and Frantoio and their synonyms is uncertain. Considering the extent of the genetic distances involved between accessions within the Corregiola/Frantoio group, and also within the Verdale group, it is likely that some of the within-group accessions are separate cultivars.

3.2 Introduction

The olive tree has been part of Mediterranean civilisation since before recorded history, and was first cultivated and traded for its oil as early as 3000 BC (Connell, 1994). However, *Olea europaea* is not native to Australia, and cultivation only commenced after commercial species were introduced in the 19th Century (Anon, 1983). Since then, numerous attempts have been made to establish a viable Australian industry, using cultivars imported from the Mediterranean and California, but due to the high cost of labour for olive picking, poor management practices, the alternate bearing habit of olives, and the relatively low cost of imported oil (Fowler, 1939) the industry has never been successful. As a result a considerable number of olive trees were uprooted and many of the remaining were abandoned, leading to uncertainty about the names of many cultivars currently being grown in Australia. The reasons for this uncertainty are firstly, records concerning the names of

the cultivars were often misplaced or lost, and secondly, some imported cultivars have been given new names and the original names have been forgotten (Dal Pero Bertini, 1960). For example, Abel, Aglanaou, and Alameno are just a few of the cultivars recorded as being introduced into Australia that are now unknown in the industry (Burr, 1997). Thirdly, olive seedlings in the past were sold labeled either as 'oil' or 'pickling' varieties, or just as 'olives' (Hartmann, 1962). Furthermore, the presence of synonyms and homonyms in olive cultivars is a long-standing problem in olive producing countries world-wide (Macdonald, 1915; Bartolini *et al.*, 1994). In most olive producing countries, hundreds of cultivars have been selected over the centuries for their adaptation to various microclimates and soil types. Bartolini *et al.* (1994) ascertained the existence throughout the world of roughly 1,200 named cultivars with over 3,000 synonyms. In some cases the synonyms are completely different, and in others only a single letter may be changed. For example, Correggiola (Roberts, 1942), Correggiolo and Correggiola (Dal Pero Bertini, 1960), and Corregiola (Fowler, 1930), all apparently refer to the same cultivar. Nevadillo is known as Nevadillo Blanco, Blanco and Picual (Barranco, 1995; Archer, 1997), whereas Frantoio is known as Correggiola, Correggiolo, Frantoiano, Razzo and Gentile (Dal Pero Bertini, 1960). In Australia, the cultivar Verdale is known as Verdale, SA Verdale, and Wagga Verdale.

The olive industry in Australia is currently being revived, and to ensure that high quality products of international standard are produced, it is necessary to identify cultivars that are recognised world-wide.

The traditional technique for identifying olive cultivars is based on morphological and agronomic characters. However, these are known to vary due to environmental and cultivation factors (Booth and Davies, 1995; Vergari *et al.*, 1996). Identification based on the analysis of gene products, such as isozymes, has been used (Pontikis *et al.*, 1980; Loukas and Krimbas, 1983), but is disadvantaged by the relatively small number of polymorphisms that are produced, and the possibility that isozyme expression can also be altered by environmental conditions (Fabbri *et al.*, 1995).

DNA based methods, including RAPD (Williams *et al.*, 1990; Welsh and McClelland, 1990), microsatellite length polymorphisms (Cregan, 1992), and AFLP (Vos *et al.*, 1995) have been used for cultivar identification in many plant species. In particular, the RAPD-PCR technique has been used by a number of authors to assess genetic similarity within and

between olive cultivars (Fabbri *et al.*, 1995; Weisman *et al.*,1998; Gemas *et al.*, 2000; Belaj *et al.*, 2001).

The level of polymorphisms produced by RAPDs is high, the cost is relatively low, and no prior knowledge is needed of the sequences being targetted (Weeden *et al.*, 1992). Furthermore, unlike RFLP and AFLP, RAPD markers are visualised without the need for labelled radioisotopes which can be a significant handling and disposal concern. In the present study, this technique has been used: (1) to determine the genetic similarities between accessions of some named commercial olives obtained from different sources, and (2) to investigate the level of genetic similarities between cultivars suspected to be synonyms or homonyms.

3.3 Materials and methods

3.3.1 Leaf material

Leaves from accessions of olive cultivars were obtained from: Foundation Plant Material Service, California, Davis, USA; Institute of Horticulture, The Volcani Centre, Bet-Dagan, Israel; CO-RIPR-OL, Pescia, Italy; and from various public and private sites within Australia. Table 3.1 shows the cultivars which were selected for the study, together with their sources and the number of accessions of each.

Table 3.1 Sources and number of accessions of the cultivars of olives tested for genetic similarities.

Accession	Source where leaves were collected	Previous source
Manzanillo - Israel	Volcani Centre, Bet-Dagan, Israel	Spain
Manzanillo - Davis	Davis, California, USA	na
Manzanillo - Vic	Victoria (Vic), Australia	Davis
Manzanillo - QLD	Queensland (QLD), Australia	Vic
Manzanillo - NSW	New South Wales (NSW), Australia	Vic
Manzanillo - CSU	Charles Sturt University (CSU), Wagga, Australia	na
Manzanillo - SA	South Australia (SA), Australia	Vic
Kalamata - Israel	Volcani Centre, Bet-Dagan, Israel	Greece
Kalamata - QLD	Queensland (QLD), Australia	Greece
Kalamata - NSW	New South Wales (NSW), Australia	Greece
Kalamata - SA	South Australia (SA), Australia	SA
Calamata - Davis	Davis, California, USA	na
Verdale - Israel	Volcani Centre, Bet-Dagan, Israel	Spain
Verdale - Davis	Davis, California, USA	na
Verdale - BEO	Blackwood Experimental Orchard (BEO), Australia	na
Verdale -SA	South Australia (SA), Australia	na
SA Verdale - Vic	Victoria (Vic), Australia	na
SA Verdale - QLD	Queensland (QLD), Australia	Vic
SA Verdale - NSW	New South Wales (NSW), Australia	Vic
SA Verdale - SA	South Australia (SA), Australia	Vic
Wagga Verdale - QLD	Queensland (QLD), Australia	CSU
Wagga Verdale - CSU	Charles Sturt University (CSU), Wagga, Australia	na
Nevadillo - Vic	Victoria (Vic), Australia	Davis
Nevadillo - Davis	Davis, California, USA	na
Nevadillo Blanco - QLD	Queensland (QLD), Australia	Vic
Nevadillo Blanco - CSU	Charles Sturt University (CSU), Wagga, Australia	na
Picual - Israel	Volcani Centre, Bet-Dagan, Israel	Spain
Picual - Davis	Davis, California, USA	na
Picual - SA (Israel)	South Australia (SA), Australia	Israel
Picual - SA (Spain)	South Australia (SA), Australia	Spain
Corregiola - QLD	Queensland (QLD), Australia	NSW
Corregiola - CSU	Charles Sturt University (CSU), Wagga, Australia	na
Corregiolo - Davis	Davis, California, USA	na
Corregiolo - SA	South Australia (SA), Australia	NSW
Frantoio - Italy	CO.RIPR.OL, Pescia, Italy	Italy
Frantoio - Israel	Volcani Centre, Bet-Dagan, Israel	Italy
Frantoio - SA	South Australia (SA), Australia	Italy
Frantojol - Davis	Davis, California, USA	Italy
Frantoja - BEO	Blackwood Experimental Orchard (BEO), Australia	na

na - information not available

3.3.2 DNA Isolation and PCR amplification

DNA isolation and DNA amplification were performed as described in Chapter 2.

3.3.3 Primer screening

Thirty ten-base oligodeoxyribonucleotides, either purchased from Operon Technologies, Alameda, CA, USA, or made by the Nucleic Acid and Protein Chemistry Unit, Adelaide University, were screened for their RAPD products on genomic DNA extracted from the following seven olive genotypes using 2.5% agarose gels (Seakem® GTG® agarose, FMC, BioProducts, Rockland, Maine): Kalamata, Manzanillo, Mission, Nevadillo Blanco, Pendulina, SA Verdale, and Wagga Verdale. A number of factors has been taken into consideration to select the best primers for DNA amplification. These includes the ability of the primers to produce polymorphisms, the clarity of the bands, the reproducibility of results, and the GC content. Primers that produce a high number of polymorphic fragments with clear RAPD profiles were compared for their reproducibility over three separate PCR runs, using PAGE, on seven Manzanillo accessions that had produced identical fingerprints during the preliminary screening.

As a further test of reproducibility of amplified fragments, four cultivars (Mission, Nevadillo Blanco, Pendulina, SA Verdale) that produced highly polymorphic bands in the preliminary screening were examined.

3.3.4 Data analysis

Silver stained gels are highly sensitive in detecting nucleic acids compared to agarose gels (Bassam *et al.*, 1991), and thus in the present study this technique was used to identify small genetic varition found between accessions of a cultivar.

Digital images of polyacrylamide gels for data analysis were obtained using a ScanJet II CX/T scanner (v.2.8, Hewlett-Packard Co. USA.). For band identification of scanned images, two computer programs were tried: Cream™ for Windows (Ken-En-Tec, Copenhagen, Denmark), and Simdex (Archer and Leung, http://www.hku.hk/zoology/simdex2). However, for both programs, the bands which were automatically scored did not match those of either the digital images or the original gels.

Both programs either identified bands which were not present on the scanned images or missed out strong bands, and the facility in those programs for manually correcting this was found to be tedious, time consuming, and also subjective. Since all accessions in each group were separated on the same gel, and each group was analysed separately, visual scoring of the presence and absence of amplification fragments was found to be easier and less subjective. The data were analysed as described in section 2.6.

3.4 Results

3.4.1 Primer screening and amplification characters

The 30 ten-base oligodeoxyribonucleotide primers tested produced a total of 329 RAPD fragments of which 48% were polymorphic. The number of amplified bands per primer varied from seven to 19, and the number of polymorphic bands varied from 0 to 10. Fifteen of the 30 primers produced a high number of polymorphic fragments with clear RAPD profiles, and were tested for their reproducibility. Six of the 15 primers produced from 92-100% reproducible DNA fragments and all had a G+C content of at least 60% (Table 3.2). These primers were used to assess genetic similarity in this study.

Except for the initial screening of primers for polymorphic bands, all accessions included in this chapter were subjected to PAGE as described in Chapter 2.

Table 3.2 Nucleotide sequence and the G + C content of the six decamer primers selected to differentiate olive accessions.

Primer	Sequence (5´-3´)	G + C (%)
GC-01	CAGGCCCTTC	70%
GC-05	AGGGGTCTTG	60%
GC-18	AGGTGACCGT	60%
GC-20	GTTGCGATCC	60%
OPZ-11	CTCAGTCGCA	60%
OPZ-13	GACTAAGCCC	60%

3.4.2 DNA amplification between duplicate DNA extracts

To test the reproducibility of the techniques being used, genomic DNA was extracted from duplicate samples of Mission and Picholine. DNA was extracted either on the same day, or on separate days, and subjected to PCR using three decamer primers (GC-01, GC-18, and OPZ-13). The three primers produced a total of 102 and 109 amplified fragments for Mission and Picholine, respectively. All primers produced identical RAPD fragments between duplicate extracts of the DNA being compared.

3.4.3 Accessions of cultivars with similar designated names

3.4.3.1 Genetic similarities between accessions of Manzanillo

Genetic similarities were investigated for seven Manzanillo accessions (Table 3.1). Using the six primers shown in Table 3.2, a total of 166 RAPD fragments were detected of which only 2 were polymorphic. The number of amplified bands per primer varied from 19 for OPZ-11 to 35 for GC-05. Two of the primers, GC-05 and GC-20, produced a single polymorphic band on the DNA extracted from the QLD accession. No polymorhism was observed on any of the Manzanillo accessions using the remaining four primers. RAPD fingerprints for the Manzanillo accessions amplified using GC-05, and a dendrogram showing genetic similarities between these accessions, are presented in Figs. 3.1 and 3.2, respectively.

The dendrogram has two clusters, one containing 6 accessions which have a genetic similarity of 100% and the other containing the single accession from QLD at 99%.

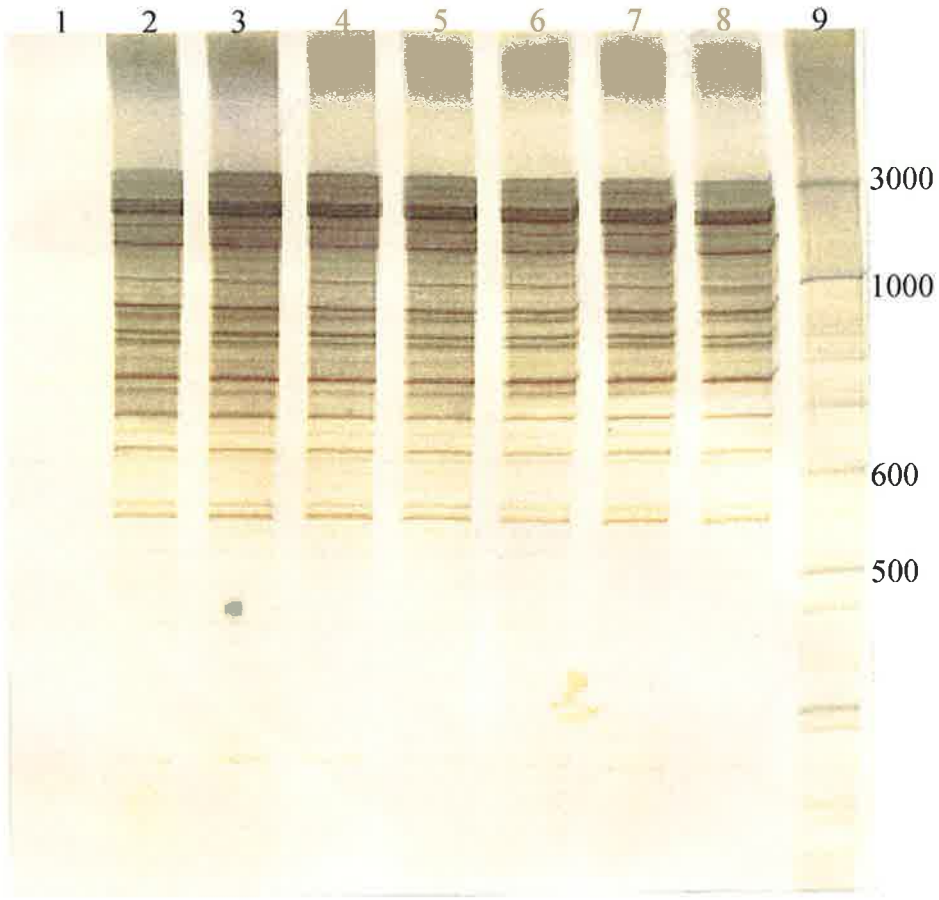


Fig. 3.1 DNA fragments amplified from seven Manzanillo accessions using primer GC-05. Lane 1: Control (no genomic DNA); lane 2: Manzanillo- Israel; lane 3: Manzanillo- Davis; lane 4: Manzanillo- SA; lane 5: Manzanillo- Vic; lane 6: Manzanillo- NSW; lane 7: Manzanillo- CSU; lane 8: Manzanillo- QLD; lane 9: 100 bp DNA ladder.

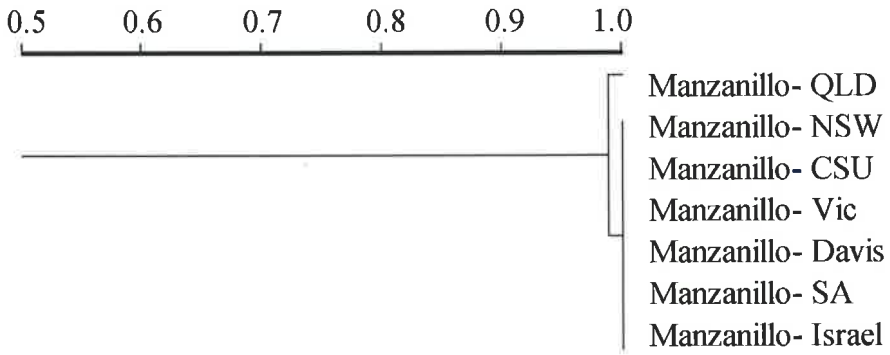


Fig. 3.2 Dendrogram derived from the genetic similarities of Manzanillo accessions using six primers.

3.4.3.2 Genetic similarities between accessions of Kalamata and Calamata

Genetic similarities were investigated for four Kalamata and one Calamata accessions (Table 3.1). Using the six primers shown in Table 3.2, a total of 211 RAPD fragments were detected of which 25% were polymorphic. The number of amplified bands per primer varied from 29 for GC-05 to 46 for OPZ-13, and the number of polymorphic bands per primer varied from seven for GC-05 to 11 for both GC-20 and OPZ-13. RAPD fingerprints for the Kalamata and Calamata accessions amplified using GC-05 and GC-20, and a dendrogram showing genetic similarities between these accessions, are presented in Figs. 3.3 and 3.4, respectively.

The dendrogram has four clusters, one containing the Calamata from Davis which has a genetic similarity of 76% to the cluster containing the Kalamatas from SA, Israel, NSW, and QLD. The Kalamata from SA showed a 98% genetic similarity to the Kalamatas from Israel, NSW, and QLD. The Kalamata from Israel had a 99.2% genetic similarity to those from NSW and QLD which were closely linked at 99.5%.

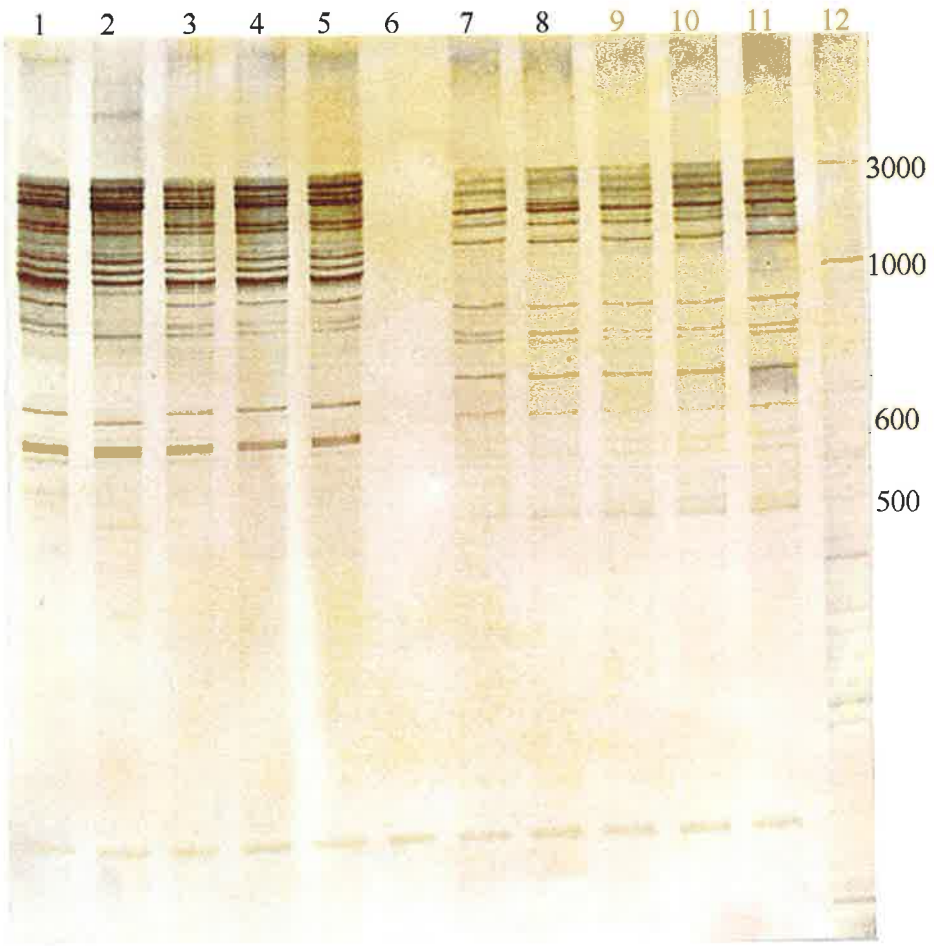


Fig. 3.3 DNA fragments amplified from Kalamata and Calamata accessions using primer GC-05 (lane 7-12) and GC-20 (lane 1-5). Lane 1 and 7: Kalamata- Israel; lane 2 and 8: Calmata- Davis; lane 3 and 9: Kalmata- SA; lane 4 and 10: Kalamata-NSW; lane 5 and 11: Kalamata-QLD; lane 6: Control (no genomic DNA); lane 12: 100 bp DNA ladder.

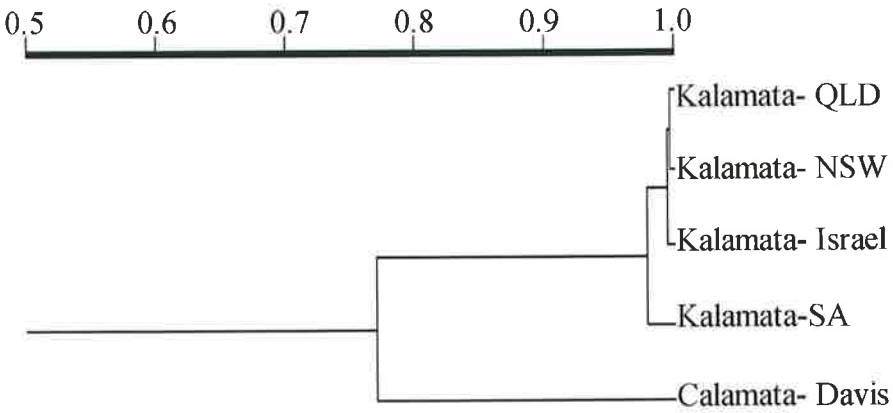


Fig. 3.4 Dendrogram derived from the genetic similarities of Kalamata and Calamata accessions using six primers.

3.4.3.3 Genetic similarities between accessions of Verdale

Genetic similarities were investigated for four Verdale accessions (Table 3.1). Using the six primers shown in Table 3.2, a total of 192 RAPD fragments were detected of which 42% were polymorphic. The number of amplified bands per primer varied from 28 for GC-18 to 36 for OPZ-13, and the number of polymorphic bands per primer varied from nine for GC-18 to 18 for GC-01. RAPD fingerprints for the Verdale accessions amplified using OPZ-13, and a dendrogram showing genetic similarities between these accessions, are presented in Figs. 3.5 and 3.6, respectively. The Verdale from Israel showed a genetic similarity of 76% to the two Verdale accessions from SA and BEO, and the Verdale from Davis. The Verdale from Davis clustered at 83% to the two Verdales from SA and BEO, which were closely linked at 92%.

3.4.3.4 Genetic similarities between accessions of SA Verdale

Genetic similarities were investigated for four SA Verdale accessions (Table 3.1). Using the six primers shown in Table 3.2, a total of 146 RAPD fragments were detected of which only four were polymorphic. The number of amplified bands per primer varied from 19 for OPZ-11 to 33 for OPZ-13. Two primers, GC-05 and OPZ-11, each produced two polymorphic bands on the DNA extracted from the QLD accession. No genetic variation was observed for any of the SA Verdale accessions using the remaining four primers. RAPD fingerprints for the Verdale accessions amplified using OPZ-13, and a dendrogram showing genetic similarities between these accessions, are presented in Figs. 3.5 and 3.6, respectively. The QLD accession of SA Verdale showed a genetic similarity of 97% to the SA Verdales from NSW, Victoria, and SA, all of which clustered at 100%.

3.4.3.5 Genetic similarities between accessions of Wagga Verdale

Genetic similarities were investigated for two Wagga Verdale accessions (Table 3.1). Using the six primers shown in Table 3.2, a total of 169 RAPD fragments were detected of which 25% were polymorphic. The number of amplified bands per primer varied from 19 for OPZ-11 to 37 for OPZ-13, and the number of polymorphic bands per primer varied from four for GC-18 to nine for both GC-05 and OPZ-13. RAPD fingerprints for the Verdale

accessions amplified using OPZ-13, and a dendrogram showing genetic similarities between these accessions, are presented in Figs. 3.5 and 3.6, respectively. The result revealed a 75% genetic similarity between the Wagga Verdale accessions obtained from QLD and CSU.

3.4.3.6 Genetic similarities between accessions of Verdale, SA Verdale, and Wagga Verdale

Genetic similarities were investigated for four Verdale, four SA Verdale, and two Wagga Verdale accessions (Table 3.1). Using the six primers shown in Table 3.2, a total of 194 RAPD fragments were detected of which 46% were polymorphic. The number of amplified bands per primer varied from 28 for both GC-18 and OPZ-11 to 38 for OPZ-13, and the number of polymorphic bands per primer varied from nine for GC-18 to 20 for OPZ-11. RAPD fingerprints for the Verdale accessions amplified using OPZ-13, and a dendrogram showing genetic similarities between these accessions, are presented in Figs. 3.5 and 3.6, respectively.

The dendrogram has 7 clusters, one containing the Verdale from Israel which has a genetic similarity of 77% to the cluster containing the Wagga Verdales from CSU and QLD, the Verdales from SA and Davis and the SA Verdales from SA, Victoria, NSW and QLD. The Wagga Verdale from CSU showed a 79% genetic similarity to the Wagga Verdale from QLD, the Verdales from SA and Davis, and the SA Verdales from SA, Victoria, NSW and QLD. The Wagga Verdale from QLD showed an 80% similarity to the Verdales from SA and Davis, and the SA Verdales from SA, Victoria, NSW and QLD. The two Verdales from SA and BEO formed a separate cluster with a genetic similarity of 92%, and these showed an 84% genetic similarity to the Verdale from Davis and the SA Verdales from SA, Victoria, NSW and QLD. The SA Verdale from QLD showed a 98% similarity to Verdale from Davis and the SA Verdales from SA, NSW and Victoria. The Verdale from Davis showed a 99% similarity to the SA Verdales from SA, Victoria and NSW, all of which clustered at 100%.

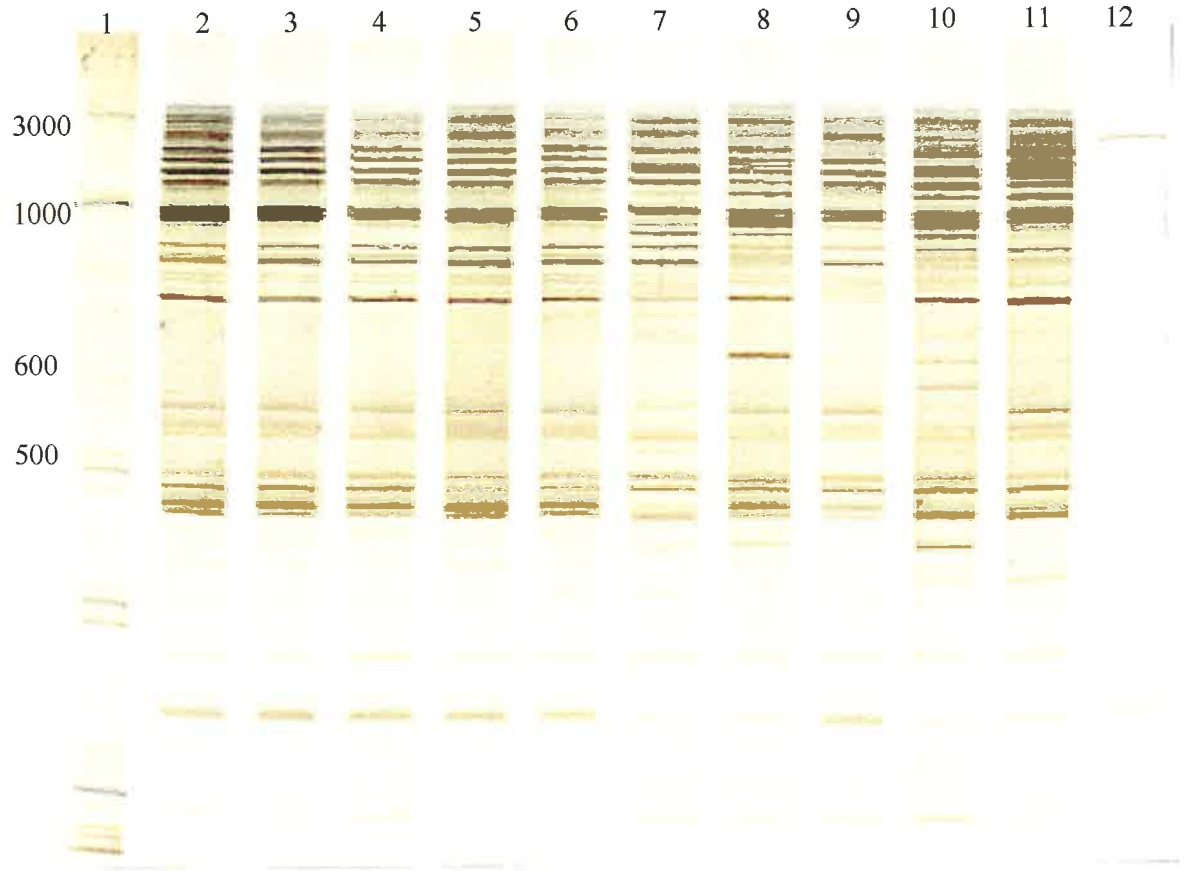


Fig. 3.5 DNA fragments amplified from 10 Verdale accessions using primer OPZ-13. Lane 1: 100 bp DNA ladder; lane 2: SA Verdale- QLD; lane 3: SA Verdale- NSW; lane 4: SA Verdale- Vic; lane 5: SA Verdale- SA; lane 6: Verdale- Davis; lane 7: Verdale- Israel; lane 8:Wagga Verdale- QLD; lane 9: Wagga Verdale- CSU; lane 10: Verdale- SA; 11: Verdale- BEO; 12: Control (no genomic DNA).

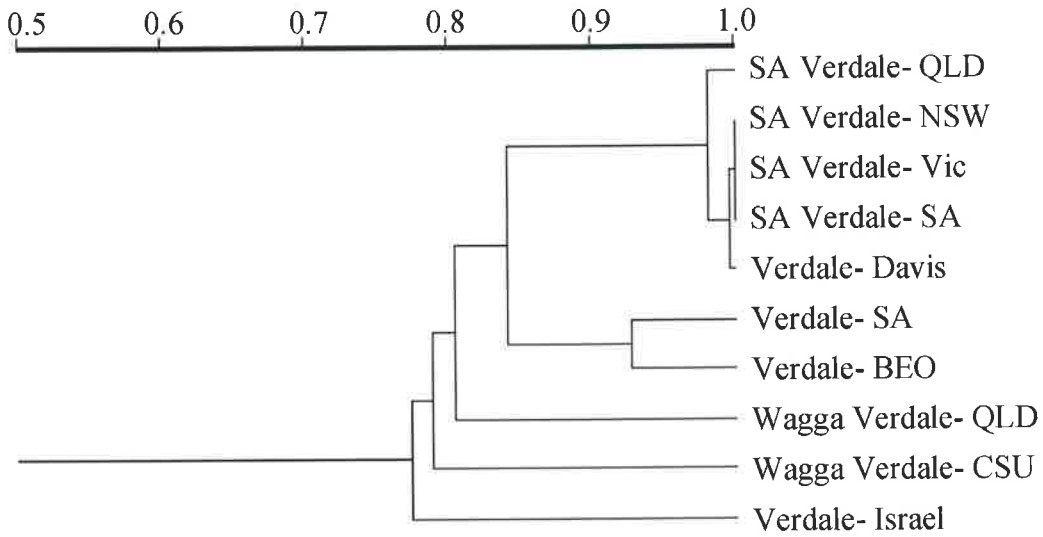


Fig. 3.6 Dendrogram derived from the genetic similarities of Verdale, SA Verdale, and Wagga Verdale accessions using six primers.

3.4.3.7 Genetic similarities between accessions of Nevadillo and Nevadillo Blanco

Genetic similarities were investigated for two Nevadillo and two Nevadillo Blanco accessions (Table 3.1). Using the six primers shown in Table 3.2, a total of 101 RAPD fragments were detected of which only two were polymorphic. The number of amplified bands per primer varied from 11 for GC-05 to 20 for OPZ-11. One primer, OPZ-11, produced two polymorphic bands on the DNA extracted from Nevadillo Blanco from CSU and Nevadillo from Victoria. No genetic variation was observed on any of the Nevadillo and Nevadillo Blanco accessions using the remaining five primers.

RAPD fingerprints for the Nevadillo and Nevadillo Blanco accessions amplified using OPZ-13, and a dendrogram showing genetic similarities between these accessions, are presented in Figs. 3.7 and 3.8, respectively. The Nevadillo from Victoria has a genetic similarity of 98% to the Nevadillo Blancos from CSU and QLD and Nevadillo from Davis. The Nevadillo Blanco from CSU had a 99% genetic similarity to the Nevadillo from Davis and the Nevadillo Blanco from QLD, both of which clustered at 100%.

3.4.3.8 Genetic similarities between accessions of Picual

Genetic similarities were investigated for four Picual accessions (Table 3.1). Using the six primers shown in Table 3.2, a total of 100 RAPD fragments were detected of which only three were polymorphic. The number of amplified bands per primer varied from 11 for GC-05 to 20 for both OPZ-11 and OPZ-13. One primer, OPZ-11, produced three polymorphic bands on the DNA extracted from accessions from Davis and Israel. No genetic variation was observed on any of the Picual accessions using the remaining five primers.

RAPD fingerprints for the Picual accessions amplified using OPZ-13, and a dendrogram showing genetic similarities between these accessions, are presented in Figs. 3.7 and 3.8, respectively. The Picual from Israel has a genetic similarity of 98% to the two Picuals from SA, and the Picual from Davis. The Picual from Davis showed a 99% genetic similarity to the two Picuals from SA, both of which clustered at 100%.

3.4.3.9 Genetic similarities between accessions of Corregiola and Corregiolo

Genetic similarities were investigated for two Corregiolo and two Corregiola accessions (Table 3.1). Using the six primers shown in Table 3.2, a total of 211 RAPD fragments were detected of which 33% were polymorphic. The number of amplified bands per primer varied from 27 for OPZ-11 to 46 for OPZ-13, and the number of polymorphic bands per primer varied from 10 for three primers GC-01, GC-05 and OPZ-11 to 15 for OPZ-13. RAPD fingerprints for the Corregiolo and Corregiola accessions amplified using OPZ-13, and a dendrogram showing genetic similarities between these accessions, are presented in Figs. 3.9 and 3.10, respectively. The Corregiolo accession from Davis has a genetic similarity of 66% to the Corregiolo from SA and the Corregiolas from CSU and QLD, all of which clustered at 100%.

3.4.3.10 Genetic similarities between accessions of Frantoio, and Frantojol

Genetic similarities were investigated for three Frantoio, one Frantojol and one Frantoja accessions (Table 3.1). Using the six primers shown in Table 3.2, a total of 203 RAPD fragments were detected of which 17% were polymorphic. The number of amplified bands per primer varied from 24 for OPZ-11 to 45 for OPZ-13, and the number of polymorphic bands per primer varied from three for GC-01 to eight for OPZ-13. RAPD fingerprints for the Frantoio and Frantojol accessions amplified using OPZ-13, and a dendrogram showing genetic similarities between these accessions, are presented in Figs. 3.9 and 3.10, respectively. The Frantoio accession from Israel has a genetic similarity of 85% to the Frantojol from Davis, the Frantoio from SA, Italy, and Frantoja from BEO. The Frantojol from Davis showed a 97% genetic similarity to the Frantoio from SA, Italy, and Frantoja from BEO which were closely linked at 99%.

3.4.4 Accessions of cultivars with suspected synonyms

3.4.4.1 Genetic similarities between accessions of Nevadillo, Nevadillo Blanco, and Picual

Genetic similarities were investigated for two Nevadillo, two Nevadillo Blanco, and four Picual accessions (Table 3.1). Using the six primers shown in Table 3.2, a total of 120 RAPD fragments were detected of which 30% were polymorphic. The number of amplified bands per primer varied from 12 for GC-05 to 26 for OPZ-13, and the number of polymorphic bands per primer varied from two for both GC-05 and GC-20 to 13 for OPZ-13.

RAPD fingerprints for the Nevadillo, Nevadillo Blanco and Picual accessions amplified using OPZ-13, and a dendrogram showing genetic similarities between these accessions, are presented in Figs. 3.7 and 3.8, respectively.

The dendrogram has two main clusters that separate Nevadillo and Nevadillo Blanco from Picual at 69% genetic similarity. The genetic similarities within these two clusters are discussed in sections 3.4.3.7 and 3.4.3.8.

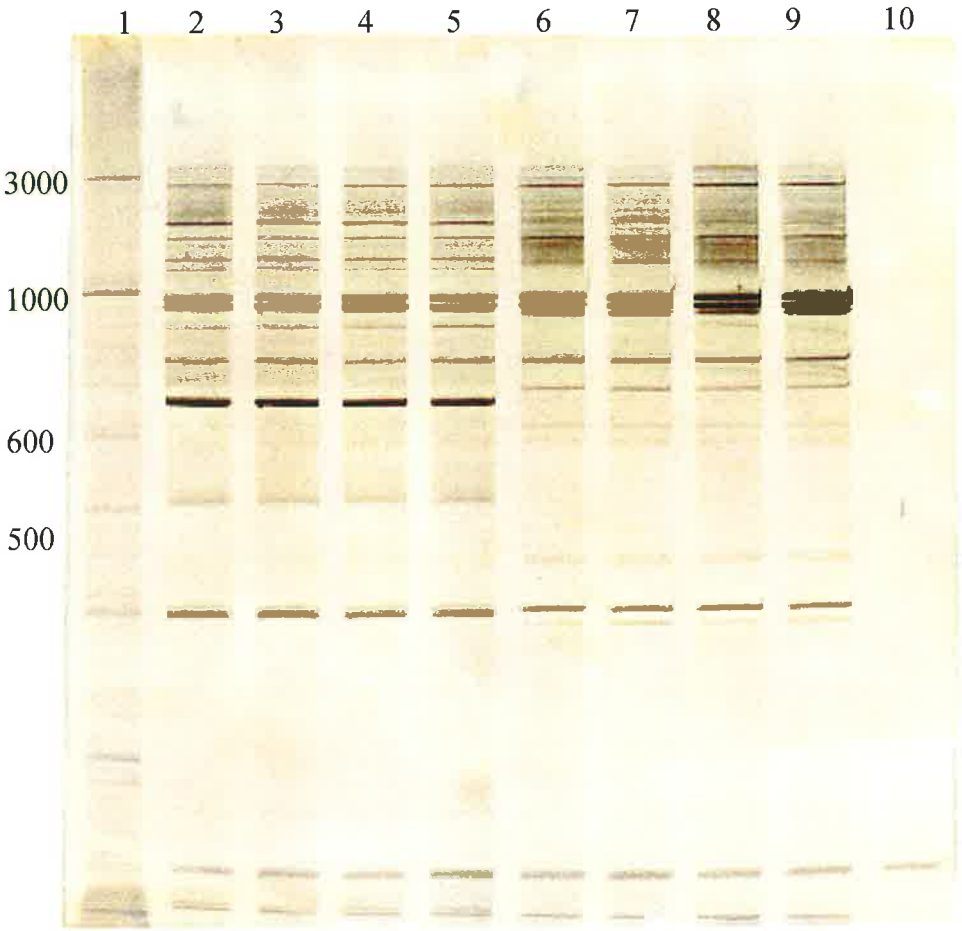


Fig. 3.7 DNA fragments amplified from accessions of Nevadillo, Nevadillo Blanco, and Picual using primer OPZ-13. Lane 1: 100 bp DNA ladder; lane 2: Nevadillo Blanco- QLD; lane 3: Nevadillo Blanco- CSU; lane 4: Nevadillo- Vic; lane 5: Nevadillo- Davis; lane 6: Picual- Davis; lane 7: Picual- Israel; lane 8: Picual- SA (Israel); and Lane 9: Picual- SA (Spain); lane 10: Control (no genomic DNA).

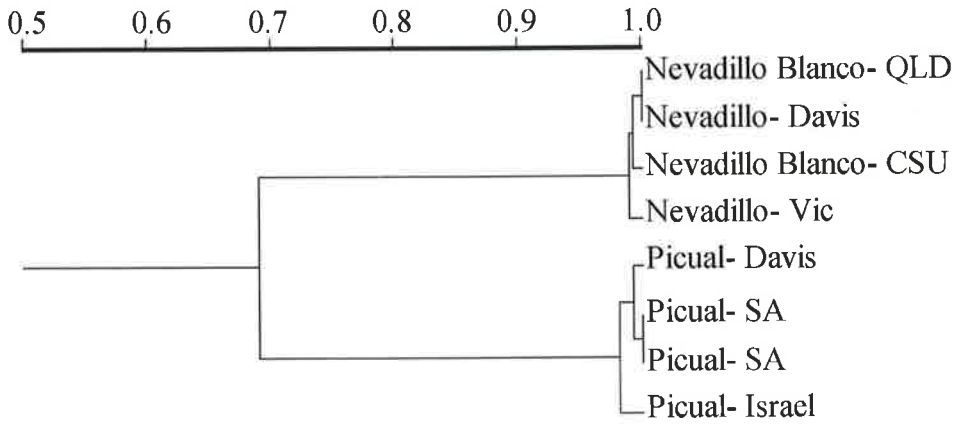


Fig. 3.8 Dendrogram derived from the genetic similarities of Nevadillo, Nevadillo Blanco, and Picual accessions using six primers.

3.4.4.2 Genetic similarities between accessions of Corregiolo, Corregiola, Frantoio, Frantoja, and Frantojol

Genetic similarities were investigated for two Corregiolo, two Corregiola, three Frantoio, one Frantoja and one Frantojol accessions (Table 3.1). Using the six primers shown in Table 3.2, a total of 221 RAPD fragments were detected of which 40% were polymorphic. The number of amplified bands per primer varied from 29 for OPZ-11 to 48 for OPZ-13, and the number of polymorphic bands per primer varied from 12 for GC-05 to 19 for OPZ-13.

RAPD fingerprints for the Corregiolo, Corregiola, Frantoio (Israel, SA), and Frantojol accessions amplified using OPZ-13, and a dendrogram showing genetic similarities between these and a Frantoio from Italy, and Frantoja accessions, are presented in Figs. 3.9 and 3.10, respectively.

The dendrogram has six clusters, one containing the Corregiolo from Davis which has a genetic similarity of 68% to the cluster containing the Frantoios from Israel, SA, and Italy, Frantoja from BEO, Frantojol from Davis, Corregiolo from SA, and the Corregiolas from CSU and QLD. Frantoio from Israel showed 85% genetic similarity to the Frantoios from SA and Italy, Frantoja from BEO, Frantojol from Davis, Corregiolo from SA, and the Corregiolas from CSU and QLD. The Frantoios from SA and Italy clustered at 99.5% and had a 99% genetic similarity to the Frantoja from BEO, all of which showed a 94% genetic similarity to Frantojol from Davis, Corregiolo from SA and Corregiolas from CSU and QLD. The Frantojol from Davis showed a 97% genetic similarity to the Corregiolo from SA and the Corregiolas from CSU and QLD, all of which clustered at 100%.

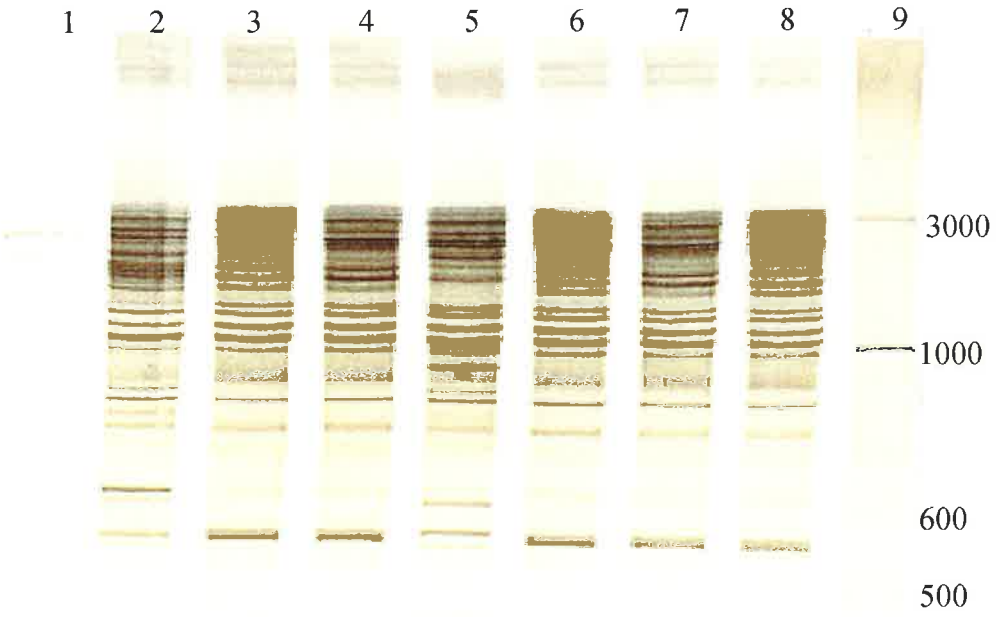


Fig. 3.9 DNA fragments amplified from accessions of Corregiola, Corregiolo, Frantoio, and Fantojol using primer GC-20. Lane 1: Control (no genomic DNA); lane 2: Frantoio- Israel; lane 3: Frantojol- Davis; lane 4: Frantoio- SA; lane 5: Corregiolo- Davis; lane 6: Corregiolo- SA; lane 7: Corregiola- CSU; lane 8: Corregiola- QLD; and lane 9: 100 bp DNA ladder.

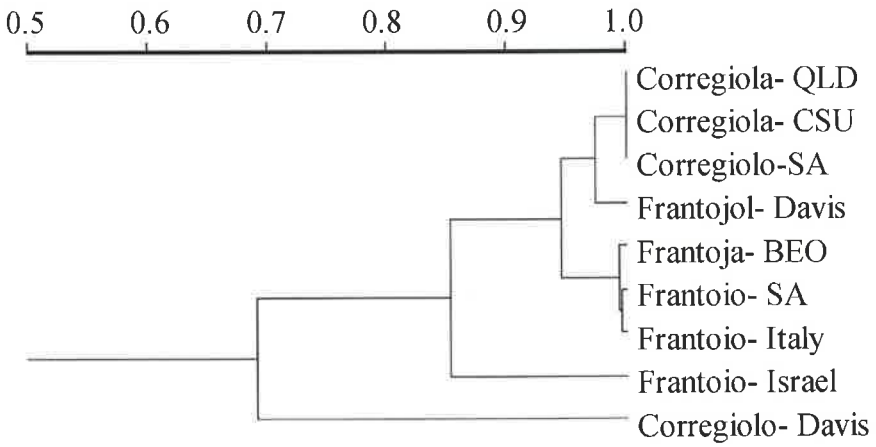


Fig. 3.10 Dendrogram derived from the genetic similarities of Corregiolo, Corregiola, Frantoio, Frantojol, and Frantoja accessions using six primers.

3.4.5 Identification of some misnamed cultivars

Preliminary DNA fingerprinting analysis was performed using a single accession each of Mission, Calamata, and Corregiolo all obtained from Davis, and three accession of Mission from NSW, BEO, and SA. It was observed that the Corregiolo accession showed high genetic similarity to the Missions from Davis, NSW, and BEO while the Calamata accession was closely linked to Mission from SA but distant from the others within the cluster. When the DNA fingerprints of Calamata and Mission from SA were compared against the fingerprints of other accessions included in this study, they were found to be similar to those of Manzanillo. Therefore, Manzanillo was included in subsequent analyses.

Genetic similarities were investigated among Corregiolo, Calamata, and Manzanillo, all obtained from Davis, and individual accessions of Mission obtained from Davis, NSW, SA, and BEO. Using the six primers shown in Table 3.2, a total of 182 RAPD fragments were detected of which 20% were polymorphic. The number of amplified bands per primer varied from 25 for OPZ-11 to 41 for OPZ-13, and the number of polymorphic bands per primer varied from 2 for GC-05 to 11 for GC-01. Fig. 3.11 shows a dendrogram derived from the similarity matrix which was calculated from the 182 RAPD fragments.

The dendrogram has two main clusters, one containing the Corregiolo from Davis which showed a genetic similarity of 95% to the cluster containing the Missions from Davis, NSW, and BEO. The Mission from Davis showed a 98% genetic similarity to the Missions from NSW and BEO, which were linked at 100%. The second cluster, containing Calamata and Manzanillo from Davis, showed 100% similarity to the Mission from SA, and about 84% to cluster 1.

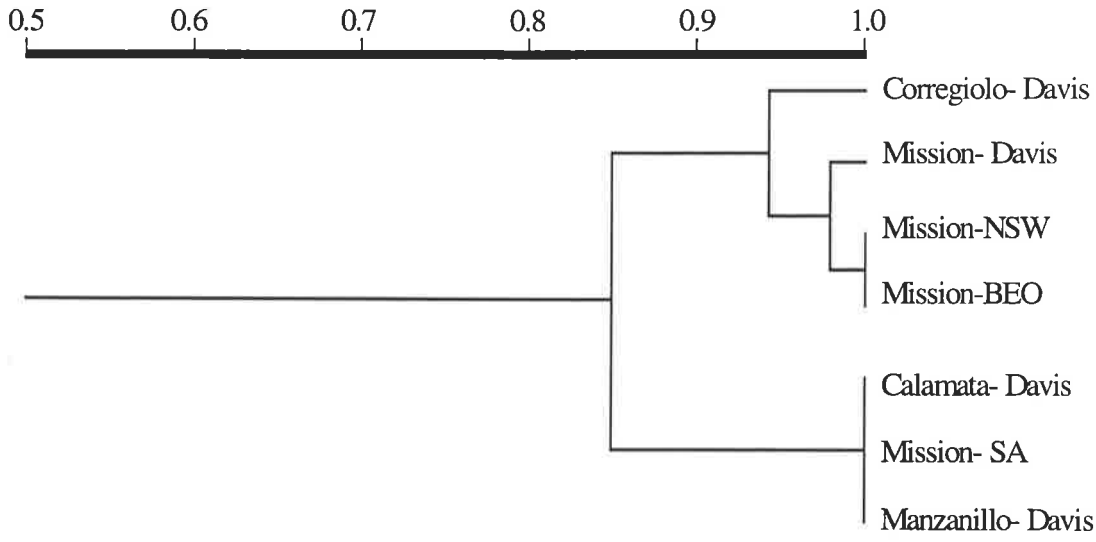


Fig. 3.11 Dendrogram derived from the genetic similarities of Corregiolo, Calamata, Manzanillo, and Mission accessions using six primers.

3.5 Discussion

3.5.1 Amplification characters of primers and reproducibility of results

RAPD analysis has proven to be a useful tool for the identification of cultivars of various crop plants, and in the estimation of genetic similarities both within and between populations (Fabbri *et al.*, 1995; Maguire *et al.*, 1997; Woolley *et al.*, 2000). The RAPD-PCR technique is performed on genomic DNA with single short oligonucleotide primers, usually 10-mers. The polymorphisms between individuals result from sequence differences between the primer binding sites, and are visible as the presence or absence of a particular RAPD band. Such polymorphisms behave as dominant genetic markers (Rafalski and Tingey, 1993). The amplified fragments are usually separated by agarose or polyacrylamide gel electrophoresis.

The major limitation of RAPD is reported to be the reproducibility of the amplified fragments (Jones *et al.*, 1997). Lamboy (1994) and Morell *et al.* (1995) argued that changes in PCR parameters such as annealing temperature, DNA purity, and ramping speeds of thermocyclers, can cause variability in RAPD banding patterns. In addition, Schierwater and Ender (1993) stated that the most important factor is the type and source of DNA polymerase. Furthermore, the use of ethidium bromide versus silver staining for

detection of products has been reported to affect the results of RAPD data (Caetano-Anolles *et al.*, 1992). A number of approaches has been adopted to improve the reproducibility of RAPD bands (Yang and Quiros, 1993; Morell *et al.*, 1995; Skroch and Nienhuis, 1995). These include the use of highly purified DNA, careful selection of primers, uniformity of amplification conditions between replicates, and analysing only those bands which are reproducible across replicate runs (Skroch and Nienhuis,1995; Oliveira *et al.*, 1999).

All of the DNA samples used in this study had ratios of absorbance at 260 nm to 280 nm greater than 1.8, which falls within the optimal range for protein and phenol free DNA (Sambrook *et al.*, 1989). The 260 nm to 230 nm ratios are also described as sensitive indicators for contaminating materials in the DNA preparation (Johnson, 1994), and only samples with ratios greater than 1.8 were used. To maintain the reproducibility of results, RAPD was carried out in replicate PCR runs, using the same thermocycler and same brand of *Taq* polymerase. RAPD bands were scored and analysed only if they appeared in all replicate PCR runs. Each of the six primers used in this study showed a reproducibility of at least 92%.

The optimum number of primers needed to unequivocally differentiate between the DNA of two or more cultivars will depend on the level of polymorphism that is expected. Belaj *et al.* (2001) found that four primers were sufficient to distinguish between 51 olive cultivars. Mulcahy *et al.* (1993) also used two decamer primers, and obtained distinctive fingerprints for several apple (*Malus* x *domestica* Borkh) cultivars. On the other hand, Woolley *et al.* (2000) used 6 primers to differentiate between 50 accessions of different almond cultivars. The six primers used in this study (Table 3.2) produced sufficient polymorphic RAPD products to differentiate between accessions of known cultivars, between known cultivars, and between cultivars with suspected synonyms.

Three of the primers used, GC-05, GC-20, and OPZ-11, produced the most polymorphisms within accessions of cultivars. Primers GC-05 and GC-20 detected unique polymorphisms between Manzanillo accessions, and OPZ-11 detected polymorphisms between Picual accessions, and between different accessions of Nevadillo and Nevadillo Blanco. Primer OPZ-13 detected a large number of polymorphisms in most of the accessions tested. The number of polymorphic bands that were detected from the other two primers varied

between different accessions. These results indicate that polymorphisms can be easily and reproducibly detected between accessions of individual cultivars of olives if reaction conditions are stringently controlled and primers are selected carefully.

3.5.2 Genetic similarity between accessions of cultivars with similar designated names

Genetic variation between accessions of cultivars has been reported in a range of cultivated plants. Singh *et al.* (1973) reported isozyme variation in accessions of three out of ten oat cultivars. Using isozyme analysis, Howard (1977, quoted in Loukas and Pontikis, 1981) suggested that minor genetic differences exist within supposedly uniform apple rootstock. Since commercial olives are propagated asexually, identical RAPD patterns were expected between accessions of individual cultivars. However, in this study some cultivars exhibited high genetic similarity, while others showed variability between accessions obtained from different sources.

According to Macdonald (1915), the Manzanillo variety was introduced originally into Australia from California, and it is possible that more than one accession was introduced. However, Manzanillo accessions obtained from seven sources showed little or no genetic variation. From a total of 166 RAPD fragments amplified, only two polymorphic bands were produced, and these were in an accession from QLD.

A similar results was found for Kalamata obtained from three sources within Australia and one from Israel. However, the accession of Calamata from Davis showed only 76% genetic similarity to the other accessions in the group (Fig. 3.4). It was assumed that Calamata was a synonym for Kalamata, but the genetic distance shown by this cultivar to the others in the group suggested that it was a different cultivar. Subsequent testing revealed that Calamata produced the same DNA fingerprints as Manzanillo.

Verdale was one of the first olive cultivars grown in South Australia and was introduced from France (Macdonald, 1915; Dal Pero Bertini, 1960). Verdale is described as a popular, widely cultivated variety which produces the highest return in South Australia (Quinn, 1920; Archer, 1997). Macdonald (1915) compared the agronomic characters of Verdales in Australia and France, and suggested that they were not the same cultivars. The origin of

SA Verdale and Wagga Verdale in Australia is unclear but they are reported by Godden (1983) to be strains of Verdale. This was confirmed by Burr (pers. comm.) who also suggested that they gained their names from the original regions where they were planted in Australia. According to Archer (1997), there is a significant variation in fruit size and oil content, flesh-to-pit ratio, and time of harvesting between Verdale, SA Verdale, and Wagga Verdale.

A high level of genetic variation was exhibited among the accessions of Verdale, SA Verdale, and Wagga Verdale included in this study. All SA Verdale accessions obtained within Australia, and the Verdale obtained from Davis, showed a 98% genetic similarity (Fig. 3.6). It is possible that the SA Verdale accessions included in this study were originally derived from Davis and were renamed at a later stage. On the other hand, Verdale from the Blackwood Experimental Orchard, South Australia (Table 3.1), is believed to be established from grafted seedlings (Quinn, 1920), and Verdale from SA showed only 86% genetic similarity to the other Verdales, SA Verdales, and Wagga Verdales. Similarly, the two Wagga Verdale accessions obtained from QLD and NSW, showed only 78% similarity and the Verdale from Israel, which was originally derived from Spain (Lavee, pers. comm.), was distinguished from the other accessions in the group at 77% genetic similarity. There is clearly a high level of genetic variability within the accessions in the Verdale group.

Nevadillo Blanco was recorded amongst the earliest introduction of olive cultivars in Australia (Macdonald, 1915), and is also sometimes called Nevadillo. All Nevadillo Blanco and Nevadillo accessions showed at least 98% genetic similarity (Fig. 3.8). Only two polymorphic bands were produced, and these were in the accession of Nevadillo Blanco from NSW, and in the accession of Nevadillo from Victoria.

Little is known about Picual and its cultivation in Australia. All Picual accessions showed about 98% genetic similarity (Fig. 3.8). Only three polymorphic bands were produced, and these were in the accessions from Israel and Davis.

Corregiola was probably one of the early introduced cultivars into Australia (Fowler, 1930). Two Corregiola and one Corregiolo accessions obtained within Australia showed no genetic variation, and have a 100% genetic similarity. One accession of Corregiolo from Davis on

the other hand showed only a 68% genetic similarity to the other accessions in the group (Fig. 3.10). All 70 polymorphic bands produced between Corregiolos and Corregiolas were generated from Corregiolo from Davis. The Corregiolo from Davis was expected to be similar to Corregiola and Corregiolo obtained from other sources, but the genetic distance shown by this cultivar to the others in the group suggests that it may be a different cultivar. It is also possible that the Corregiolo and Corregiola obtained within Australia might be misnamed and are different selections.

The accessions of Frantoio obtained from Italy and SA, Frantoja from BEO, and Frantojol from Davis showed little genetic variation, which varied from 97% for the Frantojol from Davis to 99.5% for the Frantoio from SA and Italy, and the Frantoja from BEO. These accessions may be the same cultivar. The Frantoio accession from Israel, which was expected to be similar to the other accessions, showed only 85% genetic similarity to the other accessions in the group (Fig. 3.10). Hence, it is unlikely that the Australian Frantoio was derived from Israel.

The finding that genetic variation occurs within accessions of a cultivar supports the results of other research. Using pollen from olives, Pontikis *et al.* (1980) found a 98% genetic similarity between the zymograms of two accessions of Kalamon obtained from different locations in Greece. Also, using isozyme analysis, Hilali and Antari (1994) found heterogeneity within the cultivar Picholine grown in Morocco. Furthermore, using RAPD analysis, Wiesman *et al.* (1998) found genetic similarity ranging from 95-99% between accessions of cultivar Souri grown in Israel.

Most of the olive accessions obtained from California for the present study are believed to be derived from scions grafted on Mission rootstock (B. Prins pers. comm.). It is possible that, due to the multi-stem growth habit of olives, material may have been derived from the rootstock instead of the scion. To clarify this factor, DNA fingerprints of Corregiolo and Calamata obtained from Davis were compared against accessions of Mission and Manzanillo. The results revealed that Corregiolo showed high genetic similarity to accessions of Mission obtained from Davis, NSW, and BEO, while Calamata showed high genetic similarity to accessions of Mission from SA and Manzanillo from Davis (Fig. 3.11). It is likely that the Calamata from Davis and Mission from SA were misnamed.

The extent to which accessions of cultivars can vary in their DNA fingerprints and still be considered the same cultivar is not clear. Jackson and Duff (1994) released a new variety of garlic after 4 years of selection. This new variety was found by Bradley *et al.* (1996) to have a 12% genetic difference to the original mother plant. On the other hand, Woolley *et al.* (2000) found that the genetic variability for named cultivars of almond varied by a maximum of only 4%. Cultivar discrimination may be related to the width of the gene pool. From the results of the present study, the Corregiolo and Calamata accessions from Davis, the Verdale accession from Israel, and the Wagga Verdales from QLD and NSW, which showed a 31%, 24%, 23% and a 20% genetic difference respectively to the other accessions in their groups may be different cultivars.

3.5.3 Genetic similarities between suspected synonyms

The presence of synonyms and homonyms in olive cultivars is a long-standing problem in olive producing countries world-wide (Bartolini *et al.*, 1994). The naming of some of the cultivars and their synonyms in some cases seems arbitrary, and in some cases only a single letter may be changed. For example, Correggiola (Roberts, 1942), Correggiolo and Correggiola (Dal Pero Bertini, 1960), and Corregiola (Fowler, 1930), are all apparently referring to the same cultivar. Similarly, Frantoio in Australia is reported to have more than 18 synonyms (Burr, 1997), and Nevadillo is known as Nevadillo Blanco, Nevadillo, and Picual (Davis and Booth, 1995; Barranco, 1995). It is beyond the scope of this study to compare the genetic similarities between all olive cultivars and their synonyms. However, it is aimed at answering two questions: (1) Are Nevadillo Blanco, Nevadillo and Picual genetically similar? (2) Are Corregiolo, Corregiola, Frantoio, Frantoja and Frantojol genetically similar?

Dal Pero Bertini (1960) put Picual in var. Rostrata and Nevadillo in var. Argentata. He described Picual as frost and disease resistant, with high oil content and a regular cropping habit, and Nevadillo and Nevadillo Blanco as pest and disease susceptible, frost sensitive, alternate bearing, with only fair oil. Barranco (1995), on the other hand, treats Nevadillo and Picual as synonyms, producing medium quality oil, high oleic acid content, tolerant of olive knot, but susceptible to both peacock disease and verticillium wilt. When the genetic similarities of these cultivars was examined, the accessions of Nevadillo and Nevadillo Blanco were about 99% similar but distinct from Picual at 69% (Fig. 3.8). This result is comparable with the findings of Dal Pero Bertini (1960) and Burr (1997) who found

differences in oil yield and agronomic characteristics between Nevadillo Blanco and Picual. The genetic differences of 31% between these accessions are sufficient to discriminate Nevadillo Blanco and Nevadillo from Picual, and hence Picual is probably a different cultivar.

Frantoio, Frantojol, Frantoja, Corregiolo and Corregiola, are considered by some as synonyms for the same cultivar (Dal Pero Bertini, 1960), and are described as regular heavy croppers, containing high oil content, and showing self fertility. When the genetic similarity of these accessions was examined, a high level of genetic variation was observed. The genetic similarity varied from 68% for the Corregiolo from Davis to 100% for Corregiola and Corregiolos within Australia. The three Frantoios, and the accessions of Frantoja and Frantojol showed an intermediate level of genetic similarity which varied from 85% for the Frantoio from Israel to 99.5% for the Frantoio from SA and Italy (Fig. 3.10). It is likely that the accessions of Corregiolo, Corregiola, Frantoio, Frantoja obtained within Australia, Frantoio from Italy, and Fantojol from Davis probably are the same cultivar.

3.6 Summary

Although a large number of olive cultivars has been introduced into Australia since 1836, little attention has been conducted in the past 50 years to expand the industry or even to maintain previously established plantations. The Australian olive industry has declined since the 1950s resulting in eradication of olive trees, loss of recorded histories, and confusion of cultivar names. It is questionable why Australia, which has proven to have perfect conditions for olive production and whose demand for olive products is increasing every year, should rely on imported products.

To establish a successful olive industry in Australia, the uncertainty and confusion of names needs to be rationalised. In the present study, RAPD detected a large number of polymorphisms between accessions that were expected to be identical, and between cultivars and their suspected synonyms. Based on these results the following summary has been made.

- Some olive cultivars showed highly reproducible DNA fingerprints between accessions. These cultivars included: Manzanillo, Kalamata, Nevadillo Blanco/Nevadillo, and Picual.

- Some accessions that were expected to be identical showed a high level of genetic variation compared to the other accessions in the group. These included: Calamata and Corregiolo obtained from Davis, Frantoio obtained from Israel, and the Verdale accessions obtained from all sources. However, the Calamata accession showed fingerprints identical to Manzanillo accessions, indicating misnaming of this cultivar.

- Nevadillo Blanco and Nevadillo had a 98% genetic similarity to each other, and 69% to Picual. This confirms that Picual is not a synonyms for the other two accessions, and that Nevadillo Blanco and Nevadillo are probably identical.

- Two Corregiolo and one Corregiola accessions obtained within Australia showed 100% genetic similarity. Frantoio accessions obtained from SA and Italy, and a Frantoja from BEO showed a 94% genetic similarity to those three indicating that it is probably the same cultivar. The Frantojol obtained from Davis showed a 97% similarity to the two Corregiolas and the Corregiolo, and is probably the same cultivar. However, since only a single accession of Corregiolo obtained from overseas was included in this study, the identity of Corregiola and Frantoio and their synonyms remains uncertain. It is possible that either the Corregiolo from Davis is a different cultivar, or Corregiola/Corregiolo within Australia were misnamed

Chapter Four

Genetic diversity within an isolated olive population in relation to feral spread

4.1 Abstract

Genetic variability within and between groups of olives in an isolated population within an area of about 1 km^2 was investigated using RAPD technique. Based on visual observation, three putative groups were identified: nine trees that appeared to be an original grove, 12 trees that were assumed to be planted progenies of the original grove, and several feral trees in the surrounding hills and valleys. The DNA fingerprints of these 45 trees were compared, and the genetic variability within and between groups within the population was partitioned using analysis of molecular variance (AMOVA). The AMOVA showed high genetic variation within the three putative groups of the population but there were no significant genetic differences between them. A dendrogram constructed using the simple matching coefficient with UPGMA clustering showed the presence of three molecular clusters that persisted when the data were subjected to multidimensional scaling (MDS). The three molecular clusters were significantly different. Each cluster consisted of at least one or more trees from the original grove and others from the population. Estimates of tree age were highly variable, but the overall trend supported the derivation of feral trees from the original grove. The distribution of these clusters within the population suggests that the predominant mode of feral spread was by fruit drop close to the parent trees, with occasional wider dispersal by animals or birds.

4.2 Introduction

Genetic diversity within and between plant populations results from a combination of geographical distance, population size, type of mating system (selfing or outcrossing), mode of dispersal of pollen and seed, and rate of gene flow (Loveless and Hamrick, 1984; Ellstrand and Marshall, 1986; Kaufman *et al.*, 1998).

The mating system of plants appears to play a major role in genetic diversity by determining the rate of exchange of genes (Darmency, 1997). Species that are predominantly outcrossing are reported to show lower interpopulation and higher intrapopulation

differences in genetic variation compared to species where self-fertilisation predominates (Huff *et al.*, 1993; Maguire and Sedgley, 1997). Species that are mainly self-pollinated produce fewer genotypes and show a reduced level of heterozygosity compared to outbreeders (Warwick, 1991).

The olive is a predominantly allogamous species showing a high degree of outcrossing. Most pollen transfer is by wind (Morettini and Pulselli, 1953), with insect involvement occurring to a small extent. Progeny are readily derived from crosses between cultivated varieties (cultivars), as well as between cultivars and both feral (escaped) and wild (oleaster) olives (Lavee and Datt, 1978; Fontanazza and Baldoni, 1990; Zohary, 1994; Angiolillo *et al.*, 1999). The success of outcrossing varies between cultivars. For example, cv. Koroneiki is reported set fruit to a similar extent following both self- and cross-pollination (Lavee, 1996) while cv. Moraiolo is self-sterile (Fontanazza and Baldoni, 1990). The environment where the trees are grown is also a factor. Cultivars that were self-sterile in one country or region were found to be self-fertile in others and vice versa (Lavee, 1996).

Since 1836, hundreds of olive cultivars have been introduced into South Australia, mainly from olive-growing regions in the northern hemisphere. Cross-breeding from abandoned groves over 150 years has resulted in populations of feral olives that grow in all regions of the State. These feral populations present major ecological problems, as the olive competes successfully with native Australian vegetation. An aggressive new olive industry is currently being established in Australia (Sedgley and Wirthensohn, 1999), and it is essential that the potential for future weed problems is understood and addressed.

Different techniques have been used to evaluate olive diversity. Cantini *et al.* (1999) used morphological characters such as leaf, fruit, pit, and growth form to evaluate genetic variation within and between different accessions of known and unknown olive cultivars. Isozyme analysis has also been used to examine the genetic diversity in wild and cultivated olives (Ouazzani *et al.*, 1993). The RAPD-PCR technique successfully identifies olive cultivars (Fabbri *et al.*, 1995; Mekuria *et al.*, 1999; Gemas *et al.*, 2000) and, together with analysis of molecular variance (AMOVA) (Excoffier *et al.*, 1992), has been used to study population genetics in many plants, including *Banksia cuneata* (Maguire and Sedgley, 1997), *Cedrela odorata* (Gillies *et al.*, 1997), wild bean (Cattan-Toupance *et al.*, 1998), and *Grevillea barklyana* (Hogbin *et al.*, 1998). In the present study, this technique was used to

evaluate the level of genetic variation within an isolated feral olive population, to better understand the dynamics of weed spread.

4.3 Materials and methods

4.3.1 Allocation of trees to putative groups within the population

The olive population used in this study comprised 45 individuals, and was located on an abandoned farm at Western River Cove on an isolated part of Kangaroo Island (Fig. 1A and B), South Australia (138°E, 35°S), within an area of approximately 1.25 km x 0.75 km (Fig. 2). The area had been cleared of most of the native vegetation, and consisted mainly of grassland. The nearest olive tree to this population was a single tree located 15 km east and separated from the population under study by dense natural scrub. During pollination, the prevailing winds are predominantly from the S–SE (Bureau of Meteorology, Adelaide, Australia) and the likelihood of pollen being blown towards the olive population under study is remote. Based on their location within the population, three putative groups of trees were identified (Fig. 2):

Group 1: Nine trees close to an old farm house, which were thought to be the original grove and were suspected to be the parents of the other trees in the population.

Group 2: Twelve trees, closely planted in a row along a fence, which were suspected to be derived from either seeds or cuttings taken from the original grove.

Group 3: Several trees, scattered throughout the adjacent area, suspected to be ferals derived naturally from seeds produced by groups 1 and/or 2, and possibly later by older individuals in group 3.

4.3.2 Leaf material

Leaf samples were collected for DNA isolation from the three putative groups (section 4.3.1): leaves from all trees in putative Group 1 (trees 1 - 9); all trees in putative Group 2 (trees 10 - 21), and 24 randomly selected feral trees from putative Group 3 (trees 22 - 45).

Fig. 4.1A

Fig. 4.1B

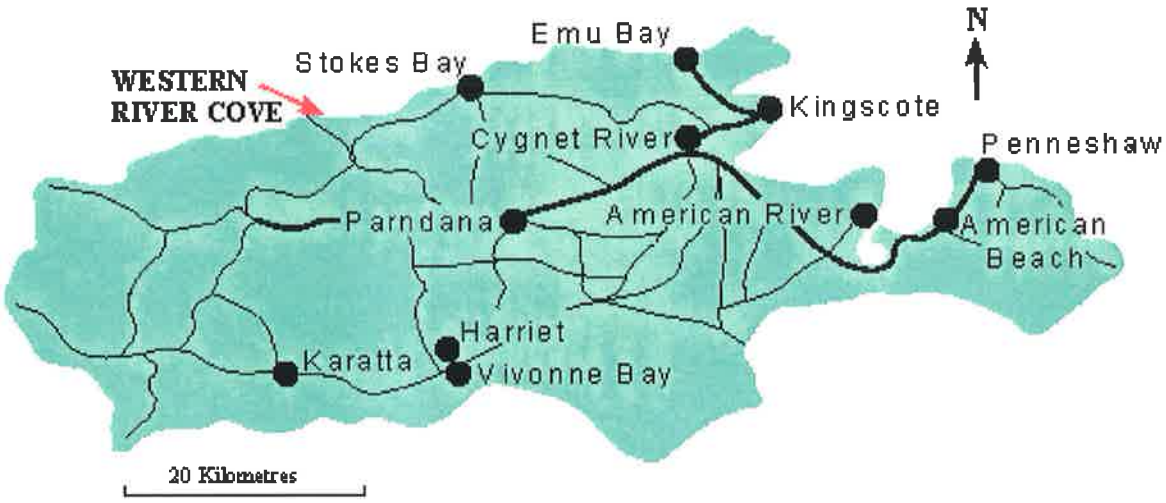


Fig. 4.1 A) The location of Kangaroo Island relative to the mainland of Australia, B) Map of Kangaroo Island (the arrow shows the site of the isolated population).

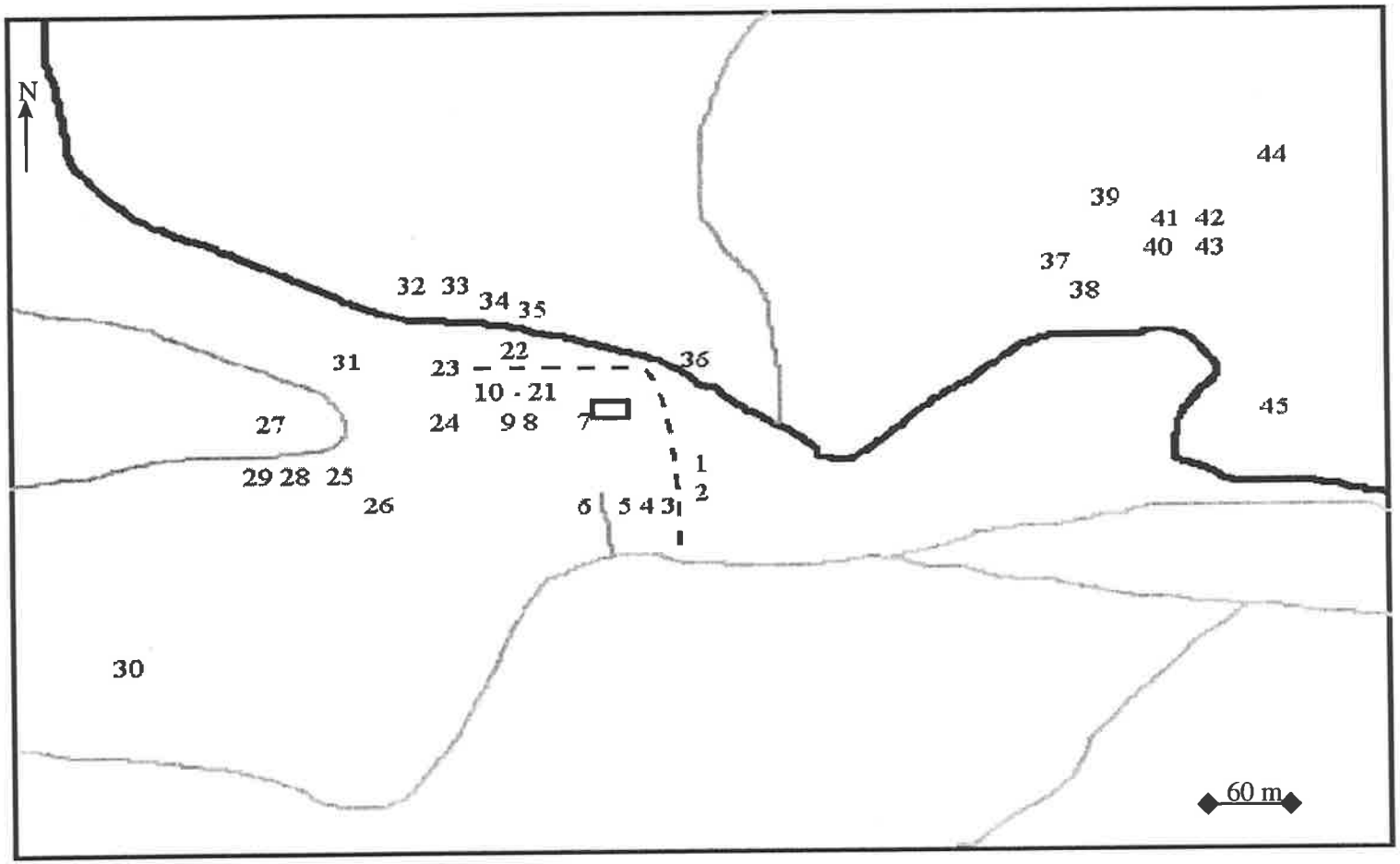


Fig. 4.2 The location of the individual trees within the population. Group 1: 1-9 original grove; Group 2: 10-21 planted fence line; Group 3: 22-45 ferals.

farm house, fence, creek, roads, Scale = 1cm:60m

4.3.3 DNA isolation and amplification

DNA isolation and DNA amplification were performed as described in Chapter 2.

4.3.4 Separation of PCR products

PCR products were separated on 1.5% (w/v) agarose gels (Seakem® GTG® agarose, FMC) in 1 x TBE buffer at 80 mA until the bromophenol blue reached approximately 1 cm from the bottom of the gel. To aid interpretation of band homology between gels, each sample was loaded with 2 µL of a standard 242 bp DNA marker, and two lanes of each gel contained a 100 bp DNA ladder (GeneWorks, Adelaide, SA, Australia). After electrophoresis, gels were stained with ethidium bromide (0.5 µg/mL), destained with milliQ water and the digital image was directly scanned under UV light using the Tekcap computer program (version 1.0. Tekram Corporation, 1998), captured using PaintShop Pro (version 5.00, Jasc Software Inc, 1998), and photographed under UV light using Polaroid 667 film.

Each sample of DNA was subjected to at least three separate PCR reactions and only bands that were reproducible were scored. The presence and absence of bands was estimated using Scion Image digital gel analyser program (Rasband, 1997). Within each lane on the gel, the mobility of the bands were calculated relative to the mobility of the 242 bp marker, and then visually checked using 100 bp DNA ladder.

4.3.5 Data analysis

The presence and absence of RAPD fragments was recorded in a binary data matrix and genetic similarities among all pairs of individuals was estimated with NTSYS-pc (Applied Biostatistics Inc. v. 2.02k) using the simple matching coefficient (Sokal and Michener, 1958) as described in Apostol *et al.* (1993).

Cluster analysis was performed with the SAHN algorithm using the UPGMA. The randomness or otherwise of the clusters was evaluated by calculating the cophenetic correlation obtained by plotting the cophenetic matrix against the similarity matrix using NTSYS-pc.

The similarity matrix was ordinated using non-metric multidimensional scaling to represent the relationships among individuals in two dimensional space. A minimum spanning tree was superimposed on the ordination to help detect outliers and to link neighbouring points.

A pairwise genetic distance matrix was generated from the binary data using AMOVA-PREP (Miller, 1998, v. 1.01). AMOVA (WINAMOVA, Excoffier *et al.*, 1992) was used to partition variability into hierarchical components of among and within groups of the population and to test the significance components. To estimate significance levels for variance components, the permutation procedure was set at 100 iterations and F statistics were calculated to test for significant differences among and within groups of the population.

4.3.6 Measurement of tree age

Data on circumference and height were collected from 26 trees (Table 4.4) within the population. Circumference was measured 1.3 m above the ground and height with measuring rods. Five trees were cut from the population and tree rings were counted in the cut stump.

The coefficients of determination (r^2) for circumference and height as a function of growth rings were 0.92 and 0.78 respectively. Hence circumference was used to estimate tree age using the linear regression equation:

$$Y = 0.5086X - 2.0367$$

where Y is the number of growth rings and X is the circumference of the tree. It was assumed that each ring represented one year of growth. The 95% confidence interval (CI) for the regression equation was calculated as described in Steel and Torrie (1960). Multi-stem trees were excluded from the analysis to reduce possible error in trunk size measurement.

4.4. Results

4.4.1 Genetic assessment

For the 45 individuals examined, using six primers, a total of 130 RAPD fragments were produced of which 119 (91.5%) were polymorphic (Table 4.1). The number of polymorphic bands per primer varied from 14 for GC-05 to 24 for OPZ-13 with a mean of 19.8. An example of RAPD fingerprints of the 45 trees studied using primer OPZ-11 is shown in Fig. 4.3.

Table 4.1 The number of total and polymorphic RAPD bands produced from 45 individual olive trees using six primers.

Primer	Primer sequence (5′ - 3′)	No. of bands produced	Polymorphic bands
GC-01	CAGGCCCTTC	24	21
GC-05	AGGGGTCTTG	16	14
GC-18	AGGTGACCGT	20	19
GC-20	GTTGCGATCC	24	20
OPZ-11	CTCAGTCGCA	21	21
OPZ-13	GACTAAGCCC	25	24
Total		130	119
Mean		21.7	19.8

4.4.1.1 Genetic similarities between and within the putative groups

The variance components from AMOVA were partitioned among individuals within (87.3%) and between (12.7%) the three putative groups based on location within the population (Table 4.2). The F statistic, calculated from the mean square distance (MSD), showed no significance differences among and within these groups. The exclusion of monomorphic bands from the data set made no differences to the significance of the F statistic.

Table 4.2 AMOVA for three putative groups of olive trees based on location within the population.

Sources of variation	d.f	SSD	MSD	Variance component	% total	*P*-value	F-statistic
Among groups	2	72.107	36.053	1.759	12.66		2.97 ns
Within groups	42	509.466	12.130	12.130	87.34	<0.01	

d.f: degree of freedom; SSD: sum of square deviation; MSD: mean square deviation; *P* value: value for level of significance at 100 permutations; ns: not significant.

Using all amplified RAPD fragments and the simple matching coefficient, genetic similarities were calculated for each of the three putative groups. The mean genetic similarities among the groups was 75% (58% - 99%), and the mean genetic similarities within the groups were 73% for group 1 (56% - 99%); 78% for group 2 (60% - 96%), and 74% for group 3 (55% - 84%).

4.4.1.2 Genetic similarities between and within the molecular clusters

Three molecular clusters were identified from the UPGMA dendrogram (Fig. 4.4). Cluster 1 included two trees (3 and 6) from the putative original grove (group 1) plus eleven of the twelve trees on the fence line (group 2). Within cluster 1 there were two sub-clusters. One included tree 3 from group 1 and trees 12, 13, 14 and 16 from group 2. The other included tree 6 from group 1 and trees 11, 15, 17, 18, 19, 20 and 21 from group 2. Cluster 2 included four trees (1, 7, 8 and 9) from the putative original grove (group 1), one tree (10) growing on the fence line (group 2), and eight ferals from group 3. All of the group 3 trees were located on the western side of the creek, with 32-36 relatively close to the house and 40, 43 and 44 more distant (Fig. 4.2). Cluster 3 comprised 16 feral trees from group 3 and one tree from group 1, the putative original grove (tree 2). The trees from group 3 were spread across the site. Two trees of the putative original grove, 4 and 5, showed low similarity to all clusters and could be considered as outliers. When the data were ordinated by multi-dimensional scaling (Fig. 4.5), the 45 trees in the population formed the same three discrete clusters as UPGMA analysis (Fig. 4.4). The value of the cophenetic correlation coefficient (Rohlf, 1993), a measure of the goodness of fit between the dendrogram and the original similarity matrix, was about 0.8 indicating the presence of clusters was more than a random event (Fig. 4.6). The mean genetic similarities within these molecular clusters,

excluding the two outlier individuals 4 and 5, was 79% for cluster 1 (range 65% - 96%), 77% for cluster 2 (range 61% - 99%), and 75% for cluster 3 (range 66% - 82%).

The trees in the population showed less than 90% similarity to each other, with the exception of trees 1 and 7 (99%) in group 1 and cluster 2, and 18 and 21 (97%) and 17 and 20 (91%) in group 2 and cluster 1 (Fig. 4.4).

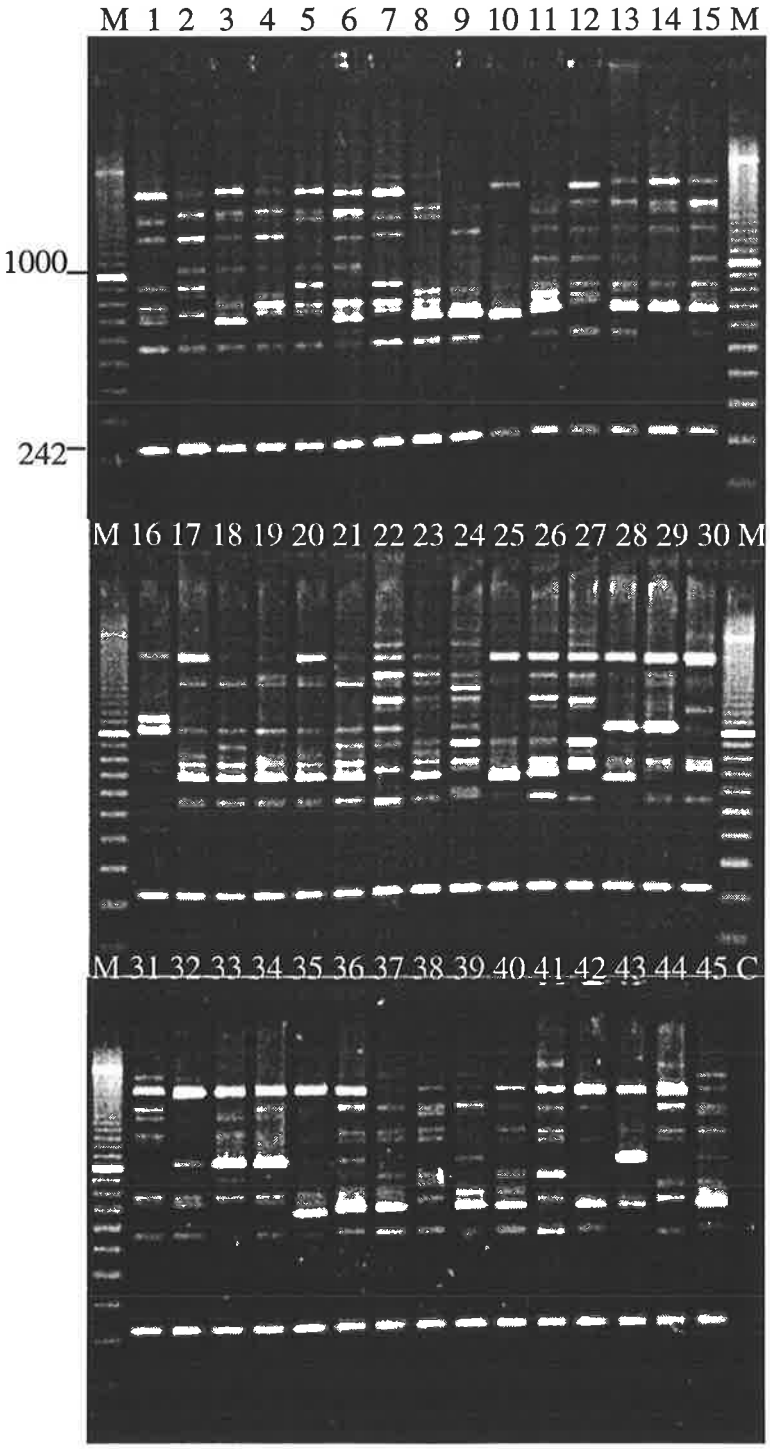


Fig. 4.3 RAPD fingerprints generated using primer OPZ-11: 1-9, group 1; 10-21, group 2; 21-45, group 3, M:100bp marker; C: control (no genomic DNA).

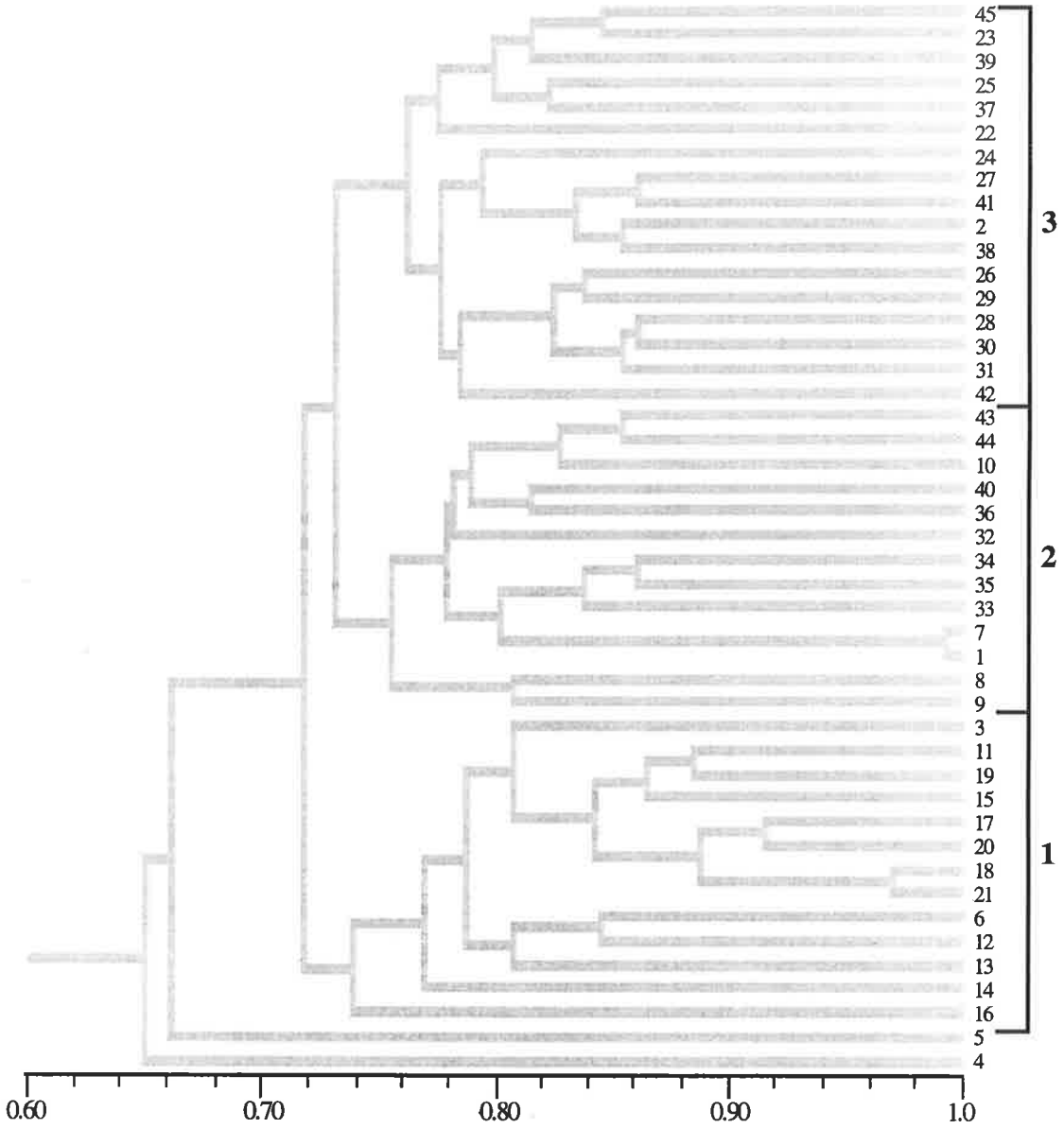


Fig. 4.4 Dendrogram showing three molecular clusters 1, 2 and 3 derived from the genetic similarities of the 45 trees in the olive population using the simple matching coefficient and UPGMA clustering: 1-9, the putative original grove (group 1); 10-21, trees on the fence line (group 2); 22-45, feral olives (group 3).
(Note that the three molecular clusters do not coincide with the three groups based on location within the population)

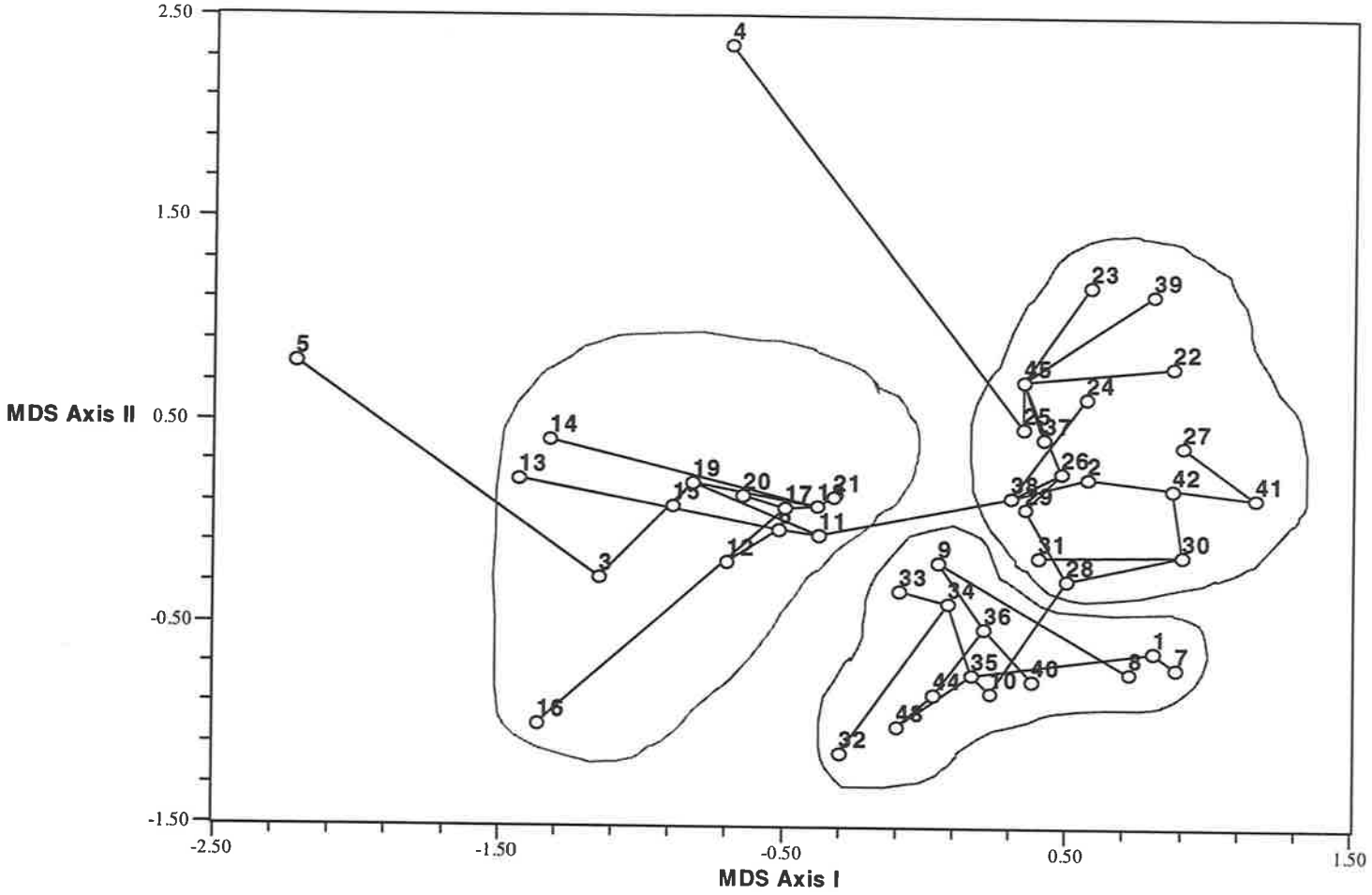


Fig. 4.5 Molecular clusters derived from the genetic similarities of the 45 trees in the olive population using two-dimensional multidimensional scaling: 1-9, the putative original grove (group 1); 10-21, trees on the fence line (group 2); 22-45, feral olives (group 3).

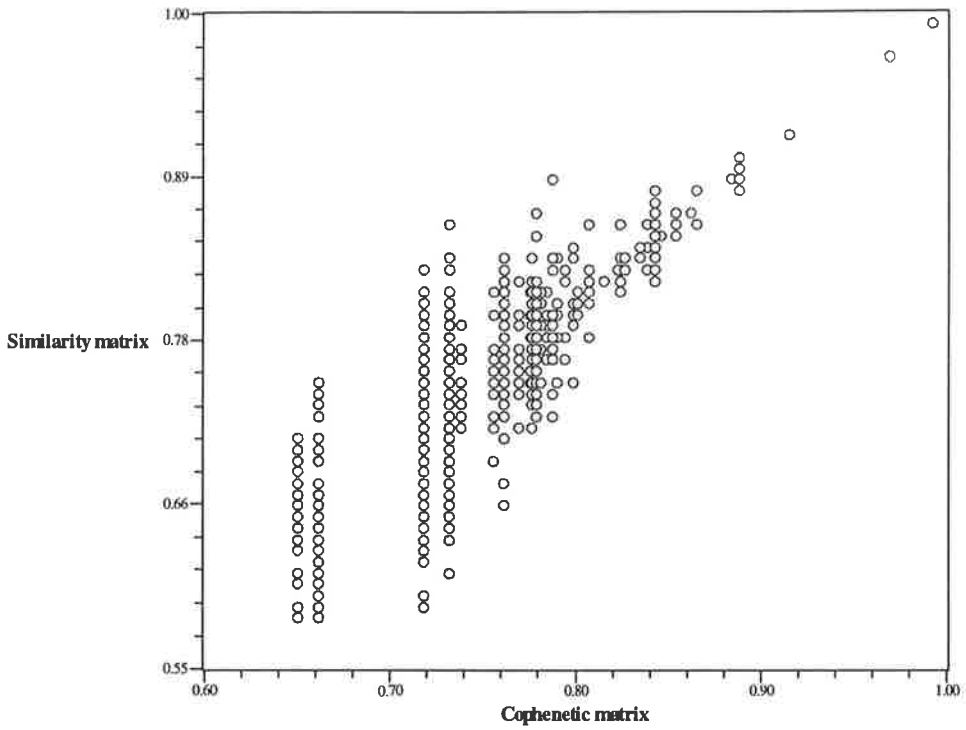


Fig. 4.6 Plot of genetic similarities against cophenetic values based on UPGMA clustering.

AMOVA conducted from the pairwise genetic distance between all bands for the three molecular clusters revealed that 76.6% of the total genetic variation was attributable to variation among individuals within the group and 23.4% was attributable to variation between the clusters (Table 4.3). Thus, the genetic variability attributable to variation between molecular clusters is almost 11% greater that between the three putative groups of the population (Table 4.2). The F statistic showed that the three molecular clusters were significantly different. When data from the two outliers was included there was no difference in the significant of the F statistic, but the total percentage of the variation attributable to among clusters of the population was reduced by 1%.

Table 4.3 AMOVA for three molecular clusters of the olive population excluding the two trees (4 and 5) regarded as outliers.

Sources of variation	d.f	SSD	MSD	Variance component	% total	*P*- value	F-statistic
Among clusters	2	117.11	58.56	3.35	23.38		5.33*
Within clusters	40	439.00	10.98	10.98	76.62	<0.01	

*significance difference

4.4.2 Assessment of tree age

4.4.2.1 Age of trees within the putative groups

The average trunk circumference for trees in the three putative groups was 172 cm for group 1, 119 cm for group 2 and 89 cm for group 3 (Table 4.4). Assuming that one ring equates to one year, the average age of the groups was estimated to be 86, 58, and 43 yrs for groups 1, 2, and 3 respectively. Within each group the derived age of the individual trees varied from 61-115 for group 1, 32-85 for group 2, and 17-110 for group 3. There was considerable age overlap, using the 95% confidence intervals, between individuals in different groups (Table 4.4).

The relationship between circumference and growth rings for the three putative groups is depicted in Fig. 4.7. The age distribution for six single trunk trees in the putative original

grove (group 1) showed that two trees (5 and 1) of estimated ages 61 and 63 years were significantly younger than the oldest tree (8) which had an estimated age of about 115 years. The youngest tree (5), 61 yrs, was also significantly younger than the second oldest tree (6), which had an estimated age of about 102 yrs.

From the age distribution for nine single trunk trees in group 2 on the fence line, three trees (14, 12, and 20) of estimated ages of 32, 42, and 43 years respectively were significantly younger than the two older trees (10 and 13) which were estimated to be 76 and 85 years old. The youngest tree (14), 32 yrs, was also significantly younger than the third (18), fourth (21) and fifth (11) oldest trees which had estimated ages of about 67, 67, and 61 yrs respectively.

The age distribution for 11 single trunk trees of group 3 feral olives showed that the oldest tree (25) with estimated age of 110 was significantly older than the remaining 10 individuals within the group. Six trees (45, 40, 36, 41, 29, and 26) of estimated ages 17, 20, 23, 24, 26, and 36 respectively were significantly younger than the second (42) and the third (22) oldest trees, which were estimated to be 66 and 62 yrs respectively. The two youngest trees (17 and 20) were also significantly younger than the fourth (39), fifth (23) and sixth (26) oldest trees which had estimated ages of 46, 42, and 36 yrs respectively.

Table 4.4 Trunk circumference, growth rings and 95% confidence interval of the single trunk trees of the three putative groups.

Tree No.	Trunk Circumference (cm)	Growth rings ±95% CI
Group 1. Original grove		
5	123	61 ± 15
1	128	63 ± 15
9	165	82 ± 20
2	183	91 ± 22
6	205	102 ± 25
8	230	115 ± 28
Mean	172	86 ± 21
Group 2. Trees on the fence line		
14	66	32 ± 8
12	87	42 ± 11
20	88	43 ± 11
16	107	52 ± 13
11	123	61 ± 15
21	135	67 ± 16
18	136	67 ± 16
10	153	76 ± 19
13	172	85 ± 21
Mean	119	58 ± 14
Group 3. Feral olives		
45	38	17 ± 5
40	44	20 ± 5
36	50	23 ± 6
41	52	24 ± 6
29	55	26 ± 7
26	75	36 ± 9
23	86	42 ± 10
39	95	46 ± 11
22	126	62 ± 15
42	134	66 ± 16
25	220	110 ± 27
Mean	89	43 ± 11

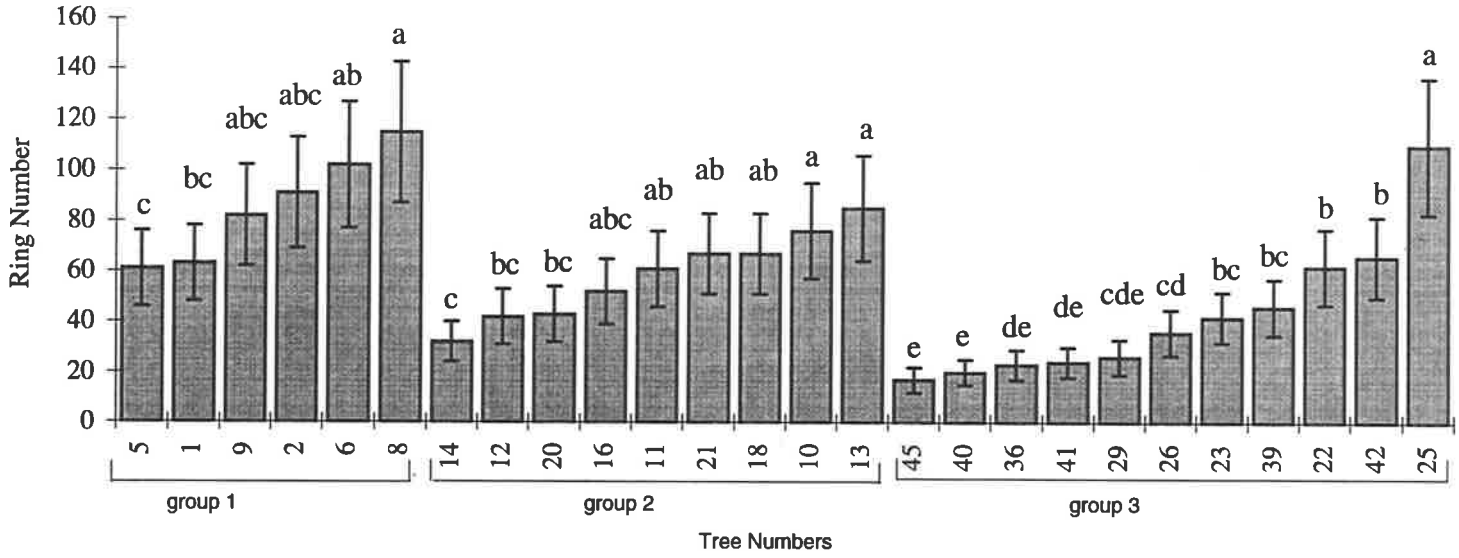


Fig. 4.7 Circumference and growth ring relationships of the three putative groups. Numbers refer to: 1-9, group 1 the original grove; 10-21, group 2 trees on the fence line; 22-45, group 3 feral trees.

4.4.2.2 Age of trees within the molecular clusters

The relationships between circumference and growth rings for trees in the three molecular clusters are shown in Table 4.5 and Fig. 4.8. From the age distribution for nine single trunk trees in cluster 1, four trees (14, 12, 20, and 16) with estimated ages of 32, 42, 43, and 52 yrs respectively were significantly younger than the two oldest trees (13 and 6) which had estimated ages of 85 and 102 yrs respectively. The youngest tree (14), 32 yrs, was also significantly younger than the third (18), fourth (21) and fifth (11) oldest trees which were estimated to be 67, 67, and 61 yrs respectively.

From the age distribution for six single trunk trees in cluster 2, the two youngest trees (40 and 36) with estimated ages of 20 and 23 yrs respectively were significantly younger than the four oldest trees (1, 10, 9 and 8) which were estimated to be 63, 76, 82 and 115 yrs respectively. The third youngest tree (1) which had an estimated age of 63 yrs was significantly younger than the oldest tree (8) which was estimated to be 115 yrs.

From the 10 single trunk trees in cluster 3, six trees (45, 41, 29, 26, 23, and 39) of estimated ages of 17, 24, 26, 36, 42 and 46 respectively were significantly younger than the two oldest trees (2 and 25) which were estimated to be 91 and 110 yrs respectively. The youngest tree (45), 17 yrs, was also significantly younger than the third (42), fourth (22), fifth (39), sixth (23), and seventh (26) oldest trees which had an estimated age of 66, 62, 46, 42, 36, and 26 yrs respectively.

Table 4.5 Trunk circumference, growth rings and 95% confidence interval of the single stem trees for the three molecular clusters.

Tree No.	Trunk Circumference (cm)	Growth rings ±95% CI
Molecular cluster 1		
14	66	32 ± 8
12	87	42 ± 11
20	88	43 ± 11
16	107	52 ± 13
11	123	61 ± 15
21	135	67 ± 16
18	136	67 ± 16
13	172	85 ± 21
6	205	102 ± 25
Mean	124	61 ± 15
Molecular cluster 2		
40	44	20 ± 5
36	50	23 ± 6
1	128	63 ± 15
10	153	76 ± 19
9	165	82 ± 20
8	230	115 ± 28
Mean	128	63 ± 16
Molecular cluster 3		
45	38	17 ± 5
41	52	24 ± 6
29	55	26 ± 7
26	75	36 ± 9
23	86	42 ± 10
39	95	46 ± 11
22	126	62 ± 15
42	134	66 ± 16
2	183	91 ± 22
25	220	110 ± 27
Mean	106	52 ± 13

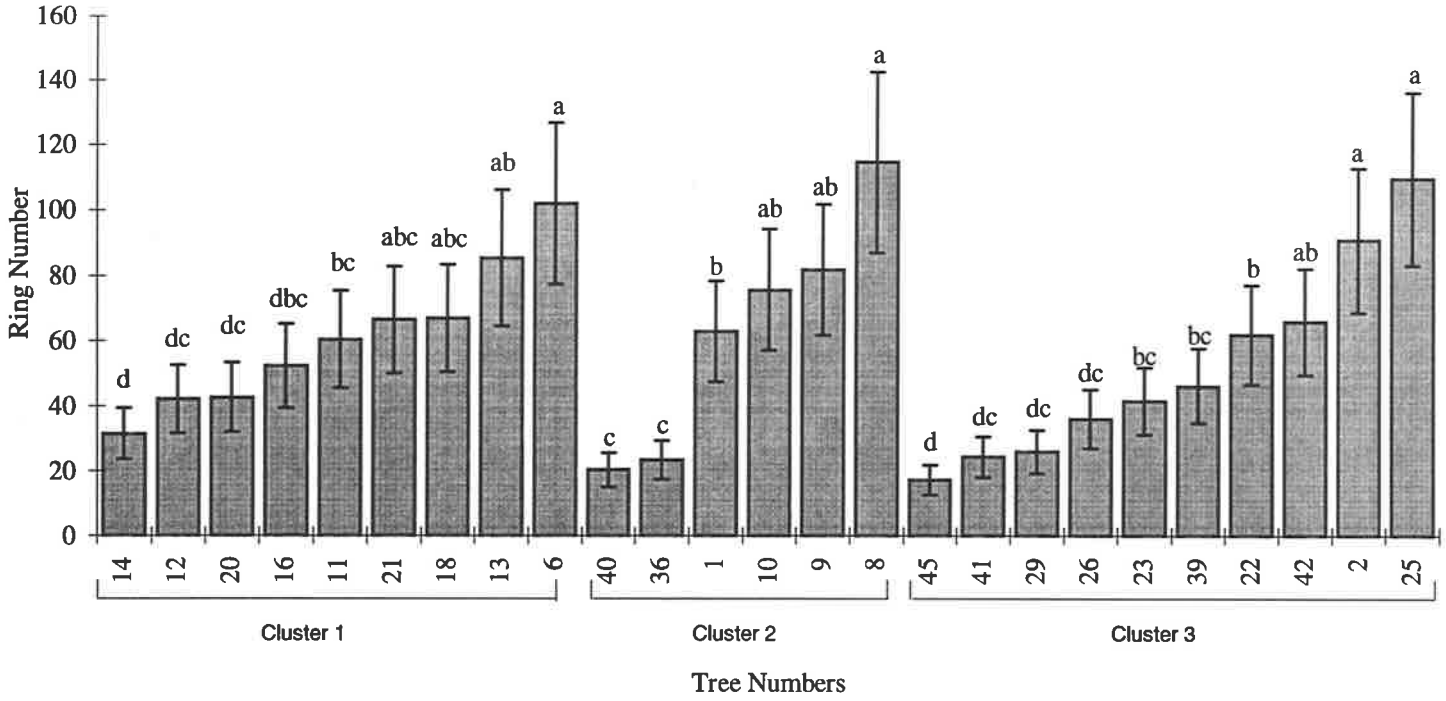


Fig. 4.8 Circumference and growth ring relationships of the three molecular clusters. Numbers refer to: 1-9, group 1 the original grove; group 2, 10-21, trees on the fence line; group 3, 22-45, feral trees.

4.5 Discussion

This study has shown spread and diversity of feral olives from an isolated grove, and from interbreeding with progeny of the grove, over a period of approximately 100 years. An isolated population of olive trees on Kangaroo Island was subdivided into three putative groups based upon visual observation. One of the groups was suspected to be an original planted grove, a second appeared to be planted along a fence line, and a third consisted of ferals located apparently at random around the other two groups.

Forty five individual trees were selected and their DNA fingerprints compared. Analysis of the molecular variance between the three putative groups (87.3% within and 12.7% between) showed that they were not significantly different. Two possible reasons can be advanced to explain this result. Firstly, that random pollen fertilisation and fruit dispersal was occurring between groups within the population. A second explanation is that, for each of the putative groups, individuals could have been drawn from more than one genetic group.

Both the dendrogram and the MDS ordination showed the existence of three molecular clusters, and the AMOVA (76.6% within and 23.4% between) indicated that these clusters were significantly different. The between cluster variability for the molecular clusters was 11% greater than for the putative groups. Therefore, the second option above is valid. That is, random gene flow has not occurred throughout the entire population, and each of the putative groups included trees from more than one of the significantly different molecular clusters.

Cluster 1 included 11 of the individuals on the fence line and two of the original grove. Since only two of the trees on the fence line (18 and 21) showed high genetic similarity to each other, this precludes the possibility that they were planted as cuttings, and it is likely that most if not all were derived from seeds. Assuming that parental trees within a cluster have a relatively high genetic contribution to the other trees within the same cluster, it is possible that individuals on the fence line may be derived either from self or cross pollination involving trees 3 and 6 of the original grove. This is particularly likely as cluster 1 included two sub-clusters, one of which included tree 3 and the other tree 6. It is also notable that the trees within cluster 1 showed greater similarity to each other than did the

trees in cluster 2 and 3. This supports the hypothesis that human intervention involved the planting of seed from trees 3 and 6, rather than chance seed dispersal.

Cluster 2 included four from group 1 (trees 1, 7, 8, and 9) and one individual from the fence line. The eight ferals also in this cluster were most likely derived from natural crosses involving these suspected parents from the original grove and from the planted fence line. Cluster 3 included one suspected parent from the original grove (tree 2), and 16 ferals possibly derived from seeds involving gene flow with this original tree.

Two trees in the original grove, 4 and 5, were outliers on both the dendrogram and MDS ordination indicating that they did not contribute to the gene flow within the population. During this study, these trees did not bear fruit and both of them were observed to be in poor health (M. Wirthensohn pers. comm.).

Geographical distance between individuals within the population was not correlated with genetic distance. Trees 40, 43, and 44 in cluster 2, and trees 37, 38, 39, 41, 42, and 45 in cluster 3 were located 300 - 400 m from other trees within the same clusters, and trees 40 and 43, and trees 41 and 42 were in separate clusters despite being only a few metres apart. Studying the geographical range of *Banksia cuneata* in Western Australia, Maguire and Sedgley (1997) also found that distance was not always a good indicator of genetic similarity, and that clustering based on DNA fingerprints provided a better indication of genetic similarities among closely related individuals regardless of their geographical distance.

The genetic similarities found between the 45 individuals studied varied from 67% to 99%, and this result is comparable with previous reports for olive. Using the RAPD technique, Fabbri *et al.* (1995) found genetic similarities between 42% to 95% for 17 olive cultivars, and, for accessions of olive cultivars with the same name but obtained from different sources, Mekuria *et al.* (1999) found genetic similarities ranging from 68 to 100%. Using AFLP analysis, Angiolillo *et al.* (1999), found genetic similarities ranging from 53% to 93% for cultivated olives, and from 51% to 87% for wild olives (oleaster). These studies demonstrate the range in the level of genetic variation that occurs in olives. For the present study, except for suspected parents 1 and 7 which showed a genetic similarity of 99% to

each other, all other suspected parents showed a similarity of 81% or less, and are therefore probably either different cultivars or were derived from seeds.

Genetic variation within and between populations can be influenced by mating system, and pollen and seed dispersal mechanisms (Loveless and Hamrick, 1984; Ellstrand and Marshall, 1986; Kaufman *et al.*, 1998). Hamrick *et al.* (1979) suggested that plant species that have long generation times, wind pollination, and outcrossing mating systems, which is probably the case in the present study, tend to maintain higher levels of within population or subpopulation variation than do species without these traits. The results of the AMOVA in this study showed that a large amount of genetic variation occurred within groups of the population, and this is consistent with previous results reported for outcrossing species. Using RAPD and AMOVA, Maguire and Sedgley (1997) found high (104.7%) genetic variation within *Banksia cuneata* species. Using similar techniques, Huff *et al.* (1993) found up to 80.5% genetic variation within a population of buffalograss, and Hogbin *et al.* (1998) found high genetic variation within an outcrossing population of *Grevillea barklyana*.

In order to better understand the ecological relationships between the trees in the olive population it was decided to estimate their ages. Growth rings have been used as a determinant of age for many evergreen or Mediterranean/subtropical trees including *Acacia* species (Eshete and Ståhl, 1999), *Agathis ovata* (Enright and Goldblum, 1998), and *Pterocarpus angolensis* (Stahle *et al.*, 1999), and in most cases the growth rings are annual. Evaluating the patterns of growth rings in *Agathis robusta*, Ash (1983) found a strong correlation between age estimated on the basis of either tree ring counts, radiocarbon dates, or growth rate data.

A search of the literature failed to find any data on the correlation between number of growth rings and tree age for olives. It was therefore decided to fell some trees in the population and examine the regression of ring number on tree morphology. Ring estimation for the 26 single stem trees, ranging in circumference from 38 to 230 cm, produced a range of estimated age from 17 to 115 yrs.

Considerable age overlap between individuals in the three putative groups was observed. The average age of the suspected original grove was 43 yrs older than the average of the

feral olives, but there was no significant age difference between the original grove and the trees on the fence line. Similarly, there was no significant difference between the average age of trees on the fence line and the feral olives. About 55% of the feral olives measured were significantly younger than the original grove. On the other hand, the age of 45% of the feral olives and 89% of trees on the fence line are comparable with the original grove.

The mean age difference was not significant between the three molecular clusters. However, individual trees within each cluster showed a range of age differences. In cluster 1, tree 6 was one of the oldest and this supports the suggestion that it was a progenitor of the others in the cluster. Suspected parent 3 in cluster 1 was not measured because it was multi-stemmed. In cluster 2, suspected parents 1, 8, and 9 were among the oldest in this cluster, and this supports the suggestion that these were possible parents. Suspected parent 7 in cluster 2 was multi-stemmed. In cluster 3, suspected parent 2 and feral tree 25 were the oldest within the cluster. Feral tree 25, because of its position within the population and its age could also be a parental tree of other ferals.

Inaccuracy in the interpretation of the age estimation might have occurred due to a cumulative effect of factors such as variation in stem size between closely-spaced trees and those found in more open areas, inaccuracy in the actual growth ring counting, variation in climate and rainfall over the 100 year period and lack of knowledge on annual growth increments between trees of different age groups. Trees of different age may not be significantly different in size once they achieve their maximum height growth. Furthermore, the assumption that xylem rings are produced on an annual basis by olives may not be valid. Multi-stemmed trees occur when the primary stem is destroyed, by drought, grazing or other damage and multiple buds shoot to form new trunks. These may be of variable age, and all would have fewer growth rings than the original trunk. Hence multi-stemmed trees were not included in the tree age analysis. However, it was impossible to tell if a single trunked tree had its original trunk, or one derived from successful competition between multiple trunks at some stage in the past. This is another possible source of inaccuracy of the age data. However, within these limitations, the findings support the original assumption regarding the position of the suspected parents and their contribution to gene flow within the population.

In this study not all suspected parents in the original grove contributed equally to gene flow. From nine suspected parents, two occurred in cluster 1, four in cluster 2, one in cluster 3, and two apparently contributed no pollen or seed to the gene pool. A number of factors may contribute to these findings. Some commercial olive cultivars can show either female sterility (Dal Pero Bertini, 1960) or male sterility (Besnard *et al.*, 2000; Shubiao *et al.*, 2000) or both (Hartmann, 1967), and thus contribute less to the gene flow in an orchard than other cultivars. In addition, trees may commence flowering and fruiting at different ages and olive genotypes do not always coincide in their bloom time (Grigger *et al.*, 1975). Any of these factors, or combinations of them, may unbalance the contribution of pollen from some individuals. Unequal pollen contribution is also reported in other plant species. Studying male reproductive success in *Cecropia obtusifolia*, Kaufman *et al.* (1998) found four (out of 47) males that did not contribute pollen. They also found that 37% of the offspring in the population studied were fathered from one or more populations outside the study area. Similarly, studying pollen dispersal in knobcone pine, Burczyk *et al.* (1996) found that the majority of mating (more than 50%) was not between neighbouring individuals. They argued that the major factors influencing mating success are distance and direction of the polleniser from the mother tree, and tree height.

Seed dispersal mechanism is reported to affect gene flow. Birds such as Sardinian warblers, and song thrushes are reported to be the major dispersers of olive seeds in the northern hemisphere (Rey *et al.*, 1997; Alcántara *et al.*, 1997) either by swallowing the fruit and voiding the seed or by carrying and dropping fruit in their bills (Rey *et al.*, 1997). In Australia, birds including blackbird and black-faced cuckoo-shrike, and animals, such as emus and foxes (by excretion), are known as olive seed dispersers (Spennemann and Allen, 2000). However, the number of seeds dispersed by birds can be affected by fruit size and ripening phenology (early ripening trees being favoured) which would bias some individuals towards greater seed dispersal, and consequently to unbalanced individual contribution to the pool of seed dispersed by the population (Alcántara *et al.*, 1997).

Olives are known to exhibit alternate bearing whereby crops consisting of a relatively few, but large fruits, alternate with those of many, smaller fruits (Lavee, 1996). This could contribute to unequal pollen flow by wind, and fruit dispersal by animals and birds from year to year.

4.6 Summary

The relative comparison of age in this study provided a broad indication of the possible range of stand ages within the population. The data appear to validate the selection of the suspected parents of the ferals, and therefore provide support for the conclusions drawn about the ecological relationships between the individuals studied.

This study revealed the range of genetic variability between individuals within an isolated population of olive trees. Significant genetic differences were found within the site indicating that dispersal of pollen and seed is not random. That is, some individuals are contributing more to the gene pool than others.

It is most likely that the major spread of trees within the three molecular clusters is by seed drop and subsequent germination within a relatively small distance from the parent trees. The lack of olive trees outside the study area (nearest is 15 km) supports this conclusion. However, occasionally some seeds can be transported to other parts of the population. The detection of two distinct molecular groups on the side of the hill about 400 m from other trees in the same molecular groups suggests that seeds were carried initially by dispersal agents, but that further spread was by seed drop, thus preserving the molecular groups.

Therefore, from these findings it can be concluded that gene flow (both pollen and seed dispersal) in an isolated olive population is not uniform among individuals, and even within an area of 1 km^2 significant genetic groups can be found. From the range in ages of the trees, it also appeared that recruitment is an ongoing and gradual process.

Chapter Five

Genetic markers linked to olive leaf spot resistance and susceptibility

5.1 Abstract

Olive leaf spot is a disease of olive (*Olea europaea* L.) caused by the fungal pathogen, *Spilocea oleaginea* Cast. The symptoms of the disease are dark green or black circular spots, that develop mainly on the leaf blades. The disease can cause a complete loss of yield of some susceptible cultivars in certain climatic regions. In the present study, progeny segregating for resistance to leaf spot disease were obtained from an olive breeding program in Israel. The progeny were obtained from crosses involving five female parents, four of which had never shown symptoms of the disease over a period of about 20 years and were designated as resistant. The fifth showed occasional infection and was designated as semi-resistant. The four male parents, each of which varied in their susceptibility, were cultivars Barnea, Manzanillo, Muhasan, and Souri. Progeny derived from these crosses were assessed in the field for eight years and classified as either resistant or susceptible. DNA from some of the progeny of this segregating population was used to identify molecular markers linked to olive leaf spot disease using the RAPD technique and bulked segregant analysis. Two DNA bulks were constructed, each containing 13 progeny showing either resistance or susceptibility for the disease, and screened for polymorphisms using 100 primers. One primer produced two polymorphic bands, one of about 700 base pairs (bp) from the susceptible bulk and the other of about 780 bp from the resistant bulk. The 780 bp marker appeared in 70.6% of the segregating progeny and 100% of parents showing resistance to leaf spot disease, while the 700 bp marker appeared in 47.1% of the segregating progeny and 100% of the parents showing susceptibility. These markers can be used as screening tools in olive improvement programs.

5.2 Introduction

Olive leaf spot, peacock spot, peacock eye, and bird's eye spot (Fig. 5.1) are alternative names for the disease caused by the pathogen, *Spilocea oleaginea* (syn. *Cycloconium oleaginum* Cast.) (Ogawa and English, 1991; Teviotdale, 1994). It is the most important leaf disease of olives in many countries around the Mediterranean basin as well as in California, South Africa, South America and Australia (Hughes 1953; Shabi *et al.*, 1994;

Sutter, 1994). The pathogen attacks mainly leaves, producing lesions and circular spots on the upper surfaces, but in rare cases fruits and fruit stems may also be attacked. Some lesions develop a yellow halo that looks like the "eye" spot on the tail feathers of a peacock, hence the names peacock spot and bird's eye spot (Teviotdale, 1994), whereas others are small spots that are usually black. Infestation is associated with rainy periods and is a particularly significant problem in damp low lying areas during wet years. The extensive defoliation that occurs, results in a marked reduction in both flower bud differentiation, and fruit set in subsequent years (Shabi *et al.*, 1994; Teviotdale, 1994). For some sensitive cultivars there can be a complete loss of yield under extreme inductive climatic conditions (Lavee *et al.*, 1999).

Susceptibility to infection varies amongst cultivars. For example, in California, Sutter (1994) found that the order of resistance amongst several cultivars from highest to lowest was Sevillano, Manzanillo, Ascolano, Barouni, and Mission, and in Israel Manzanillo is more resistant than Souri and Nabali (Lavee, 1990).

Screening progeny for phenotypic traits in a breeding program involving tree crops can take many years to accomplish. The screening process is often exacerbated for tree crops, such as olive, because phenotypic expression can be influenced by environmental conditions (Shabi *et al.*, 1994). On the other hand, the juvenile leaves of olives are more sensitive to *S. oleaginea* than mature ones, so selection for resistance can be started in the second year after planting (Lavee, 1990).

Michelmore *et al.* (1991) suggested the technique of BSA to identify genetic markers linked to specific genes of interest. BSA, when used with the RAPD-PCR technique (Williams *et al.*, 1990), requires no prior knowledge of the genomic DNA being studied, unlike other procedures such as chromosome walking (Michiels *et al.*, 1987), chromosome jumping (Rommens *et al.*, 1989), and the development of near isogenic lines (Young *et al.*, 1988). The main prerequisites are a population of plants segregating for the trait in question, and the existence of a relatively large genetic divergence between the parents in the target region (Michelmore *et al.*, 1991). In addition, BSA is a relatively rapid and simple protocol to perform. The procedure has been used successfully in many studies, including markers for the *Fom* 2 gene for resistance to fusarium wilt in muskmelon (Wechter *et al.*, 1995), freestone in peach (Warburton *et al.*, 1996), rust resistance in poplars (Villar *et al.*, 1996),

anthracnose resistance in sorghum (Boora *et al.*, 1998), glaucousness of leaves in *Eucalyptus gunnii* (Wirthensohn *et al.*, 1999), and seed coat colour in *Brassica juncea* (Negi *et al.*, 2000).

In the present study, the RAPD-PCR technique was used in conjunction with BSA to identify a genetic marker linked to resistance or susceptibility to olive leaf spot using a segregating population from a breeding program at the Volcani Center, Israel.

5.3 Materials and methods

5.3.1 Segregating population

A breeding program for resistance to *Spilocea oleaginea* has been underway at The Institute of Horticulture, Volcani Center, Bet Dagan, Israel, since the early 1980s. Trees are grown under irrigated conditions in a maritime sub-tropical climate with predominantly winter rain, and hot summers with high humidity (75-85%). The natural incidence of olive leaf spot is high, and based on the occurrence of symptoms in the field, the progeny of the breeding program are designated as resistant, semi-resistant (occasional infection), or susceptible (regular infection) (Lavee *et al.*, 1999).

The tree used as the initial parent in the breeding program for olive leaf spot resistance was selected from 17 trees, all showing low susceptibility to *S. oleaginea*, that were growing in an orchard of the cultivar Muhasan known to be infected with the disease. This particular tree was phenotypically different from the rest of the trees in the orchard, and was thought to be a seedling of cv. Chimlali since Chimlali seed had been used as the rootstock for most trees in the region of similar age to those growing in the orchard. In 1980, it was self-pollinated and the progeny were grown in the original infected area. The methods used to screen the original tree, and the pedigree of the 17 trees used in the initial screening, are reported in Lavee *et al.* (1999).

Five of the progeny were selected (Table 5.1) and observed over a period of 20 years under infective conditions. Four of the trees (MAS-21, MAS-27, MAS-29 and MAS-30) did not display any symptoms and were designated as resistant. Subsequently this was confirmed under laboratory conditions (Lavee *et al.*, 1999). The fifth tree (MAS-5) showed

occasional slight infection, and was designated semi-resistant. Since 1991, these five trees have been crossed as female parents with the commercial cultivars Barnea, Manzanillo, Muhasan, and Souri, all of which show different degrees of susceptibility to *S. oleaginea*. Barnea is the most resistant and Souri is the least resistant (Lavee *et al.*, 1999). Progeny derived from these crosses were examined for olive leaf spot under field conditions for about eight years and designated as either resistant or susceptible.

Trees that never showed infection to leaf spot, over a 20 year period in the case of the female parents, and eight years for the segregating population, were designated as resistant. Progeny that showed symptoms of the disease, regardless of the intensity of infection, were designated as susceptible, and the male parents were classified as semi-resistant or susceptible based on the level of infection (Lavee *et al.*, 1999).

5.3.2 Leaf material

For this study, leaves from the following genotypes were used: cv. Chimlali, which was assumed to be the progenitor of the female parents used in this study; five female parents, MAS-21, MAS-27, MAS-29, MAS-30 (all resistant), and MAS-5 (semi-resistant); four male parents, Barnea (B), Manzanillo (M) (both semi-resistant), Muhasan (Mu) and Souri (S) (both susceptible), and 34 progeny derived from the following parental combinations: MAS-30×S, MAS-30×Mu, MAS-30×B, MAS-29×S, MAS-27×M, MAS-21×M, MAS-5× M, and progeny derived from selfed MAS-29 (Table 5.1).

5.3.3 Bulked segregant analysis

DNA isolation was performed following the methods described in Chapter 2.

5.3.3.1 Preparation of bulked DNA samples

Two bulked DNA samples were constructed for analysis according to Michelmore *et al.* (1991). The resistant bulk consisted of a mixture of equal amounts of DNA, each diluted to 20 ng/μL, from 13 individuals designated as resistant. Similarly, DNA from 13 susceptible individuals was mixed to make the susceptible bulk. Individual progeny used to construct the two DNA bulks were derived from the same parental combinations (Table 5.1).

5.3.3.2 Primer screening

One hundred 10-mer oligoribonucleotide primers of arbitrary sequence (kits OPA, OPB, OPD, OPF, and OPZ) obtained from Operon Technologies, Almeda, CA, USA were used to screen for polymorphisms between the resistance and the susceptible DNA bulks. The two DNA bulks were subjected to PCR, using a total of 40 ng/µL genomic DNA, under the same PCR mixture and amplification conditions as described in Chapter 2. Amplification fragments were separated on 1.5% (w/v) agarose gels (Seakem® GTG® agarose, FMC, BioProducts, Rockland, Maine, USA). When a polymorphism was detected between the two bulks, the primer that produced the polymorphism was then tested in three separate PCR reactions before further analysis was made.

5.3.3.3 Confirmation of markers linked to olive leaf spot

Consistent polymorphisms found between the two bulks were tested for linkage to leaf spot resistance by examining the PCR products obtained from all parents, and all 26 F1 progeny that comprised the bulks. If a polymorphic band cosegregated among individuals within the two bulks, it was then tested on Chimlali and a further eight samples of DNA from the segregating progeny. The same primers and PCR conditions that generated the unique fragment were used for all tests. For enhanced resolution, the products were separated on 6% polyacrylamide as described in Chapter 2

5.3.3.4 Data analysis

The recombination frequency between the marker identified and the gene conferring the resistance or susceptibility to *S. oleaginea* was calculated as Rf = Rm + SM/n or Rf = Sm + RM/n respectively, where Rf = recombination frequency, Rm, SM, Sm, RM and n are the number of resistant recombinants without the resistant marker, susceptible recombinants with the resistant marker, susceptible recombinants without the susceptible marker, resistant recombinants with the susceptible marker, and total number of individuals respectively (Yang and Krüger, 1994). The observed data were fitted to the expected data using chi-square analysis (Statistica for Windows Release 5.1, StatSoft Inc., Tulsa, OK, USA).

5.4 Results

DNA from two bulked samples, each containing 13 progeny of the segregating population designated as either resistant or susceptible to leaf spot, was used as a template to identify a RAPD marker linked to olive leaf spot. A total of 100 random 10-mer oligonucleotide primers was used to screen the two bulks for polymorphisms. All primers produced amplification products and an average of nine bands was amplified per primer. One primer, OPA11 (5′-CAATCGCCGT-3′), generated two polymorphisms in the PCR products, one of about 780 bp from the resistant bulk, and the other of about 700 bp from the susceptible bulk. The presence of these polymorphisms was confirmed in three separate PCR reactions. The presence of a band in one bulk and its absence in the other is putative evidence for a marker to be linked to the olive leaf spot trait.

To test the linkage of the RAPD markers to olive leaf spot resistance, DNA prepared from 44 individuals from the breeding program was subjected to the same PCR conditions as the bulked DNA. The individuals included the nine parents, cv. Chimlali, the 26 progeny used to prepare the two DNA bulks, and another eight progeny from the segregating population, four of which were designated as resistant (Table 5.1). To further confirm the reproducibility of the results, the PCR products were separated on 6% polyacrylamide gels.

A polymorphic band of about 780 bp, produced by primer OPA11, was found to be linked to leaf spot resistance. Four of the resistant parents (MAS-21, MAS-27, MAS-29, and MAS-30), and 12 of the 17 resistant progeny from the segregating population showed the marker band. It was present also in five of the 17 susceptible progeny tested, but not in any of the susceptible or semi-resistant parents nor in cv. Chimlali. The strong band that was observed only in the resistant bulk on agarose gel, also appeared as a weak band in the susceptible bulk.

The polymorphic band of about 700 bp produced from the susceptible bulk was present in all of the susceptible or semi-resistant parents, in cv. Chimlali, and 8 of the 17 susceptible progeny of the segregating population tested. It was present also in five of the 17 resistant progeny, but absent in the resistant parents. Examples of the polymorphisms detected between the two bulks and parents, and between the two bulks, parents and progeny with OPA11 primer are shown in Figs. 5.2 and Fig. 5.3 respectively.

Table 5.2 shows the number of progeny segregating for resistance or susceptibility under field conditions and the presence or absence of the two molecular markers. Chi-square analysis showed that the numbers of segregating progeny did not vary from the expected values at $P \leq 0.05$.

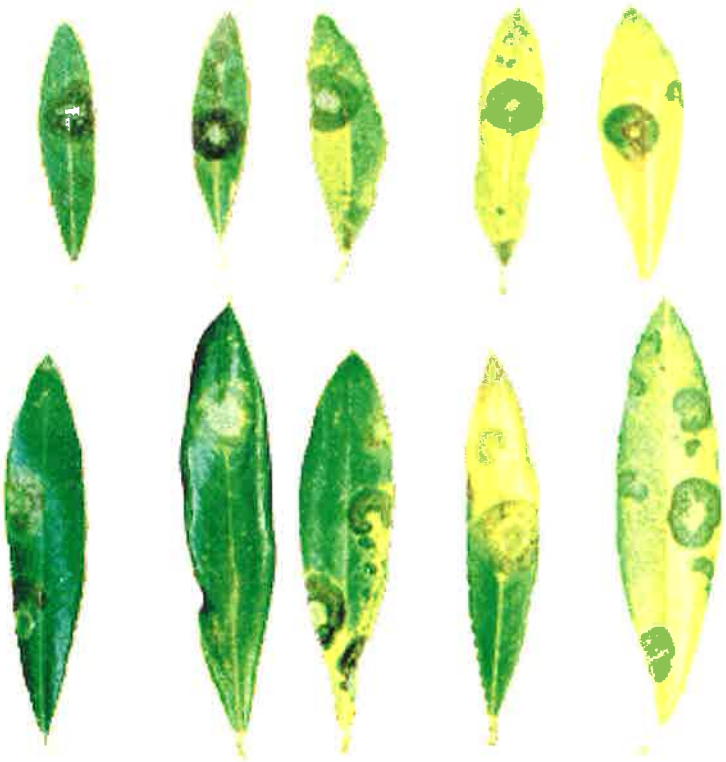


Fig. 5.1 Symptoms and different stages of olive leaf spot, on segregating population obtained from The Institute of Horticulture, Volcani Center, Bet Dagan, Israel.

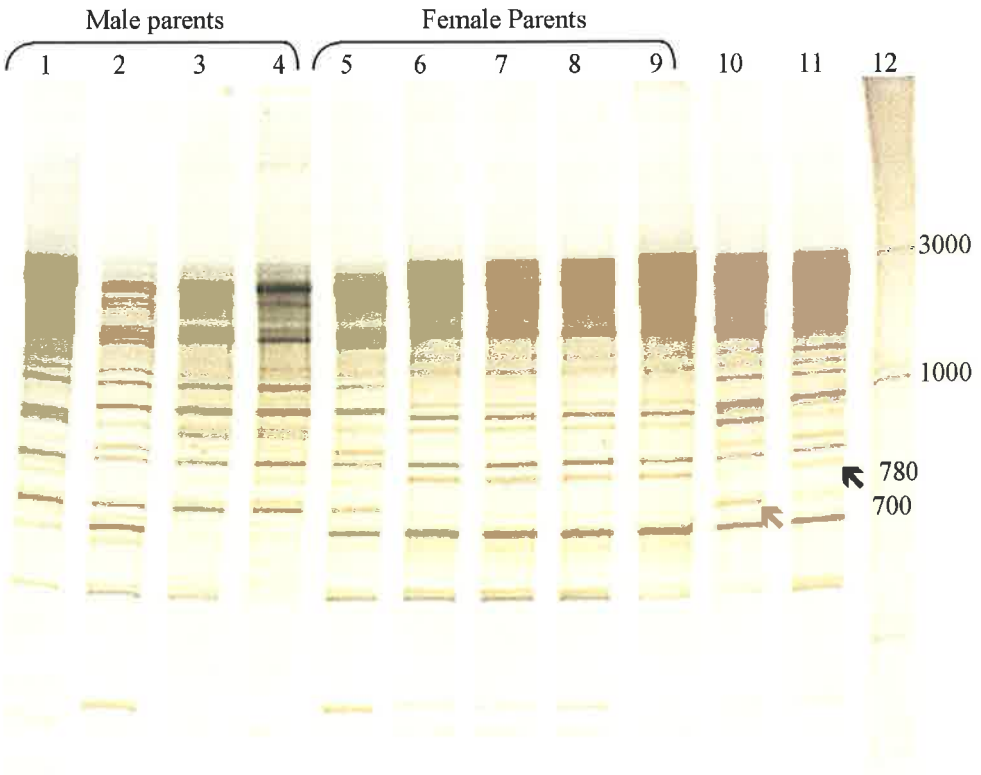


Fig. 5.2 6% polyacrylamide gel showing polymorphisms at 700 and 780 bp (arrows) between the two bulks and parents, with primer OPA11. Lane 1: Souri (susceptible); lane 2: Muhasan (susceptible); lane 3: Manzanillo (semi-resistant); lane 4: Barnea (semi-resistant); lane 5: MAS-5 (semi-resistant); lane 6: MAS-30; lane 7: MAS-21; lane 8: MAS-27; lane 9: MAS-29 (all resistant); lane 10: susceptible bulk; lane 11: resistant bulk; lane 12: 100 bp DNA ladder.

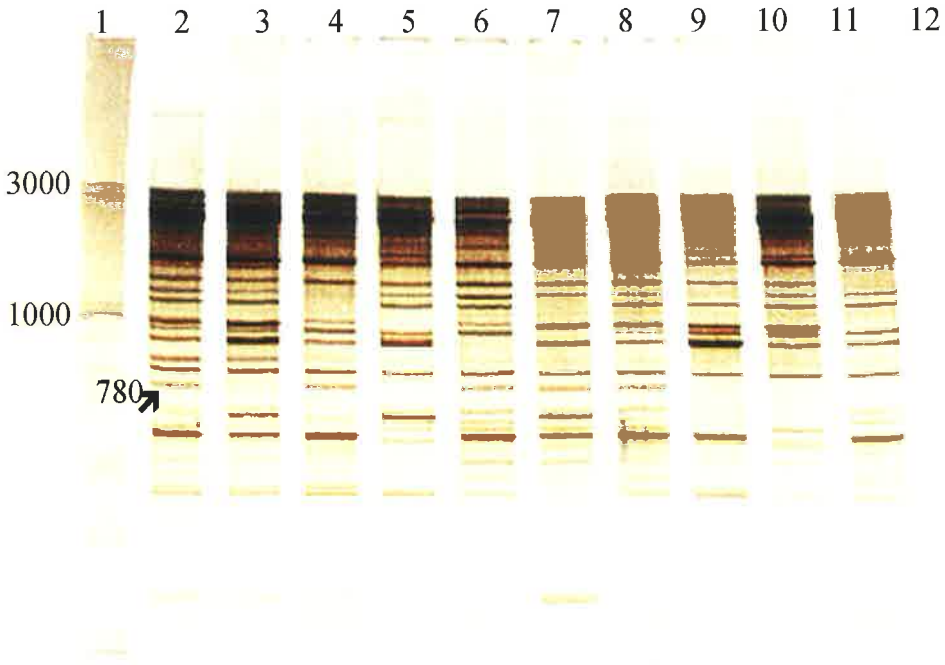


Fig. 5.3 6% polyacrylamide gel showing the polymorphism at 780 bp (arrow) between the two bulks, resistant parent and susceptible parent and progeny derived from these crosses: lane 1: 100 bp DNA ladder; lane 2: Resistant bulk; lane 3: susceptible bulk; lane 4: Resistant parent (MAS-30); lane 5: Susceptible parent (Barnea); lane 6: Resistant progeny (BD3-73); lane 7: Resistant progeny (BD3-76); lane 8: Resistant progeny (BD3-82); lane 9: Susceptible progeny (BD3-74); lane 10: Susceptible progeny (BD3-77); lane 11: Susceptible progeny (BD3-78); lane 12: Control (no genomic DNA).

Table 5.1 Segregation of olive leaf spot resistance based on field observation, and detection of markers using primer OPA11 (700 and 780 bp).

Genotypes	Used in bulk DNA samples	Field response	700 bp	780 bp
Parents				
Male				
Barnea		R-S (1)	+	–
Manzanillo		R-S (2)	+	–
Muhasan		S	+	–
Souri		S	+	–
Female (20 years field observation)				
MAS-21		R	–	+
MAS-27		R	–	+
MAS-29		R	–	+
MAS-30		R	–	+
MAS-5		R-S (1)	+	–
Chimlali-possible original parent		R-S (1)	+	–
Crosses (eight years field observation)				
MAS-30×Barnea				
BD3-73	B	R	–	+
BD3-76	B	R	+	+
BD3-82	B	R	–	+
BD3-74	B	S	–	–
BD3-77	B	S	–	–
BD3-78	B	S	+	–
MAS-27×Manzanillo				
BD2-258	B	R	–	+
BD2-260	B	R	–	+
BD2-256	B	S	+	–
BD2-257	B	S	+	–
MAS-30×Muhasan				
BD3-137	B	R	–	–
BD3-147	B	R	–	+
BD3-143	B	S	–	–
BD3-149	B	S	–	–
MAS-29×Souri				
BD2-265	B	R	+	+
BD2-268	B	R	–	–[z]
BD2-266		R	–	+
BD2-269	B	S	+	+
BD2-275	B	S	+	+

Continued

Table 5.1 continued

Genotypes	Used in bulk DNA samples	Field response	700 bp	780 bp
MAS-30×Souri				
BD3-108	B	R	–	–
BD3-105	B	S	+	–
BD3-111		S	–	+
MAS-5×Manzaillo				
BD3-88	B	R	+	–
BD3-96	B	S	–	–
BD3-89		S	+	–
BD3-95		S	+	–
MAS-21×Manzanillo				
BD3-156	B	R	–	+
BD3-165	B	R	–	+
BD3-168		R	+	–
BD3-160	B	S	–	+
BD3-164	B	S	–	+
Selfed MAS-29				
BD2-281		R	+	+
BD2-284		R	–	+
BD2-285		R	–	–

R: field resistant; S: highly susceptible; R-S: semi-resistant, 1, slightly susceptible, 2, moderately susceptible; B: individuals used in the bulk; +: marker present; –: marker absent; [z]recently showed infection.

Table 5.2 Recombination frequencies for the 780 and 700 bp markers, and observed and expected numbers of segregating progeny.

Genetic markers	Progeny numbers				Recombination frequency
	RM	Rm	SM	Sm	
780 bp	12	5	5	12	0.29
Expected	17	0	0	17	
700 bp	5	12	8	9	0.41
Expected	0	17	17	0	

R: field resistant; S: field susceptible; M: marker present; m: marker absent

5.5 Discussion

BSA, together with the RAPD-PCR technique, was used to identify genetic markers linked to leaf spot resistance. By bulking DNA from individuals, it was possible to eliminate the randomised genetic background of unlinked loci (Michelmore *et al.*, 1991), and thus the putative marker associated with leaf spot resistance could be identified by detecting polymorphisms between the two bulks. Michelmore *et al.* (1991) indicated that when the linkage of all polymorphisms is confirmed by analysis of a segregating population, only a small number of individuals in each bulk can provide enrichment for the markers linked to the target region. However, the segregating progeny used in this study were evaluated for their response to leaf spot only under natural conditions (not under controlled conditions), and hence the actual genotypes of the individuals in the two bulks were unknown. Therefore, to reduce the presence of false positives and to maximise the selection pressure for markers associated with leaf spot, a relatively large number of individuals per bulk was used.

In this study, two molecular markers, each linked to the expression of resistance or susceptibility to olive leaf spot, were identified using primer OPA11. One marker was about 780 bp and occurred in 70.6% of the resistant segregating population (field evaluated for 8 years), and 100% of the resistant parents (field evaluated for 20 years). For the combined populations tested, the band was present in 76.2% of resistant individuals, and absent in 78.3% of the individuals showing susceptibility.

Of the 34 progeny examined for the molecular markers (Table 5.1), 17 showed resistance and 17 showed susceptibility when tested under field conditions (BD2-268 recently showed susceptibility, S. Lavee, pers. comm.). These 34 individuals were derived from eight different crosses and so conclusions cannot be drawn regarding a genetic model for resistance. However, Lavee *et al.* (1999) suggest that resistance is recessive. Chi-square analysis of the data in Table 5.2 showed that the expected segregation of individuals was not significantly different from the observed values. Based on this limited data set, the recombination frequencies suggest that the 780 and 700 bp markers are 29 and 41 cM distant from the gene, respectively. Both markers are relatively distant from the gene conferring resistant or susceptibility compared to other studies (Michelmore *et al.*, 1991; Boora *et al.*, 1998).

Individuals that were classified as semi-resistant to the disease did not produce the 780 bp marker. Thus, no segregation was expected in the progeny derived from the cross between MAS-5×Manzanillo (Table 5.1) because both lack the marker for resistance, although both are designated as semi-resistant. It is clear that the segregating progeny with the marker band inherited the genetic information for leaf spot resistance from the resistant parent.

A second marker of about 700 bp was present in 60.9% of individuals designated as susceptible or semi-resistant and absent in 76.2% of resistant individuals. When the presence and/or absence of both markers was applied to all parents and progeny, 68.2% of the population could be designated as resistant and the same proportion as susceptible.

The high resolution achieved by the combination of polyacrylamide gels with silver staining allowed a faint band of 780 bp to be detected in the susceptible bulk, and likewise a faint band of 700 bp to be detected in the resistant bulk. These were not apparent on agarose gels. This is attributed to the presence of the marker for resistance or susceptibility in some of the DNA samples making up the susceptible and the resistant bulks respectively.

The gene for resistance to leaf spot in the female parents used in the breeding program was believed to be derived from the wild cultivar Chimlali. The particular tree chosen for self-pollination in 1980 was phenotypically different from the rest of the trees in the orchard that were designated as cv. Muhasan (Lavee *et al.*, 1999). It was thought to be a seedling of cv. Chimlali since Chimlali had been used as the rootstock for most of trees in the orchard. However, the marker band was not detected in DNA extracted from Chimlali. It is possible that the male parent of the seedling was resistant to the pathogen, whereas the female parent, Chimlali, was not. Alternatively, considering the high degree of genetic variability that can be found between different accessions of the same olive cultivar (Mekuria *et al.*, 1999), it is possible that the female parents were derived from a different tree of Chimlali to that from which the leaves were subsequently taken for DNA analysis. Furthermore, the marker band was not detected in DNA extracted from Muhasan.

Discrepancies occurred between the designation of trees as either resistant or susceptible to leaf spot based on field observation, and the presence or absence of molecular markers. Three possible reasons can be advanced to explain this result. Firstly, these markers identify

areas close to the genes of interest rather than the genes themselves. The 780 bp marker was more tightly linked to resistance than was the 700 bp marker to susceptibility and will be more useful in the breeding program. However, both were sufficiently distant from the gene to allow cross-over events to occur between the gene and the marker. Secondly, the results found for the 780 bp molecular marker were 100% reproducible for the DNA from parental trees, which had been subjected to field observations over a 20-year period. However, in some progeny of the segregating population, a change in the level of resistance through time was observed. For example, BD2-268 was designated as resistant until it recently displayed disease symptoms. No RAPD marker linked to resistance to leaf spot was detected for this genotype. In addition, some trees that showed field resistance produced a susceptible response in laboratory tests (Lavee *et al.*, 1999). This result suggests that if selection for resistance to olive leaf spot is made too early by field evaluation, there is a chance of falsely rating a plant as resistant. Thirdly, the phenotypic expression of resistance or susceptibility can be influenced by the environment, and hence some individuals may escape infection even when they are susceptible (Yang and Krüger, 1994; Bai *et al.*, 2000). Boora *et al.* (1998) found that plants that showed resistance to anthracnose in the field did not always show resistance in progeny tests and they concluded that this was a response of the plants to microenvironmental influences. This emphasises the importance of using molecular markers for early screening in breeding programs.

5.6 Summary

This study has detected molecular markers for olive leaf spot disease in a segregating population based on field observations of resistance and susceptibility. The 780 bp marker was found to be more closely linked to resistance to olive leaf spot than the 700 bp marker was to susceptibility. However, all of the information available about the expression of the disease on the progeny studied was based on natural infection, thus the chance of scoring individuals into the wrong phenotype may be unavoidable. The conversion of the 780 bp RAPD marker into sequence-tagged site (STS) (Williamson *et al.*, 1994) would permit a more accurate characterisation of the segregating population. Thus, future work will focus on cloning and sequencing the 780 bp marker and designing specific primers. The use of these primers will greatly assist screening for the disease in breeding programs.

Chapter Six

Sequence tagged site for the RAPD marker linked to leaf spot resistance in olives

6.1 Abstract

A RAPD band of about 780 bp was previously identified which was linked to olive leaf spot resistance. In this study the DNA band corresponding to this marker was cloned and sequenced, and sequence specific primers were designed to produce an STS marker. When the reamplified PCR product of about 780 bp was cloned, digested with restriction enzyme, and separated on agarose gel, nine different inserts were identified, each of about the same size. Three or four of these inserts appeared to be artifacts of the reamplification process, with the remainder comigrating with the original RAPD band. Based on the sequence results, 10 pairs of STS primers were selected and tested against DNA extracted from resistant and susceptible individual trees from the segregating population. Eight primer pairs amplified a product of the same size in both resistant and susceptible genotypes, and one primer pair produced a polymorphism that was unrelated to the trait in question. One primer pair, named G7F (5´-CATCACCACTCCACTCCTCTC-3´), and G7R (5´-CAGCATCTCCATAATCCTTTC-3´) produced the expected polymorphism and was tested against 44 individuals from the population segregating for resistance to olive leaf spot, and 13 commercial olive cultivars showing various levels of resistance to the disease. The STS marker was present in 71.4% of the parents and progeny that were designated as resistant, but was absent in 87% of the parents and progeny showing susceptibility. These primers were also able distinguish cultivars such as Koroneiki and Leccino, that show resistance to olive leaf spot, from Barouni and Mission, which are reported to be susceptible. The use of an STS marker will greatly assist screening olive progeny resistant to olive leaf spot in breeding programs.

6.2 Introduction

The RAPD technique, in conjunction with BSA, is efficient at generating markers linked to traits of interest (Michelmore *et al.*, 1991; Wirthensohn *et al.*, 1999). Since there is no requirement for sequence information for the plant under study, it is possible to work with a

large variety of species, and to develop markers in a relatively short period of time. However, there are some drawbacks with the RAPD-PCR technique. For example, the reproducibility of RAPD markers between different laboratories has been questioned (Jones *et al.*, 1997), polymorphisms are identified usually as present or absent without regard to the intensity of the band, and PCR products that comigrate in different lanes are considered to be the same, whereas a single RAPD band may consist of several similar-sized DNA fragments each with a different sequence (Hausner *et al.*, 1999).

To overcome these problems associated with RAPDs, and to improve their utility as markers in breeding programs, Paran and Michelmore (1993) developed sequence characterised regions (SCARs) by using longer and more specific primers developed from the sequences of the RAPD fragments. These longer primers generate a sequence-tagged site (STS) as described by Olson *et al.* (1989). Such markers have been developed for disease resistance in many plant species, such as downy mildew resistance in lettuce (Paran and Michelmore, 1993), nematode resistance in tomato (Williamson *et al.*, 1994), citrus tristeza virus resistance in trifoliate orange (Deng *et al.*, 1997), powdery mildew resistance in wheat (Hu *et al.*, 1997), and *Phytophthora* resistance in strawberry (Haymes *et al.*, 2000).

In Chapter 5, a RAPD marker linked to leaf spot resistance in olive was identified using the 10-mer primer, OPA11. This Chapter describes how the RAPD marker was cloned, sequenced and converted into a sequence-tagged site, as described in Williamson *et al.* (1994), to improve the efficiency of marker assisted selection in olive breeding programs.

6.3 Methodology

6.3.1 Isolation and purification of the RAPD marker

A number of different protocols was tested to isolate and purify the 780 bp RAPD fragment so that it could be used as a template for reamplification and further cloning:

1) Protocol I. Either the entire band was cut from agarose or polyacrylamide gels with a sterile surgical blade (Swann-Morton, England), or the band was stabbed with a micropipette tip. In each case, the gel portion was subjected to amplification, without

any further treatment, using the same PCR mix and thermal cycling regime that were previously used to produce the RAPD band. The reamplified PCR products were resolved on 1.5% agarose.

2) The entire band was excised from either agarose or polyacrylamide gels using a sterile surgical blade and the gel portion was subjected to a number of treatments:

1. Protocol II. The gel portion from a polyacrylamide gel was placed in a 1.5 mL Eppendorf tube containing 200 µL of 20% ethanol and 10% acetic acid, incubated for 30 min, and the supernatant removed. It was then washed twice with sterile water, the supernatant was discarded, and the gel debris was used as a template for PCR reamplification. This treatment was similar to the DNA fixation step used for silver staining.

2. Protocol III. The gel portion was excised either from a fresh or dried polyacrylamide gel and transferred to a 1.5 mL Eppendorf tube. 100 µL of sterile water was added, the tube incubated at RT for 10 min, and boiled for 15 min in a water bath. The sample was then centrifuged at 13,000 rpm for 5 min at RT and the supernatant was transferred to a fresh 1.5 mL tube. 10 µL of 3 M NaAc (pH 5.2) and 2½ volume (275 µL) of absolute ethanol (RT) were added to the tube and the mixture incubated for 20 min at 4°C. After centrifuging at 14,000 rpm for 20 min, the supernatent was removed, and the pellet dried by placing the tube in a heating block at 50°C for 4 min. DNA was recovered in 10 µL of TE buffer, and stored at -20°C for further use.

3. Protocol IV. The gel portion was excised from a fresh polyacrylamide gel, transferred to a 1.5 mL Eppendorf tube, and crushed with a small (0.6 x 32 mm) disposable needle in the presence of 400 µL Solution A from the HealthGene kit (Fast RNA and DNA Elution™ Kit, HealthGene Corporation, Canada). The tube was incubated for 2½ hr at RT with occasional vortexing, and centrifuged at 12,000 rpm for 10 min at RT. After transferring the supernatant to a fresh Eppendorf tube, the DNA was precipitated by adding 1 mL of Solution B from the kit, incubated at -20°C for 2½ hr, and centrifuged at 12,000 rpm for 15 min. After removing the supernatant, 50 µL of 70% ethanol was added to the pellet, and centrifuged at

12,000 rpm for 10 min at RT. The supernatent was again removed, the pellet dried by placing the tube in a heating block at 50°C for 4 min, and the DNA was recovered in 60 µL of sterile water, and stored at -20°C for further use.

4. Protocol V. The gel portion was excised from either a fresh or dried polyacrylamide gel, transferred to a 1.5 mL Eppendorf tube, and crushed with a small disposable needle. 35 µL of elution (extraction) buffer (0.5 M ammonium acetate, 10 mM magnesium acetate, 1 mM EDTA, pH 8.0, and 0.1% SDS) (Sambrook *et al.*, 1989) was added and the tube incubated on an Orbital Mixer Incubator (RATEK Instrument, Australia) at 37°C overnight. The sample was centrifuged at 12,000 rpm for 1 min at 4°C and the supernatant was transferred to a fresh Eppendorf tube. The pellet was extracted with a further 12.5 µL of elution buffer, briefly vortexed, centrifuged and the two supernatants were combined. The DNA was precipitated by adding 2 volumes of absolute ethanol (4°C), chilling on ice for 20 min, and centrifuging at 12,000 rpm for 20 min at 4°C. After removing the supernatant, the pellet was dissolved in 100 µL of TE (pH 8.0), and DNA was again precipitated by adding 10 µL of 3 M NaAc (pH 5.2) and 2 volumes of absolute ethanol (4°C). The solution was chilled on ice for 20 min, and centrifuged at 12, 000 rpm for 20 min at 4°C. After removing the supernatant, 50 µL of 70% ethanol was added to the pellet, and centrifuged at 12,000 rpm for 10 min at 4°C. The supernatent was again removed, and the pellet dried by placing the tube in a heating block at 50°C for 4 min and the DNA was recovered in 20 µL of TE buffer.

In the case of protocols III, IV, and V, 2 µL aliquots of the purified DNA (20-30 ng/µL) were reamplified using the same primer and PCR reaction conditions that were originally used to produce the RAPD fragment. The reamplified PCR products were resolved on 1.5% agarose gels together with a 100 bp DNA ladder (DMW-100M, GeneWorks, Adelaide, Australia) as a molecular weight standard. The purity of the DNA fragments was checked visually on agarose gels by considering the absence of smear background, and the intensity of the fragment of interest.

If more than one RAPD fragment was produced from the reamplified PCR products after separation on agarose, the products were reseparated by PAGE, and then excised and purified. Aliquots of the purified DNA were reamplified for a second time using the same

primer and PCR reaction conditions as before and separated on agarose gels. If more than one RAPD fragment was again amplified, the entire PCR products were cloned and the fragment of interest was selected after digesting the plasmid vector with restriction enzyme and separating the restriction digestion products on agarose.

6.3.2 DNA cloning and sequencing

6.3.2.1 DNA cloning

The reamplified PCR product (or products) was cloned into a plasmid vector, pCR® 2.1-TOPO, with the TOPO™ TA Cloning® Kit (Invitrogen Corporation, CA, USA), using the following procedure. For each DNA sample, the TOPO cloning reaction was made using 1 µL of fresh reamplified PCR product, 1 µL of pCR®-TOPO vector, and 3 µL of sterile water. The reagents were gently mixed and incubated for 5 min at RT and placed on ice. Two µL of the TOPO cloning reaction was then added to a vial of TOP10F´ competent cells, mixed, and incubated on ice for 30 min. The cells were heat shocked for 30 sec at 42°C, and incubated on ice for 2 min. After adding 250 µL of SOC medium (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM SO_4, and 20 mM glucose), the tube containing the reaction mix was placed on a shaker (Orbital Mixer Incubator) at 37°C for 1 hr. Following transformation, 100 µL of cells were spread on 9 cm LB plates (1.0% Tryptone, 0.5% Yeast Extract, 1.0% NaCl, 50 µg/mL ampicillin) and incubated overnight at 37°C. To achieve blue/white colony screening of recombinants, LB plates were coated with 16 µL of 50 mg/mL X-Gal and 33 µL of 25 mg/mL isopropylthiogalactoside (IPTG) and dried at 37°C for 30 min prior to spreading the cells.

6.3.2.2 Small scale preparation of plasmid DNA

Positive colonies (white) that contained the cloned plasmid DNA were purified using a standard protocol (Promega Protocol and Application Guide, 1991, Promega Corporation, Madison, USA).

A single white colony from the LB plate was transferred into a 10 mL screw cap tube containing 1.5 mL of LB broth (1.0% tryptone, 0.5% yeast extract, and 1.0% NaCl) and 1.5 µL of 100 mg/mL ampicillin, and incubated at 37°C overnight with vigorous shaking on a

rotating wheel. The culture was poured into a 1.5 mL Eppendorf tube and centrifuged at 14,000 rpm for 3 min. The supernatant was removed from the tube, leaving the pellet as dry as possible. The pellet was loosened by vortexing and resuspended in 90 µL of sterile 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. 130 µL of 3 M KAc, pH 4.6 was added and again gently mixed by inversion. The mixture was centrifuged at 14,000 rpm 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 each of tris-saturated phenol and chloroform were added, briefly vortexed, and centrifuged for 5 min at 14,000 rpm. The supernatant was transferred into a new tube and the DNA was precipitated with 2.5 volume of ethanol (-20°C). After incubating for 10 min at room temperature, plasmid DNA was collected by centrifuging for 15 min at 14,000 rpm, removing the supernatant, and washing the pellet with 100 µL 70% ethanol (-20°C). To remove remaining ethanol, the pellet was briefly dried on a heating block for 4 min at 50°C, and DNA was recovered with 30 µL of 0.1 mM ETDA, pH 8.0, and stored at -20°C for further analysis.

The cloned DNA was analysed by cleavage with restriction enzyme using 1 µL of cloned DNA, 6.8 µL of sterile water, 2 µL of 10 x restriction enzyme buffer (1 M Tris acetate, pH 7.8, 5 M KAc, 1 M MgAc, 0.1 M spermidine, and 0.1 M dithioerythritol) and 0.2 µL of *Eco*RI (10 U/µL) (Boehringer, Mannheim, GmbH, Germany), incubated for 1 hr at 37°C and separated by electrophoresis on 1.5% agarose in TBE buffer. When the presence of an insert of the appropriate size was confirmed, the cloned DNA was then prepared for sequencing.

6.3.2.3 Preparation of cloned DNA for sequencing

PCR was performed with the dye deoxy terminator sequencing kit of Applied Biosystems, using 2 µL of the cloned DNA (about 200 ng), 3 µL (4 pmoles/µL) of M13 forward primer 5′-TGTAAAACGACGGCCAGT-3′, 8 µL of terminator ready reaction mix, and 7 µL of sterile water. The same reaction mixture was made using M13 reverse primer (4

pmoles/μL) 5′-CAGGAAACAGCTATGACC-3′. Amplification was performed using the following program: 25 cycles of 96°C for 10 s, 50°C for 5 s, and 60°C for 4 min.

After amplification, the PCR products were precipitated by adding 2 μL of 3 M sodium acetate (pH 5.2), and 50 μL of absolute ethanol (RT), vortexing, and incubating on ice for 10 min. The mixture was centrifuged for 30 min at 14,000 rpm, the supernatant removed, and the pellet washed with 100 μL of 70% (RT) ethanol and stored at -20°C.

The DNA sequence of the cloned PCR products was determined from 26 colonies. To check reproducibility of the sequencing results, some samples were sequenced in duplicate. Sequencing was carried out by either the Nucleic Acid and Protein Chemistry Unit, Adelaide University, using an Applied Biosystem, Model 337A, or Division of Molecular Pathology, the Institute of Medical and Veterinary Science, Adelaide, Australia, using ABI PRISM 3700 DNA Analyser.

Sequences of the cloned RAPD products were aligned with ClustalX (Thompson *et al.*, 1997) and BioEdit Sequence Alignment Editor ver. 4.8.1 (North Carolina State University, USA).

6.3.2.4 Design and analysis of sequence specific primers

Primers were designed, based on the sequencing results, using the following methods:

1) Automatic selection of primers using a computer program PRIMER (version 0.5, Whitehead Institute for Biomolecular Research, USA). This program selects the best forward and reverse primers from the sequence of the DNA fragment in question.
2) By visual observation: 10-14 bases were added to the 3′ end of the sequence of the original 10-mer, primer.
3) NetPrimer (Primer Biosoft International, Palo Alto, CA). Using the NetPrimer computer program, primers were designed initially by adding 8 to 11 bases to the 3′ end of the sequence of the original 10-mer, as described by Paran and Michelmore (1993). However, when analysis of the primer sequences by NetPrimer indicated potential interfering interactions between or within primers, they were designed using internal sequences without regard to the original 10-mer primer (Williamson *et al.*, 1994). A number of factors including primer length, GC content, and melting temperature was

taken into account to avoid possible primer dimer or secondary structure formation, to match melting temperatures, and to achieve appropriate internal stability.

Primers were screened using individuals from the olive population segregating for resistance to peacock spot disease previously described in Section 5.3.2. The reaction mixture for PCR was identical to that used for RAPD amplifications except that the specific forward and reverse primers were used at 0.25 µM each. Amplification was performed according to the following program: 95°C for 3 min, followed by 35 cycles of 30 s at 95°C, 45 s at 58°C, 1 min at 72°C, with final extension of 10 min at 72°C, although the actual annealing temperature was optimised for each primer pair. The PCR products were separated on 3% agarose gels (Nusieve:Seakem LE agarose, 3:1) in TBE buffer, stained with ethidium bromide, and visualised under UV light.

When a sequence specific primer pair produced a polymorphism between resistant and susceptible individuals in the initial screening process, verification of the sequence tagged site was carried out on all parents including the possible parent Chimlali, and all 34 progeny that were tested originally with the 10-mer primer OPA11 (Section 5.3.3.3). The primer pair was also tested on 13 samples of DNA, extracted from commercial olive cultivars: Koroneiki, Leccino, Corregiola, Frantoio, SA Verdale, Sevillano, Ascolano, Kalamata, Picual, Mission, Novo, Barouni, and Attica which are reported to show various levels of resistance to olive leaf spot. Thus, promising primer pairs were tested for their usefulness on a total of 57 individuals.

6.3.2.5 Purification of PCR products amplified with sequence specific primers

When it was necessary, PCR products amplified using the sequence specific primers (section 6.3.2.4) were purified using the BRESAspin PCR Purification kit (GeneWorks, Adelaide, Australia). PCR products were mixed with 100 µL of Spinbind buffer (BSB) in 1.5 mL Eppendorf tubes, applied to BRESAspin filter units, and centrifuged for 30 s at 13,000 rpm. The liquid in the collection tube was discarded, 300 µL of Spinclean buffer (BSC) was added, and centrifuged for 30 s at 13,000 rpm. After removing the solution, the BRESAspin filter was transferred to a clean DNA elution tube, and 20 µL of elution buffer

(BSE) was added and centrifuged for 30 s at 13,000 rpm. Eight μL aliquots of the cleaned PCR product were separated on agarose gels.

6.4 Results

The RAPD band of about 780 bp that was amplified with the 10-mer primer, OPA11, and found to be linked to olive leaf spot resistance was recovered from either agarose or polyacrylamide gels, reamplified, cloned, and sequenced with the objective of converting into a sequence tagged site.

6.4.1 Isolation and purification of the RAPD marker

A number of protocols were tested for their ability to isolate and purify the 780 bp DNA fragment from either agarose or polyacrylamide so that the fragment could be reamplified by PCR. The following results were found:

Protocol I

Gel portions and stabs were taken from the 780 bp fragment separated either on agarose or polyacryalmide, and used as the template for PCR reamplification. For the stab taken from agarose, a number of other bands, in addition to that at about 780 bp, was produced, most of them being faint. For the stab taken from polyacrylamide, no PCR products were obtained. No products were reamplified from the gel portions excised from either agarose or polyacrylamide that were used directly for PCR.

Protocol II

No PCR products were reamplified when gel portions excised from polyacrylamide were fixed with ethanol and acetic acid, and then washed with sterile water.

Protocol III

No consistent results were found when the DNA fragment isolated using Protocol III was used as a template for PCR reamplification. The 780 bp DNA fragment was sometimes

present and at other times absent (between different lanes), and either many non-specific fragments were formed or no amplification products were detected.

Protocol IV

The DNA recovered from Protocol IV consistently produced a strong fragment of 780 bp when the reamplified PCR products were separated on agarose gels. However, many other PCR products were also produced, many with the same intensity as the 780 bp marker.

Protocol V

The DNA recovered from Protocol V produced two strong fragments when reamplified, one of about 780 bp, and the other about 800 bp. In addition, a number of faint low molecular weight fragments were also produced. Protocol V was found to be superior to the others tested in terms of producing a few consistent, clear, strong bands, with a small number of weakly amplified bands, and hence further work was performed using this method. When the annealing temperature was increased from 35 to 42°C, all non-specific or faint fragments were removed, leaving only the two strong bands at 780 and 800 bp. In an attempt to obtain a single band, the reamplified PCR products were separated by PAGE, and the 780 bp fragment was excised and reamplified for the second time. However, the two strong bands at 780 and 800 bp were observed again when the PCR products were separated on agarose gel (Fig. 6.1). These PCR products were used for cloning.

6.4.2 Cloning and sequencing of the RAPD marker

Eight separate reamplified DNA fragments from Protocol V (Section 6.4.1) were cloned into pCR® 2.1-TOPO vector. A total of 112 white colonies were selected from eight LB plates and plasmid mini-preps were prepared. The plasmid DNA was digested with *Eco*RI restriction enzyme, and the products separated on 1.5 % agarose gels. 103 positive recombinants were found, the remainder being false positives. After separation on agarose, a number of different inserts, each of about 780-800 bp could be detected. One insert, representing about 28% of recombinants, resolved into four distinct bands, indicating that the insert contained three internal *Eco*RI restriction sites (Fig. 6.2). The total size of the four bands was about 800 bp.

From visual observation, it was apparent that a number of different inserts, each of about the same size, had been cloned. Because it was not possible to readily identify the resistant marker, sequencing was performed on all different size cloned fragments. A total of 26 cloned fragments was sequenced. The results showed that nine different sequences had been cloned from eight reamplified PCR products (Fig. 6.1) produced by the 10-mer primer, OPA11. These ranged in size from 765 to 814 bases. Four of the cloned fragments produced a similar sequence of 765 bp (Group 1), six were 776 bp (Group 2), two were 777 bp (Group 3), four were 814 bp (Group 4), three were 774 bp (one was less by 10 bp, Group 5), two were 765 bp (Group 6), three were 777 bp (one was less by 4 bp, Group 7), and two others had sequences of 769 and 779 bases.

An example of three different similar-sized sequences identified from the resistant bulk is shown in Fig. 6.5.

6.4.3 Analysis of sequence specific primers

A total of 10 pairs of STS primers was selected from the sequences determined for Groups 1 to 7 in Section 6.4.2. For the sequences in Groups 1, 2, and 3, primers were selected either by the PRIMER computer program or by visual observation, and for Groups 4, 5, 6, and 7, primers were selected using the NetPrimer computer program. Genomic DNA extracted from 5 to 8 individuals within the population studied were used for PCR amplification with each pair of STS primers. When promising primers were found, DNA from more individuals was tested.

6.4.3.1 STS primers designed using PRIMER computer program

The primer pairs were named as G1F and G1R (Group 1), G2F and G2R (Group 2), G3F and G3R (Group 3). Using these primer pairs, PCR was performed at different annealing temperatures and the results are summarised in Table 6.1.

Table 6.1 Results of the STS primers designed using the PRIMER computer program.

Primer pair	Annealing Temperature (°C)	Size of Main product* (bp)	Usefulness as STS	Presence of other products
G1F/G1R	40	300 (594)	Poor	Present
	53	594 (594)	Poor	Present
	58	594 (594)	Poor	Present
	62	-	-	Absent
G2F/G2R	40	500 (580)	Poor	Present
	53	500 (580)	Poor	Absent
	58	500 (580)	Poor	Absent
	62	-	-	Absent
G3F/G3R	40	615 (615)	Poor	Present
	53	615 (615)	Poor	Present
	58	615 (615)	Poor	Present
	62	615 (615)	Poor	Absent

* Expected size shown in parentheses

Each of the primers G1F and G1R was tested either singly or in combination with the 10-mer primer, OPA11. However, the results were variable, and the expected product of about 780-800 bp did not appear.

When primer G2F was used singly for PCR amplification, a single band about 500 bp was produced in both resistant and susceptible individuals. No PCR products were identified when primer G2R was used singly. Neither of these primers was tested in combination with OPA11.

When primers G3F and G3R were tested either singly or in combination with the 10-mer primer, OPA11, the results were variable, and the expected product of about 780-800 bp did not appear.

6.4.3.2 The STS primers selected by visual observation

Three primer pairs were synthesised by simply adding 10-14 bases to the 3´ end of the original 10-mer sequence. The primers were made for Group 1, Group 2, and Group 3 sequences and the primer pairs were named AG1F and AG1R, AG2F and AG2R, and AG3F and AG3R, respectively. Using these primer pairs, PCR was performed at different annealing temperature and the results are summarised in Table 6.2.

Table 6.2 Results of the STS primers selected by visual observation.

Primer pair	Annealing Temperature (°C)	Size of Main product* (bp)	Usefulness as STS	Presence of other products
AG1F/AG1R	55	765 (765)	Poor	Present
	60	765 (765)	Poor	Absent
AG2F/AG2R	55	776 (776)	Poor	Absent
	60	776 (776)	Poor	Absent
AG3F/AG3R	55	777 (777)	Poor	Absent
	60	777 (777)	Poor	Absent
	65	777 (777)	-	Absent

Note: At 60°C, primers AG1F/AG1R amplified in only one lane out of 5.

All primer pairs selected by visual observation produced a PCR product of the expected length but did not generate a polymorphism between the resistant and susceptible samples tested.

6.4.3.3 The STS primers designed using NetPrimer computer program

Four primer pairs of 18-21 bases were synthesised using NetPrimer computer software from both ends of the sequence. The primers were synthesised for sequences from Groups 4, 5, 6, and 7, and were named G4F and G4R, G5F and G5R, G6F and G6R, G7F and G7R, respectively. Using these primer pairs, PCR was performed at different annealing temperature and the results are summarised in Table 6.3.

Table 6.3 Results of the STS primers designed using NetPrimer computer program.

Primer pair	Annealing Temperature (°C)	Size of Main product* (bp)	Usefulness as STS	Presence of other products
G4F/G4R	55	810 (810)	Poor	Absent
	58	810 (810)	Poor	Absent
	62	810 (810)	Poor	Absent
G5F/G5R	55	768 (768)	Poor	Absent
	58	768 (768)	Poor	Absent
	62	768 (768)	Poor	Absent
	68	-	-	Absent
G6F/G6R	55	630 (610)	Poor	Present
	58	630 (610)	Poor	Present
	62	630 (610)	Poor	Present
G7F/G7R	55	651 (641)	Useful	Present
	58	651 (641)	Useful	Present
	62	651 (641)	useful (weak)	Present

The primer pair, G6F and G6R, amplified a polymorphic band (together with two monomorphic fragments) at an annealing temperature of 55°C between some of the resistant and some susceptible individuals of the segregating population. When the annealing temperature was raised to 62°C, only two bands were produced one of which was polymorphic. However, the primers were not useful for an STS marker because the polymorphism did not appear in the resistant parents, it was present in some susceptible parents, and produced variable results in the resistant and susceptible progeny.

The primer pair, G7F and G7R, amplified a polymorphic band between the resistant and susceptible individuals that appeared to be linked to resistance to olive leaf spot disease. Other minor bands were also present (Fig. 6.3). This primer pair was therefore tested further on the segregating population described in Section 5.2.1.2.

6.4.3.4 Analysis of the STS primer pair G7F and G7R

The G7F and G7R primers were selected from the RAPD sequence of 773 bases produced from the cloned fragments that had three internal *Eco*RI restriction sites (lane 10, Fig. 6.2).

The sequences of the primers are:
G7F 5′-CATCACCACTCCACTCCTCTC-3′, 21-mer, located between bases 34-54 and, G7R 5′-CTTTCCTAATACCTCTACGAC-3′, 21 mer, located between bases 119-99. These primers were expected to amplify the sequence from base 34 to base 674, and the product would therefore be shorter than the original RAPD marker by 132 bp.

The annealing temperature was optimised at 58°C and PCR was performed using genomic DNA from nine parents, including the possible original parent Chimlali, and 34 progeny from the segregating population (Table 6.4). The result showed that four of the resistant parents, (MAS-21, MAS-27, MAS-29, and MAS-30), and 11 of the 17 progeny designated as resistant produced the STS marker. It was present also in three of the 17 progeny designated as susceptible, but not in any of the susceptible parents, nor in Chimlali.

The primers were further tested using DNA from some commercial olive cultivars that are reported to show various levels of resistance to olive leaf spot (Table 6.4). Among the cultivars tested, the marker band was present in cultivars Koroneiki (Greece), Leccino (Italy), Corregiola (Australia), Frantoio (Australia), and SA Verdale (Australia), but absent in cultivars Sevillano (Davis, USA), Ascolano (Davis, USA), Kalamata (Australia), Picual (Israel), Mission (Davis, USA), Novo (Israel), Barouni (Australia), and Attica (Australia). The identities of these cultivars (except for cultivar Sevillano, Nova, Frantoio, Barouni and Attica) had been confirmed previously by comparing their DNA fingerprints with different accessions of the same cultivars obtained from different sources (J. Guerin, unpublished; Mekuria *et al.*, 1999).

From Fig 6.3, it can be seen that the STS marker produced by primers G7F and G7R appeared to be about 680 bp, whereas the expected product was 641 bp. This effect occurred with different types of agarose, including Agarose LE, Analytical Grade (Promega Corporation, Madison, USA), and Nusieve® GTG® agarose (Rockland, ME, USA), at

concentrations of 1.5% - 3%. A possible reason for the apparent difference in size was differences in salt content between the solutions containing the 100 bp ladder and the STS marker that might affect the mobility of the bands. To decrease this differential, the PCR product was purified using the Bresaspin PCR Purification kit (section 6.3.2.7), and then

1) Dissolved in water and separated on agarose using the 100 bp ladder as a size marker,
2) Both the 100 bp DNA ladder and the purified PCR product were diluted with BSE elution buffer from the Bresaspin PCR Purification kit, and separated on agarose.

Neither of these treatments changed the apparent size of the STS marker on agarose, and therefore two STS fragments, one from a resistant parent (MAS-30) and the other from a resistant progeny (BD3-82), were cloned, and five inserts were sequenced as described in Section 7.3.2, and the sequences compared to that of the original RAPD marker.

All five STS fragments showed an extra 10 bp between bases 264 to 273 (5′-3′) of the RAPD sequence. They showed high homology (99.1%) to each other, but less (98%) to the original RAPD band (excluding the extra 10 bp) (Fig. 6.6).

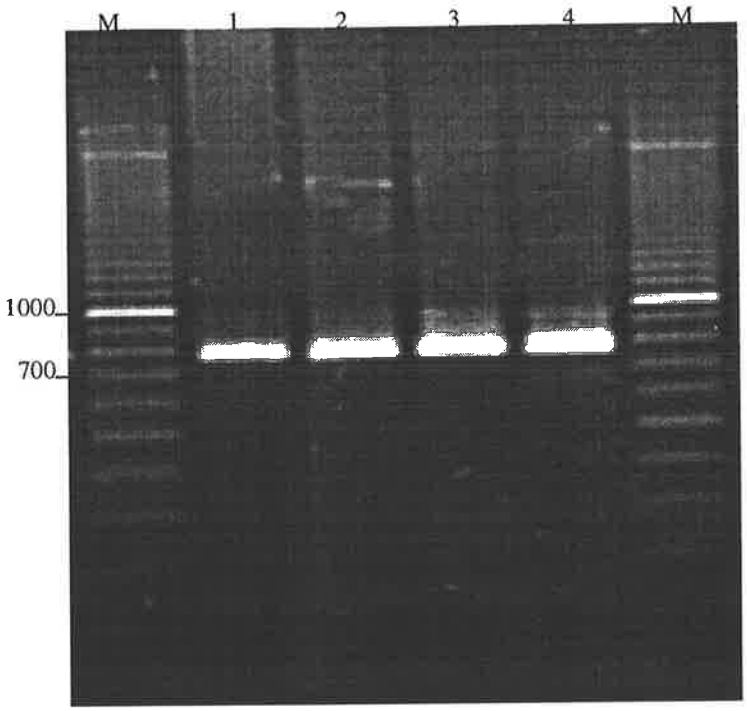


Fig. 6.1 RAPD band at about 780 bp excised from polyacrylamide, reamplified, and separated on 1.5% agarose. Lane M: 100 bp ladder; lane 1: resistant bulk; 2: resistant parent; 3: resistant progeny; 4: progeny that showed the RAPD marker for resistance but showed susceptibility under field conditions.

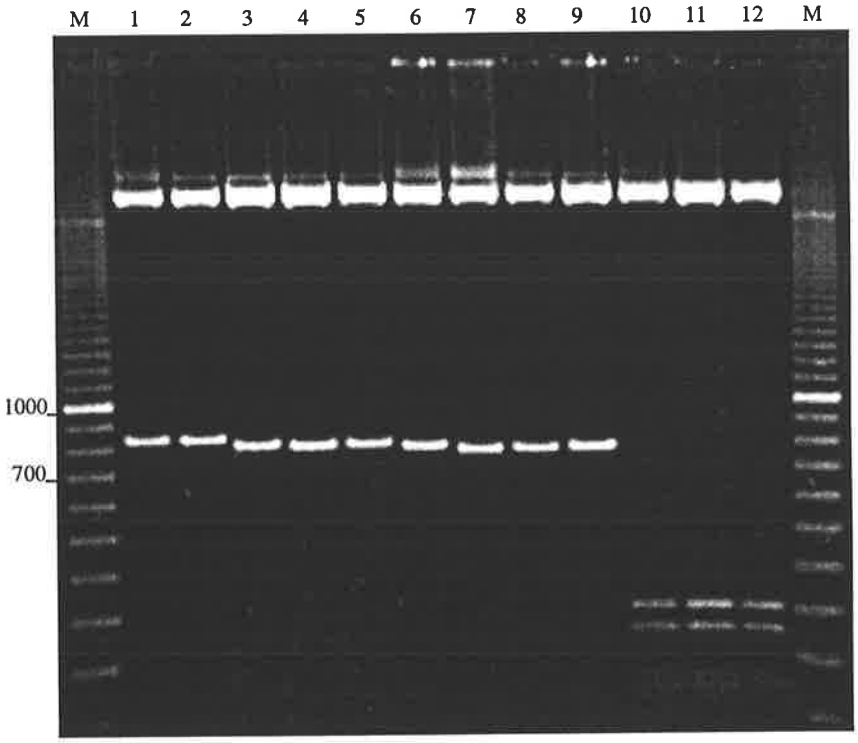


Fig. 6.2 The RAPD marker of about 780 bp inserted into TOPO vector, digested with restriction enzyme, and separated on 1.5% agarose. Lane M: 100 bp marker; 1-12: recombinant DNA showing different size fragments.

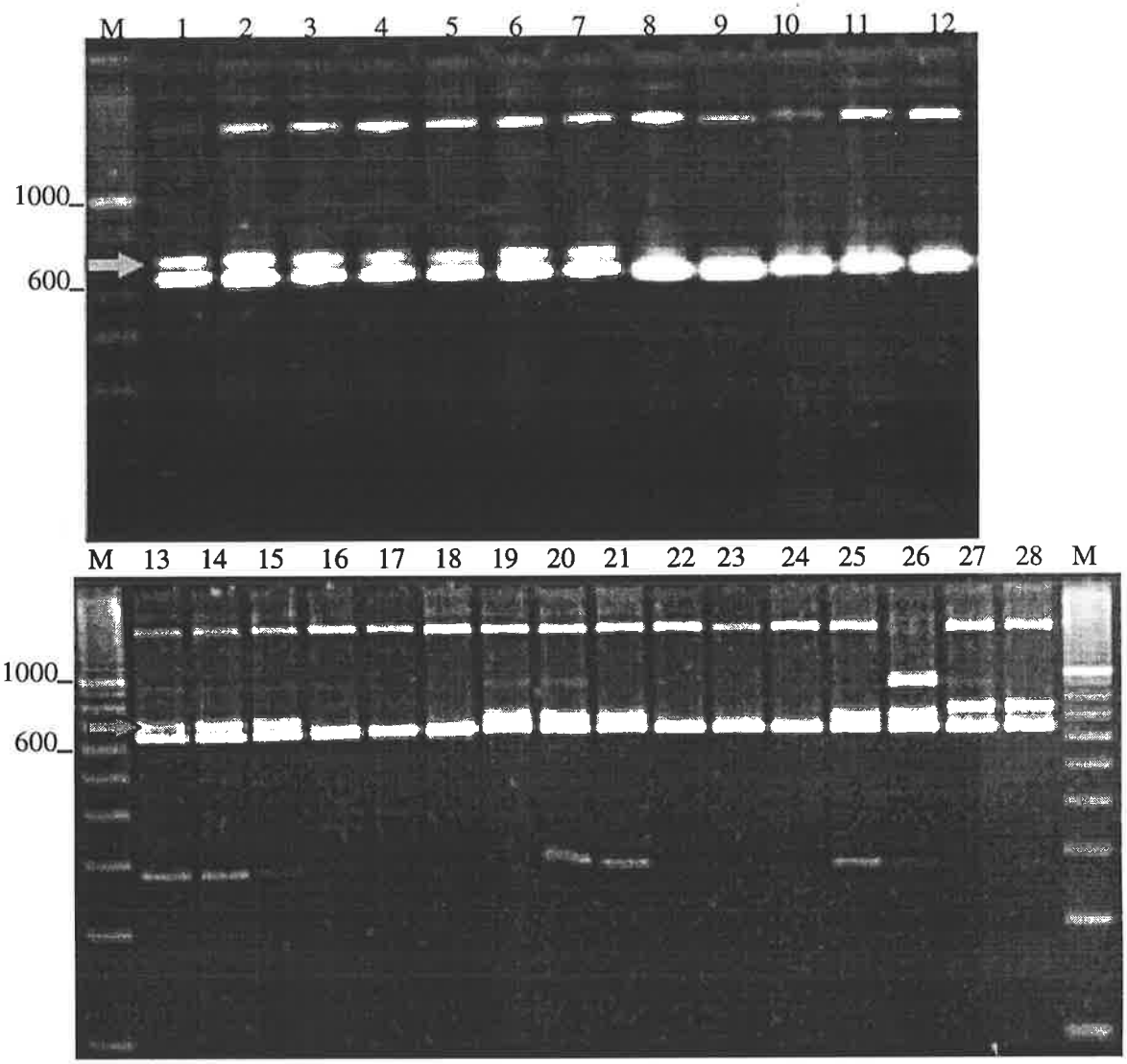


Fig. 6.3 STS marker (arrow) amplified with primers G7F and G7R and separated on 3% agarose. Lane M = 100 bp ladder; 1, 13-15, resistant parents; 2-7, 19-21, resistant progeny; 16-18 susceptible parents; 8-12, 22-24 susceptible progeny; 25, 'Koronieki' (resistant); 26, 'Leccino' (resistant); 27, 'Attica' (susceptible); 28, 'Mission' (susceptible).

Table 6.4 Segregation of olive leaf spot resistance based on field observation, and detection of markers using primer OPA11 (780 bp), and STS.

Genotypes	Field response	780 bp	STS
Parents			
Male			
Barnea	R-S (1)	–	–
Manzanillo	R-S (2)	–	–
Muhasan	S	–	–
Souri	S	–	–
Female (20 years field observation)			
MAS-21	R	+	+
MAS-27	R	+	+
MAS-29	R	+	+
MAS-30	R	+	+
MAS-5	R-S (1)	–	–
Chimlali-possible original parent	R-S (1)	–	–
Crosses (eight years field observation)			
MAS-30×Barnea			
BD3-73	R	+	+
BD3-76	R	+	+
BD3-82	R	+	+
BD3-74	S	–	–
BD3-77	S	–	–
BD3-78	S	–	–
MAS-27×Manzanillo			
BD2-258	R	+	+
BD2-260	R	+	+
BD2-256	S	–	–
BD2-257	S	–	–
MAS-30×Muhasan			
BD3-137	R	–	–
BD3-147	R	+	+
BD3-143	S	–	–
BD3-149	S	–	–
MAS-29×Souri			
BD2-265	R	+	+
BD2-268	R	–[z]	–
BD2-266	R	+	+
BD2-269	S	+	–
BD2-275	S	+	+

Continued

Table 6.4 Continued

Genotypes	Field response	780 bp	STS
MAS-30×Souri			
BD3-108	R	–	–
BD3-105	S	–	–
BD3-111	S	+	+
MAS-5×Manzaillo			
BD3-88	R	–	–
BD3-96	S	–	–
BD3-89	S	–	–
BD3-95	S	–	–
MAS-21×Manzanillo			
BD3-156	R	+	+
BD3-165	R	+	+
BD3-168	R	–	+
BD3-160	S	+	–
BD3-164	S	+	+
Selfed MAS-29			
BD2-281	R	+	–
BD2-284	R	+	–
BD2-285	R	–	–
Commercial olive cultivars			
Koronieki	R		+
Leccino	R		+
Corregiola	R		+
Frantoio	R		+
Sevillano	na		–
Ascolano	na		–
SA Verdale	na		+
Kalamata	na		–
Picual	S		–
Mission	S		–
Barouni	S		–
Attica	S		–
Novo	S		–

R: resistant; S: highly susceptible; R-S: semi-resistant, 1: slightly susceptible, 2: moderately susceptible; +: marker present; –: marker absent; [z]recently showed infection; na: information not available.

Fig. 6.5 Multiple sequences of a RAPD band amplified from the resistant bulk.

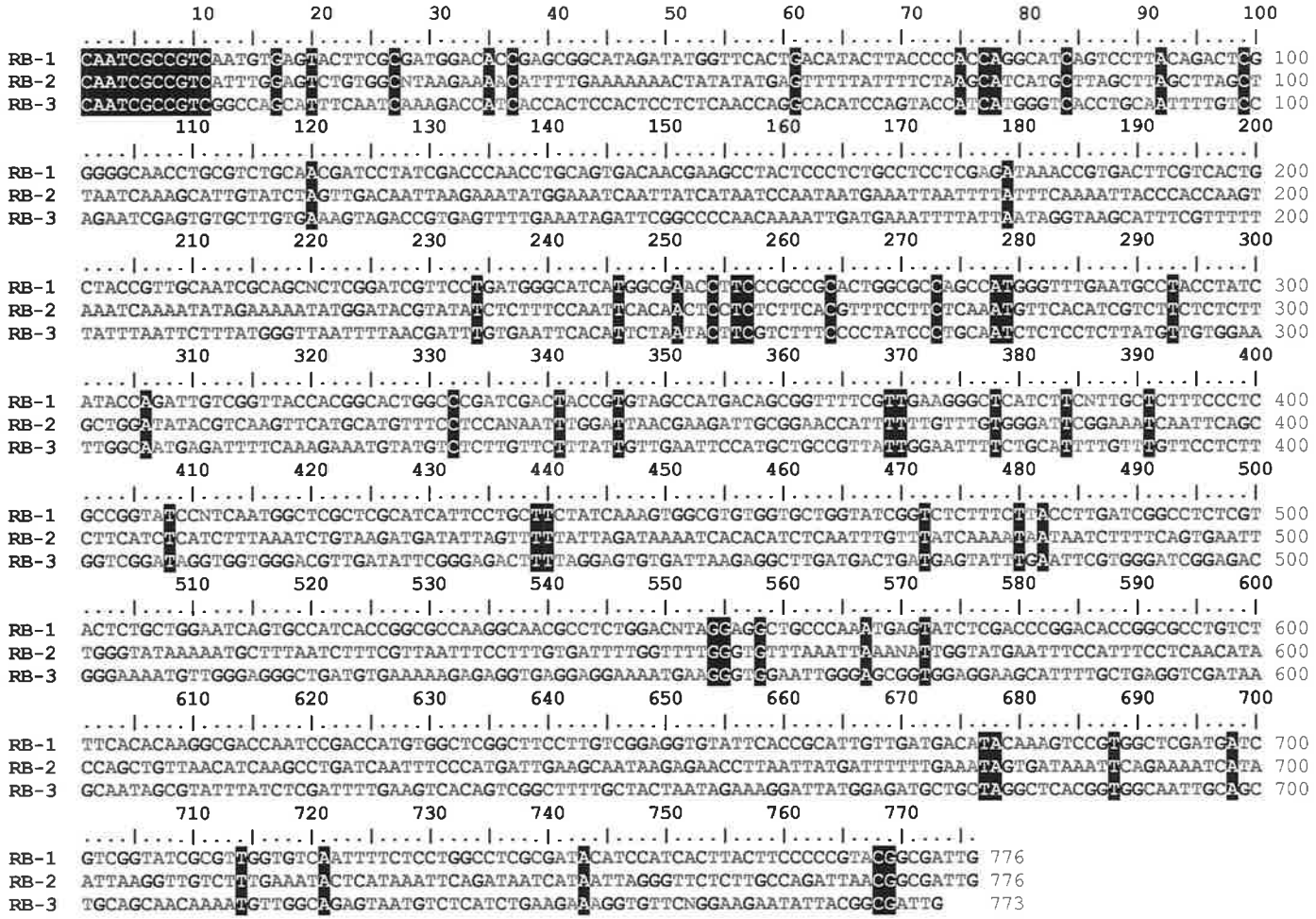
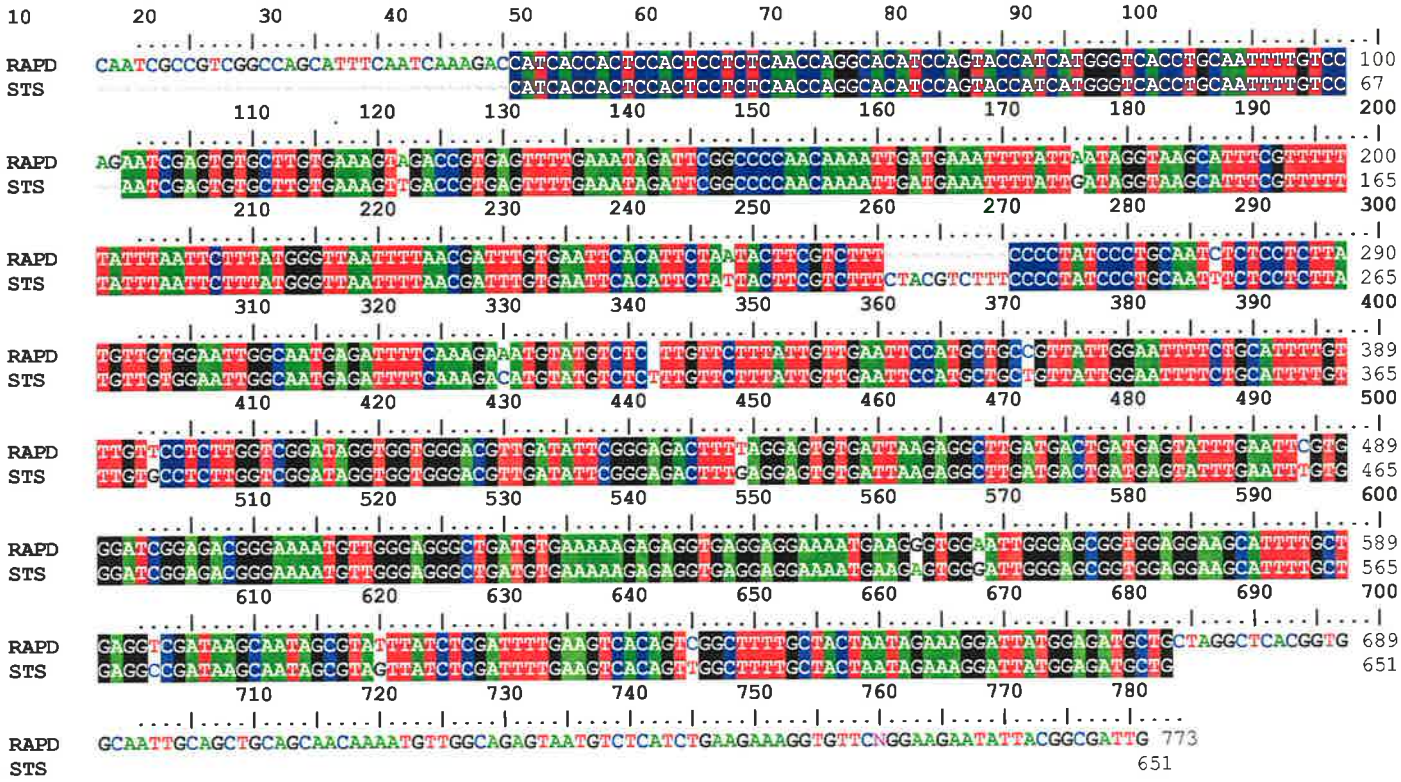


Fig. 6.6 Sequence homology between the STS marker and the original RAPD sequence.



6.5 Discussion

In Chapter 6, a RAPD marker of about 780 bp, linked to olive leaf spot resistance, was identified with the 10-mer primer, OPA11. Progeny obtained from crosses involving five female parents, four of which were resistant, and four male parents showing susceptibility were used in the analyses. In this study the objective was to isolate and clone the 780 bp marker and produce an STS marker that could be used reliably as a selection tool in olive breeding programs.

6.5.1 Isolation and purification of the RAPD marker

A number of protocols was tested for their ability to isolate reamplifiable DNA fragments from agarose and polyacrylamide gels. Most of them failed, either because no PCR products were formed, or multiple PCR products were produced, or the reamplification was unreliable. One protocol, taken from Sambrook *et al.* (1989) consistently produced two bands, one of which was the size of the expected product, and this protocol was adopted for the cloning and sequencing reactions used to identify the RAPD marker.

6.5.2 Cloning and sequencing of the RAPD marker, and analysis of the STS

Nine different sequences, varying from 765 to 814 bp, were identified from the reamplified PCR products originating from the marker band produced by OPA11. Some of these were apparently produced as artifact during the reamplification process, but it is assumed that five sequences from, 773 to 779 bp, were comigrating as a single RAPD band. This finding confirms those of Brady *et al.* (1996) and Hausner *et al.* (1999) that a single RAPD band may consist of several similar-sized DNA fragments each with a different sequence.

Based on the sequencing results, 10 pairs of sequence specific primers were synthesised for the seven different sequences (Group 1 to Group 7). Primers designed by either the PRIMER computer program or by visual observation (adding 10-14 bases to the 10-mer sequence) were not successful in producing a consistent polymorphism between resistant and susceptible samples tested. On other hand, of the four primer pairs designed using the NetPrimer computer program, one pair produced a polymorphism which was found to be useful as an STS marker. This is attributed to the ability of this program to help the user to

avoid potential interfering interactions between or within primers for factors such as GC content, presence of dimers, cross-dimers, and hairpins.

The polymorphic band produced by the most successful primer pair, G7F and G7R, consistently appeared longer than the expected size when the PCR products were separated on agarose. Subsequently, the STS marker was cloned and sequenced, and found to be 10 bp longer than that of the original RAPD fragment. The reason for this extra sequence is not known, and the 10 bp difference does not adequately account for the apparent size of the product being larger than expected when separated on agarose. A similar disparity was reported by Barret *et al.* (1998), and they suggested that the inconsistency between the size of a DNA fragment on the gel, and the size of the fragment after sequencing, is probably due to the imprecision of the measurement of the ladder used. In the present study, the salt concentration of both DNA fragment and ladder were matched, but the disparity persisted.

In addition to the marker for olive leaf spot resistance, other products were produced during PCR with the primer pair, G7F and G7R. Amplification of multiple PCR products from sequence specific primers was also found in other studies (Olsen and Eckstein, 1989; Brady *et al.*, 1996; Lu *et al.*, 2000; Cao *et al.*, 2001). This phenomenon could be due to incomplete extension by the polymerase (Love *et al.*, 1990) or slippage events, which occur during the PCR replication process (Tautz, 1989). In addition, Lu *et al.*, (2000) suggested that monomorphic products may serve as an internal control to the PCR reaction, by distinguishing between the absence of PCR amplification due to genetic factors and those due to technical problems.

6.5.2.1 Testing of the STS marker produced by the primer pair G7F and G7R

These primers were used to test for the presence of the marker for resistance to olive leaf spot disease in 44 olives from the population segregating for olive leaf spot, and 13 commercial olives showing various level of resistance to the disease.

The STS marker occurred in 65% of the resistant segregating progeny (field evaluated for 8 years), and 100% of the resistant parents (field evaluated for 20 years). When the presence of the STS marker was applied to all parents and progeny designated as resistant, the marker was present in 71.4% cases. On the other hand, 87% of the parents and progeny showing susceptibility to the disease did not produce the STS marker.

The primers were further used to test the DNA from 13 olive cultivars that showed various responses to olive leaf spot. Cultivars such as Koroneiki and Leccino that show resistance to olive leaf spot were able to be distinguished from Barouni and Mission, which are reported to be susceptible (Anon, 1996).

6.5.2.2 Comparison of results obtained with the 10-mer primer, OPA11 and the STS primer pair, G7F and G7R

When the STS primers were compared with the 10-mer primer, OPA11 (Chapter 5) the following results were found.

Both OPA11 and the STS primers produced a marker with the four parents that were designated as resistant under field conditions, but not with the parents that were designated as either susceptible or semi-resistant. Two field resistant progeny (BD2-281 and BD2-284), and two field susceptible progeny (BD2-269 and BD3-160), which produced the marker band with OPA11, did not produce it with the STS marker. On the other hand, the STS primers produced a marker for the resistant progeny BD3-168, but no marker was produced by OPA11.

Overall, 88.6% of the results obtained using the STS primers matched the 10-mer primer. Five progeny designated as field resistant, BD3-137, BD2-268, BD3-108, BD3-88, and BD2-285, produced no marker for resistance with either OPA11 or the STS primers, and so these could be considered as susceptible. As already observed for progeny BD2-268 (originally designated as field resistant but recently reviewed as susceptible, Chapter 5), these progeny may be scored as resistant on the basis of relatively short-term field observation, but may actually be susceptible. Three progeny (BD2-275, BD3-111, and BD3-164) that were designated as field susceptible produced the marker band with both OPA11 and the STS primers. These progeny showed infection only after many years from planting (S. Lavee, pers. comm.). Possible reasons for this result could include the involvement of a number of genes (not identified yet) that modify the expression of resistance under field conditions, experimental errors such as mistakes in labelling, and the existence of more than one genotype of the causal organism.

No segregation was detected in the progeny derived from the cross between MAS-5 × Manzanillo. Although both parents are designated as semi-resistant, they did not produce the marker for resistance with either the 10-mer primer or the STS primers.

6.6 Summary

Screening progeny for resistance to olive leaf spot, based on field observation alone, takes many years, whereas using a genetic marker linked to this trait, resistance can easily be identified, making it possible to select resistant progeny at an early stage.

In this study an STS marker linked to resistance to olive leaf spot was developed using sequence specific primers designed from the sequence of a RAPD marker. This marker was present in 71.4% of individual trees classified as resistant on the basis of field observation, and absent in 87% of individuals showing susceptibility. The marker will help to speed up the selection of progeny resistant to olive leaf spot in breeding programs.

Chapter Seven

General discussion

During the early colonisation of Australia, a considerable number of new plants and animals were introduced. Unfortunately, many of these introductions adapted so well that they became pests and invasive weeds (Williams and West, 2000). One of these successful introductions is the olive (Robertson, 1994; Anon, 1999). In fact, the spread of olives in southern Australia has been so successful that some local councils have proclaimed it as a weed, and instigated eradication programmes (Sedgley and Wirthensohn, 1999).

The cultivation of olives and trade in olive oil is thought to have originated in Syria around 3000 B.C. and then spread through North Africa and the Mediterranean. The olive finally reached Australia in early 1800s, and eventually South Australia in 1836. The policy makers in the mid to late 1800s were aware of the adaptability of the olive in Australia and offered the new colonists many incentives to set up olive groves. Many of the world's best olive varieties were introduced, and collections of these were established, and the early history of olive growing in SA seemed to be very encouraging (Perkins, 1917).

However, the industry did not thrive for a variety of reasons, and consequently many of the groves were either abandoned completely, or replaced with other fruit trees, and any olives that were left were often neglected. Government subsidies and support gradually dwindled, and finally disappeared in 1950. This was about the time when large numbers of migrants, whose diet was based around olive oil, came from Europe to Australia. Consequently, the demand for olive products began to escalate, and imports now amount to between $100 m and $120 m each year (Anon, 1998), mainly from countries bordering the Mediterranean. The money spent on importing olive products has naturally attracted many investors, and there is now an expansion in olive growing Australia-wide.

Meanwhile, the olive trees that had been abandoned and neglected for 100 to 150 years became the nucleus for a large natural breeding program. The feral progeny from crosses between the best varieties collected from around the world have been naturally producing the world's largest gene pool of olives (Sedgley and Wirthenshon, 1999).

Olive improvement and production in other countries is also expanding (Anon, 2000). To be competitive, the Australian industry will need to emphasise quality by growing trees with known identity, and by developing cultivars that are both uniquely Australian and well-adapted to local conditions. This will be achieved by a combination of cultivar identification (Anon, 1998), selection of high quality progeny from the feral population (Sedgley and Wirthenshon, 1999), and the use of molecular markers for plant improvement.

This research program was aimed at clarifying the identity of some important commercial cultivars, evaluating the genetic diversity of feral olives, and developing a molecular marker linked to leaf spot disease in olives.

DNA fingerprinting analysis, using molecular techniques, such as AFLP, microsatellites, and RAPD, has many applications including the identification of cultivars and molecular markers useful for selection strategies (Brady *et al.*, 1996). The RAPD technique is relatively easy to employ, requires only very small amounts of DNA, and markers can be visualised without the need for labelled radioisotopes. However, reproducibility of results can be a limitation of RAPDs, unless reaction conditions are stringently standardised.

Olive cultivars are vegetatively propagated, and accessions within a cultivar could be expected to have identical genotypes. However, the results in Chapter 3 showed that while some cultivars, such as Manzanillo, exhibited little genetic variation, others such as Verdale showed a high level of genetic variation. Small genetic variation between accessions of the same cultivar has also been found in other studies, including olives (Wiesman *et al.*, 1998), oats (Singh *et al.*, 1973), and apple (Howard, 1977), suggesting that they are probably selections from the same variety. On the other hand, the level of genetic variation found between the Verdale accessions in this study (ranging from 77% to 100%) was comparable with the genetic variation reported to occur between different cultivars. For example, Belaj *et al.* (2001) found genetic similarities ranging from 71% to 96% among the 51 olive cultivars studied, and Fabbri *et al.* (1995) found genetic similarities ranging from 42% to 95% for 17 olive cultivars. Although a minimum genetic difference to determine the level of a cultivar has not yet been adopted for any horticultural crop, it appears likely that some of the accessions within the Verdale group are different cultivars and were either misnamed, or errors were made during propagation. The misnaming of cultivars was clearly demonstrated for the accession of Calamata from Davis in California. DNA testing of this

accession revealed that it had the same genotype as Manzanillo. Some olive seedlings in Australia were sold in the past as true seedlings of Mission, but some growers are suspicious about the identity of this cultivar by its morphological and fruit characters (M. Burr pers. comm.). This study confirmed that the accession of Mission obtained from SA was apparently misnamed at some time in the past, as it also had the same genotype as Manzanillo.

The results shown in Chapter 3 have helped to clarify the identities of some important commercial cultivars grown in Australia. The data have also provided the foundation for the database of DNA fingerprints that has been developed within the Department of Horticulture, Viticulture and Oenology at Adelaide University to clarify the identities of many other olive cultivars that are grown in Australia. However, the present study evaluated only a subset of many cultivars introduced in Australia and future work in the area will involve expanding the database by adding genotypes from other collections around the world to verify all of the important commercial cultivars grown in Australia.

An olive improvement program has been established recently (Sedgley and Wirthensohn, 1999), focusing on the selection of feral olives with superior qualities. The broader the genetic diversity, the more likely it is that significant gains can be made using selection criteria for crop improvement. The research that forms the subject of Chapter 4 was designed to quantify the extent of genetic variability, and to evaluate the rate of spread of feral olives in an isolated olive population in Kangaroo Island. This population (45 trees in total) consisted of three putative groups: several trees, assumed to be part of an original grove, that were adjacent to an abandoned farmhouse, a row of trees that appeared to be planted along a fence line, and numerous ferals on the surrounding areas.

The RAPD data revealed a high level of genetic diversity, ranging from 67% to 99%, between the 45 olive genotypes. No previous genetic diversity study has been conducted using feral olives in Australia, but the results found between the feral olives in this study were comparable with the level found between olive cultivars (Angiolillo *et al.*, 1999; Belaj *et al.*, 2001). The variation in genotypes parallels the findings of Sedgley and Wirthensohn (1999) that oil yield varied from 1.6% w/w to 37.3% w/w, and oleic and linolenic acid levels varied from 40.9% to 87.7%, and 0.2% to 2.1%, respectively. In addition, the high level of diversity supports the conclusion that this is a feature of plants that display

outbreeding (Huff *et al.*, 1993; Maguire and Sedgley, 1997). The recruitment of individuals from seeds for many generations would be the most probable reason for the high genetic diversity exhibited in this study. In any breeding program, the appearance of individuals that are superior to the parents depends to a large extent on the size of the breeding population. The number of ferals growing throughout southern Australia is in the tens of thousands of trees, and future research is aimed at identifying a subset of the most promising of these to develop olive cultivars that are of high quality and uniquely adapted to the local environment.

Because the olive is predominantly outcrossing, and the fruit is reported to be dispersed by animals and birds (Zohary, 1994; Spennemann and Allen, 2000), it was expected that gene flow would be random. However, in the isolated population under study, three significantly different molecular clusters were identified, indicating that dispersal of pollen and seed is not random. Each cluster included some putative parent trees and some ferals, indicating that the ferals within each cluster had originated from fruits that were shed close to the parent trees. Two of the molecular clusters were also identified in feral trees located some distance from the main clusters. This was interpreted as indicating that seeds had been carried by birds at some stage, and that the trees that developed then shed seeds within a relatively small area, so that the molecular clusters were preserved.

Maguire and Sedgley (1997) found no significant genetic differences between outcrossing populations of *Banksia cuneata* over an area greater than 14,000 km^2, whereas Jain and Bradshaw (1966) found significant genetic differences between outcrossing populations of the self-incompatible perennial, *Agrostis tenuis,* located within a distance of 100 m of each other. Wiesman *et al.* (1998) found significant genetic variability within the local olive germplasm in Israel. Therefore, the significance of genetic variation detected within a population may correspond with the putative history of introduction of cultivars with diverse genetic bases.

Olive seeds are known to be difficult to germinate (Sotomayor Leon and Caballero, 1990), and this feature, together with the finding for the population on Kangaroo Island that seed spread is relatively slow, questions the decision made by some local Councils to ban olive growing because of the danger of spread by ferals. Future research is needed to improve the knowledge of gene flow in olives by examining groves located close to populated areas,

where the soil is more disturbed and possibly more conducive to the germination of olive seeds.

Breeding olive genotypes with a trait of interest such as disease resistance is one of the major challenges to olive improvement due to the long period of juvenility (Fontanazza and Baldoni, 1990), and the interaction of phenotype and genotype (Rafalski and Tingey, 1993). Breeding programs can therefore be significantly improved by adopting molecular markers for marker assisted selection. Olives are relatively free of pests and diseases, but one pest that is well known in olive groves, and reported to cause considerable crop losses in the northern hemisphere, is olive spot, or peacock spot, caused by the fungal pathogen, *Spilocea oleaginea* (Shabi *et al.*, 1994). The extent of crop losses due to this disease in Australia has not been investigated but its presence for more than 50 years has been reported (Hughes, 1953), and it has the potential to be an important disease of olives in Australia. This study focussed on the identification of molecular markers linked to leaf spot in olives using two approaches, firstly using RAPD and BSA analysis (Chapter 5), and secondly developing a sequence-tagged site for leaf spot resistance (Chapter 6).

Individuals that showed either resistance or susceptibility to leaf spot disease in a breeding program in Israel were selected, and two RAPD markers, one linked to leaf spot resistance and the other to susceptibility were identified (Chapters 5). A marker for resistance was detected in 76.2% of the trees that showed field resistance, and a marker for susceptibility was detected in 60.9% of trees that showed susceptibility in the field. Thus, the marker linked to resistance has considerable potential to be used in marker assisted selection for breeding and improvement of olives.

In order to obtain a specific marker that could be easily and widely used in breeding programs, the RAPD marker for resistance was converted to an STS (Chapter 6). This was achieved by cloning, and sequencing the RAPD fragment, and designing specific primers from the sequence. The separation of the RAPD fragment was carried out on polyacrylamide, and after the band was purified from the gel, it was expected that a single fragment would be cloned. However, after the cloned fragments were digested from the multiple-cloning site on the vector, multiple fragments were detected. This character of RAPDs has been reported in other studies (Hausner *et al.*, 1999). Out of the multiple fragments amplified one was linked to leaf spot resistance, and the pair of primers designed

from this sequence amplified all parents, and 65% of the progeny that were designated as resistant to leaf spot on the basis of field data. Nine recombinant plants (six resistance plants lacking the resistance marker, and three susceptible plants exhibiting the resistance marker) were detected among the 34 progeny tested. That means the probability of misclassification of the segregating progeny to the wrong genotype is between 17-35%, and this is relatively higher than the findings of Plieske and Struss (2001). In developing a STS marker linked to *Phoma* resistance genes in oilseed rape breeding lines, Plieske and Struss (2001) found 5-10% misclassification and suggested environmental conditions are the reasons.

Since the parents used in this study had been observed over a period of 20 years, compared to only eight years for the progeny, the inconsistencies that were observed between the molecular marker and the data from field observations were ascribed to insufficient testing. In fact, one of the progeny that was designated as resistant at the start of the research program was subsequently reclassified as susceptible. This may indicates that the expression of leaf spot resistance is highly affected by environmental conditions, and confirms the significance of molecular markers for an early screening of resistance genotype.

Both the RAPD and the STS markers linked to leaf spot resistance will be used as selection tools in the current olive improvement programs in Australia. They will provide a timely and less costly way of identifying individuals possessing the trait for leaf spot resistance without the necessity of the time-consuming task of field testing. Future study in the area should be aimed at understanding the host-pathogen interaction, especially possible differences in virulence strains, and the importance of semi-resistance. Other markers that should be the focus for future research include oil quality, oleic acid content, and linoleic acid content.

In conclusion, this research program has contributed information to three areas of significant importance for olive production and improvement in Australia, some of which will be of benefit to other olive producing countries.

References cited

Ahn S. and S.D. Tanksley. (1993). Comparative linkage maps of the rice and maize genomes. *Proceedings of the National Academy of Science of the United States of America*, 90:7980-7984.

Alcántara J.M, P.J Rey, F. Valera, A.M. Sánchez-Lafuenete, and J.E. Gutiérrez .(1997). Habitat alteration and plant intra-specific competition for seed dispersers. An example with *Olea europaea* var. *sylvestris*. *OIKOS*, 79:291-300.

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

Anon (1899). Olives and Manufacture of olive oil. *Journal of Agriculture and Industry*, 2:972-974.

Anon (1964). Agricultural Research centres. *Annual report of the Department of Agriculture* 1963-64, pp23.

Anon (1983). Olive, pp204. The Australian Encyclopaedia, 4th edn. The Grolier Society of Australia pty Ltd, Sydney, Australia.

Anon (1992). The Olive Tree The Oil The Olive, pp 23-24, 30-32. International Olive Oil Council, Principle de Vergara, MADRID.

Anon (1996) Olive Germplasm: cultivars and world-wide collections. FAO. Plant production and Protection Division (http://www.fao.org/ag/agp/agps/data.htm).

Anon (1998). Plant for the Australian Olive Industry. Rural Industries Research and Development Corporation, ISSN 1440-6845, ACT.

Anon (1999). Risk assessment and management of olives. Animal and Plant Control Commission, Adelaide, South Australia.

Anon (2000). The world market for olive oils: current situation report. *Olivæ*, 84:29-33.

Apostol B.L, W.C Black, B.R. Miller, P. Reiter, and B.J. Beaty. (1993). Estimation of the number of full sibling families at an oviposition site using RAPD-PCR markers: applications to the mosquito *Aedes aegypti*. *Theoretical and Applied Genetics*, 86:991-1000.

Apuya N.R., B.L Frazier, P. Keim, E.J. Roth, and K.G. Lark. (1988). Restriction fragment polymorphisms as genetic markers in soybean. *Glycine max* (L.) Morr. *Theoretical and Applied Genetics*, 75:889-901.

Archer J. (1997). Information on Australian olive cultivars. Olives Australia: http://www.oliveaustralia.aust.com/Olive_Variety.html.

Ash J. (1983). Growth rings in *Agathis robusta* and *Araucaria* cunninghamii from tropical Australia. *Australian Journal of Botany*, 31:269-275.

Bai G.H., G. Shaner, and H. Ohm. (2000). Inheritance of resistance to *Fusarium graminearum* in wheat. *Theoretical and Applied Genetics*, 100:1-8.

Barranco D. (1995). The choice of varieties in Spain. *Olivæ*, 59:54-58.

Barret P., R. Delourme, N. Foisset, and M. Renard. (1998). Development of a SCAR (sequence characterised amplified region) marker for molecular tagging of the dwarf BREIZH (*Bzh*) gene in *Brassica napus* L. *Theoretical and Applied Genetics*, 97:828-833.

Bartolini G., C, Messeri, and G. Prevost. (1994). Olive tree germplasm: Descriptor list of cultivated varieties in the world. *Acta Horticulturae*, 356:116-118.

Barua U.M, K.J. Chalmers, C.A. Hackett, W.T.B. Thomas, W. Powell, and R. Waugh. (1993). Identification of RAPD markers linked to a *Rhynchosporium secalis* resistance locus in barley using near-isogenic lines and bulked segregant analysis. *Heredity*, 71:177-184.

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

Becker J., P. Vos, M. Kuiper, F. Salamini, and M. Heun. (1995). Combined mapping of AFLP and RFLP markers in barley. *Molecular and General Genetics*, 249:65-73.

Beckmann J.S., and M. Soller. (1990). Toward a unified approach to the genetic mapping of eukaryotes based on sequence tagged microsatellite sites. *Bio/Technology*, 8: 930-932.

Belaj A, I. Trujillo, R. de la Rosa, L. Rallo, and M.J. Giménez. (2001). Polymorphism and discrimination capacity of randomly amplified polymorphic markers in an olive Germplasm Bank. *Journal of the American Society for Horticultural Science*, 126:64-71.

Bertin P., D. Grégoire, S. Massart, and D. de Froidmont. (2001). Genetic diversity among European cultivated spelt revealed by microsatellites. *Theoretical and Applied Genetics*, 102:148-156.

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

Blázquez J.M. (1996). The origin and expansion of olive cultivation. pp19-20. World Olive Encyclopaedia, International Olive Oil Council, Spain.

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

Boora K.S., R. Frederiksen, and C. Magill. (1998). DNA-based markers for a recessive gene conferring anthracnose resistance in sorghum. *Crop Science*, 38:1708-1709.

Booth N. and G. Davies. (1995). An introduction to olive growing in Southern Australia. Primary Industries South Australia, Adelaide, Australia, pp4-6.

Bradley K.F., M.A. Rieger, and G.G. Collins. (1996). Classification of Australian garlic cultivars by DNA fingerprinting. *Australian Journal of Experimental Agriculture*, 36:613-618.

Brady J.L, N.S. Scott, and M.R. Thomas. (1996). DNA typing of hops (*Humulus lupulus*) through application of RAPD and microsatellite marker sequences converted to sequence tagged sites (STS). *Euphytica*, 91:277-284.

Burczyk J., W.T. Adams, and J.Y. Shimizu. (1996). Mating patterns and pollen dispersal in a natural knobcone pine (*Pinus attenuata* Lemmon.) stand. *Heredity*, 77:251-260.

Burr M. (1997). Australian olives. A guide for growers and producers of virgin oils. 2nd edn. OLTECH Pty Ltd, South Australia, Australia, pp70-75, 128-132.

Caballero J.M., C. Del Río, and J. Eguren. (1990). Further agronomic information about a world collection of olive cultivars. *Acta Horticulturae*, 286:45-48.

Caetano-Anollés G., B.J. Bassam, and P.M. Gresshoff. (1992). Primer-template interactions during DNA amplification fingerprinting with single arbitrary oligonucleotides. *Molecular and General Genetics*, 235:157-165.

Cantini C., A. Cimato, and G. Sani. (1999). Morphological evaluation of olive germplasm present in Tuscany region. *Euphytica*, 109:173-181.

Cao W., G.R. Hughes, H. Ma, and Z. Dong. (2001). Identification of molecular markers for resistance to *Septoria nodorum blotch* in durum wheat. *Theoretical and Applied Genetics*, 102:551-554.

Cattan-Toupance I., Y. Michalakis, and C. Neema. (1998). Genetic structure of wild bean populations in their South-Andean centre of origin. *Theoretical and Applied Genetics*, 96:844-851.

Chevalier A. (1948). In Fontanazza G. and L. Baldoni. (1990). Proposed programme for genetic improvement of the olive. *Olivæ*, 34:32-40.

Ciferri R. (1950). In Fontanazza G. and L. Baldoni. (1990). Proposed programme for genetic improvement of the olive. *Olivæ*, 34:32-40.

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

Connell J.H. (1994). History and scope of the olive industry, pp1-9. *In*: Ferguson, L., G.S. Sibbett, and G.C. Martin. (1994). Olive production manual. University of California, Division of Agriculture and Natural Resources, CA. Publication 3353. USA.

Cregan P.B. (1992). Simple sequence repeat DNA length polymorphisms. *Probe*, 2:18-22.

Crouse J. and D. Amorese. (1997). Ethanol precipitation: ammonium acetate as an alternative to sodium acetate. *Focus*, 19:13-16.

Dal Pero Bertini G.V. (1960). Olive Growing and Processing. Institute of Rural Industries, Florence, Italy, pp35-52, 105-116.

Darmency H. (1997). Gene flow between crops and weeds: Risk for new herbicide resistant weeds? p239-248. *In*: De Prado R., J. Jorrín, and L. García-Torres. (1997). Weed and crop resistance to herbicides. Kluwer Academic Publishers, Dordrecht, The Netherlands.

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

Duval M.F., J.L. Noyer, X. Perrier, G. Coppens d'Eeckenbrugge, and P. Hamon. (2001). Molecular diversity in pineapple assessed by RFLP markers. *Theoretical and Applied Genetics*, 102:83-90.

Elisiário P.J, E.M. Justo, and J.M. Leitão. (1999). Identification of mandarin hybrids by isozyme and RAPD analysis. *Scientia Horticulturae*, 81:287-299.

Ellstrand N.C and D.L. Marshall. (1986). Patterns of multiple paternity in populations of *Raphanus sativus*. *Evolution*, 40:837-842.

Enright N.J. and D. Goldblum. (1998). Stand structure of the emergent conifer *Agathis ovata* in forest and maquis, Province Sud, New Caledonia. *Journal of Biogeography*, 25:641-648.

Eshete G. and G. Ståhl. (1999). Tree rings as indicators of growth periodicity of acacias in the Rift Valley of Ethiopia. *Forest Ecology and Management*, 116:107-117.

Excoffier L., P.E. Smouse, and J.M. Quattro. (1992). Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. *Genetics*, 131:479-491.

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

Field B. (1997). The use of microsatellite to detect genetic variation among olive (*Olea europea*) cultivars. Honours Thesis, University of Adelaide.

Fontanazza G. (1988). Growing for better quality oil. *Olivæ*, 24:31-39

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

Fontanazza G., M. Patumi, M. Solinas, and A. Serraiocco. (1994). Influence of cultivars on the composition and quality of olive oil. *Acta Horticulturae*, 356:358-361.

Fowler R. (1930). Annual Reports 1924-1930. Department of Agriculture South Australia, pp27-29.

Fowler R. (1939). The olive in South Australia. *Journal of The Department of Agriculture of South Australia*, 62: 812-814.

Garkava L.P., K. Rumpunen, and I.V. Bartish. (2000). Genetic relationships in *Chaenomeles* (*Rosaceae*) revealed by isozyme analysis. *Scientia Horticulturae*, 85:21-35.

Gemas V.J., M.J. Rijo-Johansen, R. Tenreiro, and P. Fevereiro. (2000). Inter-and intra-varietal analysis of three *Olea europaea* L. cultivars using the RAPD technique. *Journal of Horticultural Science and Biotechnology*, 75:312-319.

Gillies A.C.M., J.P. Cornelius, A.C. Newton, C. Navarro, M. Hernández, and J. Wilson. (1997). Genetic variation in Costa Rican populations of the tropical timber species *Cedrela odorata* L., assessed using RAPDs. *Molecular Ecology*, 6:1133-1145.

Godden G.D. (1983). Growing olives. Agnote, ISSN 0155-0217, Department of Agriculture, Victoria.

Goodfellow P.N. (1993). Microsatellites and the new genetic maps. *Current Biology*, 3:149-151.

Greer R. and C. Blau. (1995). Olive Oil. The Good Heart Protector. Souvenir Press Ltd. London, pp97-99.

Griggs W.H., H.T. Hartmann, M.V. Bradley, B.T. Iwakiri, and J.E. Whisler. (1975). Olive pollination in California. *California Agricultural Experiment Station Bulletin*, 869:3-50.

Haley S.D., P.N. Miklas, J.R. Stavely, J. Byrum, and J.D. Kelly. (1993). Identification of RAPD markers linked to a major rust resistance gene block in common bean. *Theoretical and Applied Genetics*, 86:505-512.

Hartmann H.T. (1957). Olive Production in California. The College of Agriculture, University of California, pp50-59.

Hartmann H.T. (1962). Olive growing in Australia. *Economic Botany*, 16: 31-43.

Hartmann H.T. (1967). 'Swan Hill'- new ornamental fruitless olive for California. *Californian Agriculture*, 21:4-5.

Hausner G., K.Y. Rashid, E.O. Kenaschuk, and J.D. Procunier. (1999). The identification of a cleaved amplified polymorphic sequence (CAPS) marker for the flax rust resistance gene M^3. *Canadian Journal of Plant Pathology*, 21:187-192.

Haymes K.M, W.E. Van de Weg, P. Arens, J.L. Maas, B. Vosman, and A.P.M. Den Nijs. (2000). Development of SCAR markers linked to a *Phytophthora fragariae* resistance gene and their assessment in European and North American strawberry genotypes. *Journal of American Society for Horticultural Science*, 125:330-339.

Hilali S. and A. El Antari. (1994). Varietal polymorphism in fruit-bearing olive cultivars in Marrakesh: A Study. *Olivæ*, 50:45-47.

Hill M., H. Witsenboer, M. Zabeau, P. Vos, R. Kesseli, and R. Michelmore. (1996). PCR-based fingerprinting using AFLPs as a tool for studying genetic relationships in *Lactuca* spp. *Theoretical and Applied Genetics*, 93:1202-1210.

Hobman F. (1993). Olive Information Package. Department of Primary Industries, South Australia - Loxton Research Centre.

Hogbin P.M., D.J. Ayre, and R.J. Whelan. (1998). Genetic variation and reproductive success of road verge populations of the rare shrub *Grevillea barklyana* (*Proteaceae*). *Heredity*, 80:180-186.

Howard B.H. (1977). *In*: Loukas M. and C.A. Pontikis. (1981). Is the phenotypic variation observed within cultivars of *Olea europaea* L. reflected in isozyme banding patters? *Journal of Horticultural Science*, 56:377-379.

Hu J. and C.F. Quiros. (1991). Identification of broccoli and cauliflower cultivars with RAPD markers. *Plant Cell Reports*, 10:505-11.

Hu X.Y., H.W. Ohm, and I. Dweikat. (1997). Identification of RAPD markers linked to the gene *PM1* for resistance to powdery mildew in wheat. *Theoretical and Applied Genetics*, 94:832-840.

Huff D.R., R. Peakall, and P.E. Smouse. (1993) RAPD variation within and among natural populations of outcrossing buffalograss [*Buchloë dactyloides* (Nutt.) Engelm.]. *Theoretical and Applied Genetics*, 86:927-934.

Hughes S.J. (1953). Some foliicolous hyphomycetes. *Canadian Journal of Botany*, 31:560-576.

Jackson K., and A. Duff. (1994). Improvements made the Queensland way. 'Growing in the 1990's' Australian Garlic Industry Seminar, Hay, NSW.

Jain S.K. and A.D. Bradshaw. (1966). Evolutionary divergence among adjacent plant populations: The evidence and its theoretical analysis. *Heredity*, 21:407-441.

Johnson J.L. (1994) Similarity analysis of DNAs. *In*: Gerhardt P, Murray RGE, Wood WA, Krieg NR, (1994) Methods for general and molecular bacteriology. American Society for Microbiology, Washington, D.C.

Jones C.J, K.J. Edwards, S. Castaglione, M.O. Winfield, F. Sala, C. van de Wiel, G. Bredemeijer, B. Vosman, M. Matthes, A. Daly, R. Brettschneider, P. Bettini, M. Buiatti, E. Maestri, A. Malcevschi, N. Marmiroli, R. Aert, G. Volckaert, J. Rueda, R. Linacero, A. Vazquez, and A. Karp (1997). Reproducibility testing of RAPD, AFLP and SSR markers in plants by a network of European laboratories. *Molecular Breeding*, 3:381-390.

Kailis S. (2000). Table olives - a national and international approach to quality, pp25-27. The Olive Press, Australian Olive Association, ISSN 1442-357X, Spring 2000.

Kaufman S.R., P.E. Smouse, and E.R. Alvarez-Buylla. (1998). Pollen-mediated gene flow and differential male reproductive success in a tropical pioneer tree, *Cecropia obtusifolia* Bertol. (Moraceae): a paternity analysis. *Heredity*, 81:164-173.

Keim P., B.W. Diers, T.C. Olson, and R.C. Shoemaker. (1990). RFLP mapping in soybean: Association between marker loci and variation in quantitative traits. *Genetics*, 126:735-742.

Kiritsakis A.K. (1998). Olive oil: From the tree to the table, 2nd edition, Food and Nutrition Press, Inc. Trumbull, Connecticut, USA. pp19-24.

Lagercrantz U., H. Ellegren, and L. Andersson. (1993). The abundance of various polymorphic microsatellite motifs differs between plants and vertebrates. *Nucleic Acids Research,* 21:1111-1115.

Lamboy W.F. (1994). Computing Genetic Similarity Coefficients from RAPD Data: The Effects of PCR Artifacts. PCR Methods and Application Cold Spring Harbor Laboratory Press ISSN 1054-9803, 31-37.

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

Lavee S. (1996). Biology and physiology of the olive, pp61-110. World Olive Encyclopaedia, International Olive Oil Council, Spain.

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

Lavee S., H. Harshemesh, A. Haskal, V. Meni, M. Wodner, A. Ogrodovich, B. Avidan, Z. Wiesman, N. Avidan, and A. Trapero Casas. (1999). 'Maalot' a new orchard-resistant cultivar to peacock eye leaf spot (*Spilocaea oleagina* Cast.). *Olivæ*, 78:51-59.

Lin J.J., J. Kuo, J. Ma, J.A. Saunders, H.S. Beard, M.H. MacDonald, W. Kenworthy, G.N. Ude, and B.F. Matthews. (1996). Identification of Molecular Markers in Soybean Comparing RFLP, RAPD and AFLP DNA Mapping Techniques. *Plant Molecular Biology Reporter*, 14:156-169.

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.

Loukas M. and C.B. Krimbas. (1983). History of olive cultivars based on their genetic distances. *Journal of Horticultural Science*, 58:121-127.

Love J.M., A.M. Knight, M.A. McAleer, and J.A. Todd. (1990). Towards construction of a high resolution map of the mouse genome using PCR-analysed microsatellites. *Nucleic Acids Research*, 18:4123-4130.

Loveless M.D. and J.L. Hamrick. (1984). Ecological determinants of genetic structure in plant populations. *Annual Review of Ecology and Systematics*, 15:65-95.

Lu J., M.R. Knox, M.J. Ambrose, J.K.M. Brown, and T.H.N. Ellis. (1996). Comparative analysis of genetic diversity in pea assessed by RFLP-and PCR-based methods. *Theoretical and Applied Genetics*, 93:1103-1111.

Lu Y.H., J.M. Melero-Vara, J.A. García-Tejada, and P. Blanchard. (2000). Development of SCAR markers linked to the gene Or5 conferring resistance to broomrape (*Orobanche cumana* Wallr.) in sunflower. *Theoretical and Applied Genetics*, 100:625-632.

Macdonald L. (1915). The Olive. *The Journal of The Department of Agriculture of Victoria*, 12:154-160, 221-228, 499-503.

Maguire T.L. and M. Sedgley. (1997). Genetic diversity in *Banksia* and *Dryandra* (Proteaceae) with emphasis on *Banksia cuneata*, a rare and endangered species. *Heredity*, 79:394-401.

Maguire T.L., G.G. Collins, and M. Sedgley. (1994). A modified CTAB DNA extraction procedure for plants belonging to the family *Proteaceae. Plant Molecular Biology Reporter*, 12:106-109.

Maheswaran M., P.K. Subudhi, S. Nandi, J.C Xu, A. Parco, D.C. Yang, and N. Huang. (1997). Polymorphism, distribution, and segregation of AFLP markers in a doubled haploid rice population. *Theoretical and Applied Genetics*, 94:39-45.

Markert C.L. and F. Møller. (1959). Multiple forms of enzymes: tissue, ontogenetic and species specific patterns. *Proceedings of the National Academy of Science of the United States of America,* 45:753-763.

Martin G.C. (1994). Botany of the olive, pp19-21. *In*: Ferguson, L., G.S. Sibbett, and G.C. Martin. (1994). Olive production manual. University of California, Division of Agriculture and Natural Resources, CA. Publication 3353. USA.

Maughan P.J., M.A.S. Maroof, and G.R. Buss. (1995). Microsatellite and amplified length polymorphisms in cultivated and wild soybean. *Genome*, 38:715-723.

Maughan P.J., M.A.S. Maroof, G.R. Buss, and G.M. Huestis. (1996). Amplified fragment length polymorphism (AFLP) in soybean: species diversity, inheritance, and near-isogenic line analysis. *Theoretical and Appied Genetics*, 93:392-401.

Meksem K., D. Leister, J. Peleman, M. Zabeau, F. Salamini, and C. Gebhardt. (1995). A high-resolution map of the vicinity of the R1 locus on chromosome V of potato based on RFLP and AFLP markers. *Molecular and General Genetics*, 249:74-81.

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

Michelmore R.W., I. Paran, and R.V. Kesseli. (1991). Identification of markers linked to disease resistance genes by bulked segregant analysis: A rapid method to detect markers in specific genomic regions by using segregating populations. *Proceedings of the National Academy of Science of the United States of America*, 88:9828-9832.

Michiels F., M. Burmeister, and H. Lehrach. (1987). Derivation of clones to *met* by preparative field inversion gel electrophoresis. *Science*, 236:1305-1308.

Miller M.P. (1998). AMOVA-PREP Computer program for the preparation for AMOVA input files from dominant marker raw, Department of Biological Sciences Northern Arizona University.

Morell M.K., R. Peakall, R. Appels, L.R. Preston, and H.L Lloyd. (1995). DNA profiling techniques for plant variety identification. *Australian Journal of Experimental Agriculture*, 35:807-819.

Morettini A. and A. Pulselli. (1953). *In*: Lavee S. (1996). Biology and physiology of the olive, pp 61-110. World Olive Encyclopaedia, International Olive Oil Council, Spain.

Mulcahy D.L., M. Cresti, S. Sansavini, G.C. Douglas, H.F. Linskens, G.B. Mulcahy, R. Vignani, and M. Pancaldi. (1993). The use of random amplified polymorphic DNAs to fingerprint apple genotypes. *Scientia Horticulturae*, 54:89-96.

Negi M.S., M. Devic, M. Delseny, and M. Lakshmikumaran. (2000). Identification of AFLP fragments linked to seed coat colour in *Brassica juncea* and conversion to a SCAR marker for rapid selection. *Theoretical and Applied Genetics*, 101:146-152.

Nei M. and W.H. Li. (1979). Mathematical model for studying genetic variation in terms of restriction endonucleases. *Proceedings of the National Academy of Science of the United States of America*, 76:5269-5273.

Ogawa J.M. and H. English. (1991). Diseases of temperate zone tree fruits and nut crops. University of California, Division of Agriculture and Natural Resources, Oakland, Calif Publ 3345, pp344-347.

Ohmori S.T., M. Murata, and F. Motoyoshi. (2000). Molecular characterization of the SCAR markers tightly linked to the *Tm-2* locus of the genus *Lycopersicon*. *Theoretical and Applied Genetics*, 101:64-69.

Oliveira C.M., M. Mota, L. Monte-Corvo, L. Goulão, and D.M. Silva. (1999). Molecular typing of *Pyrus* based on RAPD markers. *Scientia Horticulturae*, 79:163-174.

Olsen D.B. and F. Eckstein. (1989). Incomplete primer extension during *in vitro* DNA amplification catalyzed by *Taq* polymerase; exploitation for DNA sequencing. *Nucleic Acids Research*, 17:9613-9620.

Olson M., L. Hood, C. Cantor, and D. Botstein. (1989). A common language for physical mapping of the human genome. *Science*, 245:1434-1435.

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

Paran I. and R.W. Michelmore. (1993). Development of reliable PCR-based markers linked to downy mildew resistance genes in lettuce. *Theoretical and Applied Genetics*, 85:985-993

Perkins A.J. (1917). On the scope in South Australia for the extension of olive groves. Department of Agriculture of South Australia, Bulletin, 111:3-22.

Plieske J. and D. Struss. (2001). STS markers linked to *Phoma* resistance genes of the *Brassica* B-genome revealed sequence homology between *Brassica nigra* and *Brassica napus*. *Theoretical and Applied Genetics*, 102:483-488.

Pontikis C.A., M. Loukas, and G. Kousounis. (1980). The use of biochemical markers to distinguish olive cultivars. *Journal of Horticultural Science*, 55:333-343.

Prezioso V.R. (2000). General notes on primer design in PCR. http://www.eppendorfsi.com/PCR_appl_primer.html.

Quinn G. (1920). Notes on olive growing in South Australia. *Journal of the Department of Agriculture of South Australia*, 23:603-606.

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

Rallo L. (1995). Selection and breeding of olive in Spain. *Olivæ*, 59:46-53.

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

Rasband W. (1997). NIH Image for Macintosh, http://www.scioncorp.com. Scion Corporation, National Institutes of Health, USA.

Rey P.J, J.E. Gutiérrez, J. Alcántara, and F. Valera. (1997). Fruit size in wild olives: implications for avian seed dispersal. *Functional Ecology*, 11:611-618.

Roberts R. (1942). Olive Culture. *The Journal of the Department of Agriculture of Victoria* Australia, 60:295-300.

Robertson M. (1994). Stop bushland weeds, pp101. A guide to successful weeding in South Australia's bushland. The Nature Conservation Society of South Australia Inc.

Rohlf F.J. (1993). NTSYS-pc. Numerical taxonomy and multivariate analysis system. Ver. 1.80 Department of Ecology and Evolution, State University of New York. Exeter Publ. Ltd., Setauket, N.Y.

Rommens J.M., M.C. Iannuzzi, B.S. Kerem, M.L. Drumm, G. Melmer, M. Dean, R. Rozmahel, J.L. Cole, D. Kennedy, N. Hidaka, M. Zsiga, M. Buchwald, J.R. Riordan, L.C. Tsui, and F.S. Collins. (1989). Identification of the Cystic fibrosis gene: Chromosome walking and jumping. *Science*, 245:1059-1065.

Sambrook J., E.F. Fritsch, and T. Maniatis. (1989). Molecular Cloning. A Laboratory Manual 2nd ed. Cold Spring Harbor Laboratory Press.

Schierwater B. and A. Ender. (1993). Different thermostable DNA polymerases may amplify different RAPD products. *Nucleic Acids Research*, 21:4647-4648.

Sedgley M. and M. Wirthensohn. (1999). Better olive cultivars for Australia, pp12-14. The Olive Press, *Journal of the Australian Olive Association*, Spring 1999.

Shabi E., R. Birger, and S. Lavee. (1994). Leaf spot (*Spilocaea oleaginea*) of olive in Israel and its control. *Acta Horticulturae*, 356:390-394.

Shubiao W., M. Wirthensohn, G. Collins, and M. Sedgley. (2000). Olive trees need the right pollinator, pp13-15. The Olive Press, *Journal of the Australian Olive Association*, ISSN 1442-357X, Winter 2000.

Singh R.S., S.K. Jain, and C.O. Qualset. (1973). Protein Electrophoresis as an aid to oat variety identification. *Euphytica*, 22:98-105.

Skroch P. and J. Nienhuis. (1995). Impact of scoring error and reproducibility of RAPD data on RAPD based estimates of genetic distance. *Theoretical and Applied Genetics*, 91:1086-1091.

Sokal R.R. and C.D. Michener. (1958). A statistical method for evaluating systematic relationships. *The University of Kansas Science Bulletin*, 38:1409-1438.

Sokal R.R. and P.H.A. Sneath. (1963). Principles of Numerical Taxonomy. W.H. Freeman and Company, San Francisco, CA.

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

Spennemann D.H.R., and L.R. Allen. (2000). Feral olives (*Olea europaea*) as future woody weeds in Australia: a review. *Australian Journal of Experimental Agriculture*, 40:889-901.

Stahle D.W., P.T. Mushove, M.K. Cleaveland, F. Roig, and G.A. Haynes. (1999). Management implications of annual growth rings in *Pterocarpus angolensis* from Zimbabwe. *Forest Ecology and Management*, 124:217-229.

Steel R.G.D. and J.H. Torrie. (1960). Principles and procedures of STATISTICS with special reference to the biological sciences. McGraw-Hill Book Company Inc. NY.

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

Stiles J.I., C. Lemme, S. Sondur, M.B. Morshidi, and R. Manshardt. (1993). Using randomly amplified polymorphic DNA for evaluating genetic relationships among papaya cultivars. *Theoretical and Applied Genetics*, 85:697-701.

Sun C.Q., X.K. Wang, Z.C. Li, A. Yoshimura, and N. Iwata. (2001). Comparison of the genetic diversity of common wild rice (*Oryza rufipogon* Griff.) and cultivated rice (*O. Sativa* L.) using RFLP markers. *Theoretical and Applied Genetics*, 102:157-162.

Sutter E.G. (1994). Olive cultivars and propagation, pp23-25. *In*: L. Ferguson, G.S. Sibbett, and G. Martin (eds.). Olive production manual. University of California, Division of Agriculture and Natural Resources, CA. Publication 3353. USA.

Sweeney S. (2000). Olive Tree numbers 2000, pp25-26. The Olive Press, *Journal of the Australian Olive Association*, ISSN 1442-357X, Summer 2000.

Tautz D. (1989). Hypervariability of simple sequences as a general source for polymorphic DNA markers. *Nucleic Acids Research*, 17:6463-6471.

Teviotdale B.L. (1994). Diseases of olives, pp107-109. *In*: L. Ferguson, G.S. Sibbett, and G.C. Martin (eds.). Olive production manual. University of California, Division of Agriculture and Natural Resources, CA. Publication 3353. USA.

Thompson J.D., T.J. Gibson, F. Plewniak, F. Jeanmougin, and D.G. Higgins. (1997). The Clustal_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research*, 25:4876-4882.

Tingey S.V., J.A, Rafalski, and J.G.K Williams. (1992). Genetic analysis with RAPD markers.pp3-8. *In* Application of RAPD technology to plant breeding, Plant Breeding Symposia Series, American Society of Horticultural Science.

Trujillo I., L. Rallo, E.A. Carbonell, and M.J. Asins. (1990). Isoenzymatic variability of olive cultivars according to their origin. *Acta Horticulturae*, 286:137-140.

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

Villar M., F. Lefèvre, H.D. Bradshaw Jr., and E. Teissier du Cros. (1996). Molecular genetics of rust resistance in poplars (*Melampsora larici- populina* Kleb/*Populus* sp.) by bulked segregant analysis in a 2 X 2 factorial mating design. *Genetics*, 143:531-536.

Vos P., R. Hogers, M. Bleeker, M. Reijans, T. Van de Lee, M. Hornes, A. Frijters, J. Pot, J. Peleman, M. Kuiper, and M. Zabeau. (1995). AFLP: a new technique for DNA fingerprinting. *Nucleic Acids Research*, 23:4407-4414.

Wang Z., J.L. Weber, G. Zhong, and S.D. Tanksley. (1994). Survey of plant short tandem DNA repeats. *Theoretical and Applied Genetics*, 88:1-6.

Warburton M.L., V.L. Becerra-Velásquez, J.C. Goffreda, and 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.

Warwick S.I. (1991). The influence of intraspecific variation on the biology and control of agricultural weeds. *Brighton Crop protection Conference-Weeds*, 8A-1:997-1006.

Watson J.C., and W.F. Thompson. (1986). Purification and restriction endonuclease analysis of plant nuclear DNA, pp 57-75. In: A. Weissbach and H. Weissbach (1986). Methods in Enzymology, 118: 57-75.

Wechter W.P., M.P. Whitehead, C.E. Thomas, and R.A. Dean. (1995). Identification of a randomly amplified polymorphic DNA marker linked to the *Fom 2* Fusarium wilt resistance gene in muskmelon MR-1. *Phytopathology*, 85:1245-1249.

Weeden N.F., G.M. Timmerman, M. Hemmat, B.E. Kneen, and M.A. Lodhin. (1992). Inheritance and reliability of RAPD markers, pp12-17. *In*: Application of RAPD technology to plant breeding, Plant Breeding Symposia Series, American Society for Horticultural Science.

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

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

Williams J.A. and C.J. West. (2000). Environmental weeds in Australia and New Zealand: issues and approaches to management. *Australian Ecology*, 25:425-444.

Williams J.G.K., A.R. Kubelik, K.J. Livak, J.A. Rafalski and S.V. Tingey. (1990). DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Research*, 18:6531-6535.

Williamson V.M., J.-Y. Ho, F.F. Wu, N. Miller, and I. Kaloshian. (1994). A PCR-based marker tightly linked to the nematode resistance gene, *Mi*, in tomato. *Theoretical and Applied Genetics*, 87:757-763.

Wirthensohn M.G., G. Collins, G.P. Jones, and M. Sedgley. (1999). Variability in waxiness of *Eucalyptus gunni* foliage for floriculture. *Scientia Horticulturae*, 82:279-288.

Woolley F.M., G.G. Collins, and M. Sedgley. (2000). Application of DNA fingerprinting for the classification of selected almond [*Prunus dulcis* (Miller) D.A. Webb] cultivars. *Australian Journal of Experimental Agriculture*, 40:995-1001.

Yang H. and J. Krüger. (1994). Identification of a RAPD marker linked to the *Vf* gene for scab resistance in apple. *Plant Breeding*, 112:323-329.

Yang X. and C. Quiros. (1993). Identification and classification of celery cultivars with RAPD markers. *Theoretical and Applied Genetics*, 86:205-212.

Young N.D., D. Zamir, M.W. Ganal, and S.D. Tanksley. (1988). Use of isogenic lines and simultaneous probing to identify DNA markers tightly linked to the Tm-2a gene in tomato. *Genetics*, 120:579-585.

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

Zohary D. and P. Spiegel-Roy. (1975). Beginnings of fruit growing in the old world. *Science*, 187:319-27.

Appendix

Poster and papers from thesis research

Posters

Mekuria G., G. Collins, and M. Sedgley. (2000). Genetic diversity of a feral population of olive trees surrounding an abandoned grove. International Symposium under the aegis of the Commission Biotechnology of ISHS Montpellier, France, 6-8 March, 2000.

Papers

Collins G., M. Sedgley, and G. Mekuria. (2000). Establishing a database of DNA fingerprints to identify olive cultivars. The 4th International Symposium on Olive Growing. Bari, Italy, 25-30 September, 2000, pp249-251.

Guerin J., M. Wirthensohn, G. Mekuria, K. Neaylon, G. Collins, and M. Sedgley. (2000). Oil yield and the olive DNA database, pp16-18. The Olive Press, *Journal of the Australian Olive Association*, ISSN 1442-357X, Summer 2000.

Mekuria G.T., G.G. Collins, and M. Sedgley. (1999). DNA fingerprinting: distinguishes differences both between and within named olive cultivars, pp13-16. The Olive Press, *Journal of the Australian Olive Association*, ISSN 1442-357X, Summer 1999: a version of this paper also published in *Australian Olive Grower* Issue 16 April 2000, pp16-19.

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

Mekuria G., G. Collins, S. Lavee, M. Sedgley. (2000). Identification of a RAPD marker linked to tolerance to peacock disease (*spilocea oleaginea*) in olives (*Olea europaea*). International Symposium under the aegis of the Commission Biotechnology of ISHS Montpellier , France, 6-8 March, 2000, pp565-567.

Mekuria G.T, G. Collins, S. Lavee, and M. Sedgley. (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. Collins, and M. Sedgley. (2001). Genetic diversity within an isolated olive population in relation to feral spread. *Scientia Horticulturae* (submitted).

Mekuria G.T., M. Sedgley, S. Lavee, and G. Collins. (2001). 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* (submitted).

Mekuria, G.T., Collins, G. G. & Sedgley M. (1999). Genetic variability between different accessions of some common commercial olive cultivars. *Journal of Horticultural Science and Biotechnology, 74*(3), 309-314.

Mekuria, G. T., Collins, G., Sedgley, M. & Lavee, S. (2001). Identification of genetic markers in olive linked to olive leaf spot resistance and susceptibility *Journal of the American Society for Horticultural Science, 126*(3), 305-308.