Genetic Analysis of Resistance to Powdery Mildew (*Podosphaeara leucotricha*) in Apple (*Malus* x *domestica* Borkh.).

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Thesis submitted in partial fulfilment of the requirements for the degree of Master Scientiae at the Department of Biotechnology, University of the Western Cape.

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ABSTRACT

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M.Sc thesis, Department of Biotechnology, Faculty of Science, University of the Western Cape.

Apple powdery mildew, caused by *Podosphaera leucotricha*, is one of the major diseases of the cultivated apple in the Western Cape, causing severe losses in yield and affecting fruit quality. This affects the deciduous fruit industry as costs are incurred on the management of this disease. The application of fungicides is one of the strategies mostly used to control the spread of the pathogen. Since consumers demand high quality fruit with no harmful chemical residues, alternative approaches that minimize the reliance on agri-chemicals for the control of this disease must be developed. Marker-assisted breeding for the development of new varieties that exhibit durable resistance to the fungus is the best and safest way to reduce damage caused by powdery mildew. The availability of SSR markers, tightly linked to genes for resistance to this disease is a major break-through. Using these molecular markers is an efficient approach for breeding resistance to *Podosphaera leucotricha*, and for the improvement of some complex traits having environmental relationships. However, these markers needed optimization. In this study, a set of ninety-nine primer pairs, from literature and GenBank, were optimized and

DEDICATED TO MY FAMILY AND MY LOVELY WIFE ZANELE

DECLARATION

I herewith declare that 'Genetic Analysis of Resistance to Powdery Mildew (*Podosphaeara leucotricha*) in Apple (*Malus* x *domestica* Borkh.)' is my own work and has not been submitted for any degree or examination in any other university, and that all the sources I have used or quoted have been indicated and acknowledged by complete references.

Zolani Ellias Simayi December 2008

Signed:.....

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ABBREVIATIONS

AFLP	Amplified fragment length polymorphism
APS	Ammonium persulphate
ARC	Agricultural Researc Council
BAC	Bacterial artificial chromosome
bp	Base pair
cDNA	Complementary Deoxyribonucleic acid
cM	Centimorgans
cpDNA	Chloroplast Deoxyribonucleic acid
dNTPs	Deoxyribonucleotide triphosphate
DFPT	Deciduous Fruit Producers Trust
DNA	Deoxyribonucleic acid
DARE	Durable apple resistance
EAGMP	European apple genome mapping project
EMBL	European Molecular Biology Laboratory
ESTs	Expressed sequence tags
et al	Et alii
FPEF	Fresh Produce Exporters' Forum
GDP	Gross domestic Product
HiDRAS	High-quality disease resistant apples for sustainable agriculture
LG	Linkage group
LOD	Logarithm of odds
MAB	Marker-assisted breeding

MAS	Marker-assisted selection
µl	Microliter
mtDNA	Mitochondrial Deoxyribonucleic acid
mM	Millimolar
NBS	Nucleotide-binding site
nDNA	Nuclear Deoxyribonucleic acid
OABS	Optimal Agricultural Business Systems
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
QTL	Quantitative trait loci
RAPD	Randomly amplified polymorphic DNA
RFLP	Restriction fragment length polymorphism
SCAR	Sequence characterized
SSR	Simple sequence repeat
V/cm	Volts per centimetre
v/v	Volume per volume
w/v	Weight per volume

1 General Introduction

1.1 The Deciduous fruit industry and the South African economy

South Africa is a net exporter of agricultural products in rand value. The largest export groups are wine; citrus; raw sugar; fresh grapes; wine, maize and deciduous fruit such as apples, pears, peaches and apricots. Currently, the commercial sector of agriculture contributes about 2,5% to the gross domestic product (GDP), a drop from 2,8% in 2007. It also contributes about 8% to formal employment (South African Yearbook, 2007/08). The deciduous fruit industry creates employment for over 100 000 employees (converted to permanent equivalents), and more than 400 000 dependents are sustained by the industry (see Table 1.1).

Table 1.1: On-farm employment

Fruit	No. of Labourers*	No. Dependents
Apples	25,878	103,514
Grapes	36,941	147,796
Pears	14,588	58,352
Peaches	10,516	42,064
Plums	6,014	24,055
Apricots	4,522	18,087
Nectarines	2,024	8,096
Total	**100.491**	**401,964**

* Casual labour converted to permanent equivalents Source: OABS 2006

The South African deciduous fruit industry's agricultural and foreign exchange earnings make important contributions to the economy of the country. The export earnings of the deciduous fruit industry represent 16% of South Africa's total earnings from agricultural

exports in 2007 (South African Yearbook, 2006/07). According to the Fresh Produce Exporters Forum, the South African deciduous fruit is mainly exported to the markets in the UK and Central Europe. It is also gaining access to markets of countries such as USA, Far and Middle East, and Asia. Markets have also opened in Africa (see figure 1.1 below).

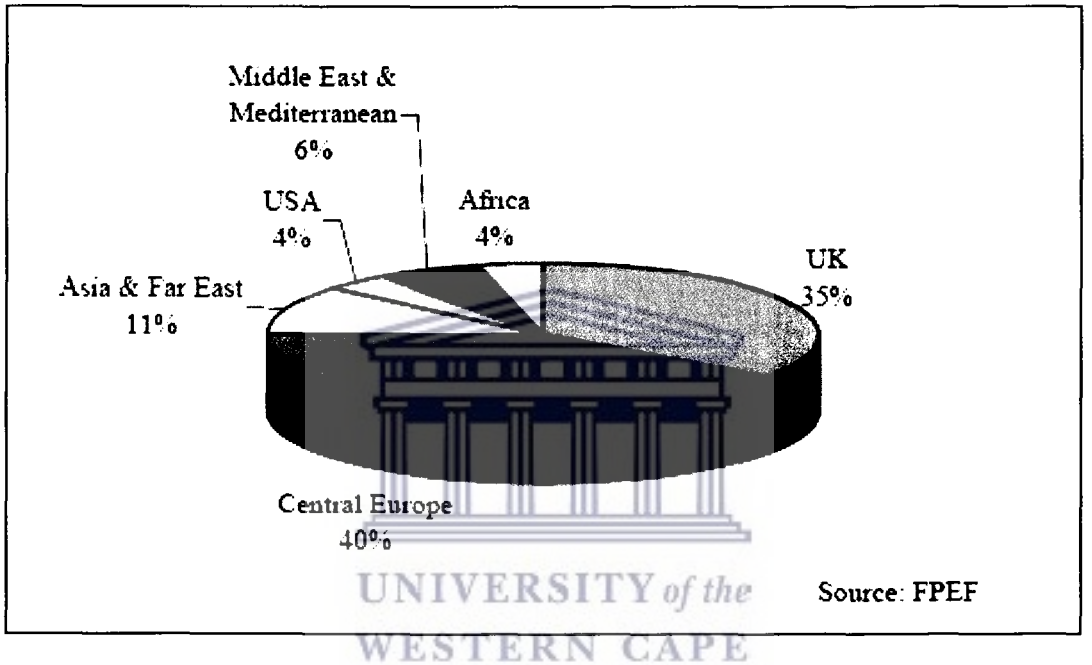


Figure 1.1: The graph indicates deciduous fruit export destinations during the 2005 season: (FPEF, 2005).

Locally, the wine industry contributes R163 billion a year to South Africa's GDP and employs 257 000 people directly and indirectly, while an additional R4,2 billion is generated annually through wine tourism (South African Yearbook, 2007/08).

With its mild Mediterranean climate and fertile valleys, the Western Cape is ideal for deciduous fruit trees and grape vines. In 2005/06, about 73% of the total crop was produced in the Western Cape, 12% in the Northern Cape, 11% in the Eastern Cape and 2% in Limpopo (South African Yearbook, 2007/08). In 2003/04 the income increased from deciduous fruit to R5 266 million, but the industry experienced a drop of income for the 2004/05 period (from R5 266 to R4 633 million) (South African Yearbook, 2004/5, 2005/6 and 2006/7).

1.1.1 Apple production in South Africa

Apples are the major crop of the South African deciduous fruit industry. According to the agricultural census for 2004/05, apples made up the largest percentage of the deciduous fruit crop (35%), followed by table grapes (33%), while pears totaled at 24% (South African Yearbook, 2006/07). This crop is mainly grown in the Western Cape. Other production areas include Northern Cape, Eastern Cape, Northern Province, Free State, Gauteng and Mpumalanga (see figure 1.2). In Table 1.2, Groenland; Ceres; Langkloof East and Villiersdorp / Vyeboom have 6 332; 4 863; 3 992 and 3 396 hectares of planted areas, respectively. These are major districts of apple production in South Africa.

Figure 1.2. Represents the Geographical spread of pome fruit in South Africa (DFPT, 2006).

Table 1.2: Production Areas of Apples in South Africa

District	Number of Trees	Area (ha)
Groenland	7,271 718	6, 332
Ceres	5,432 530	4,863
Langkloof East	3,585 654	3,992
Villiersdorp / Vyeboom	3,383 203	3,396
Langkloof West	467,580	468
Picketberg	436,230	332
Free State	390,542	267
Little Karoo	207,150	265
Southern Cape	308,269	225
Mpumalanga	249,273	164
Somerset West	198,479	126
Wolseley / Tulbagh	79,285	65
Berg River	34,234	36
North East Free State	50,937	32
Stellenbosch	23,601	19
Worcester	26,082	16
Eastern Cape	3,739	15
Northern Province	11,286	7
Gauteng	10,872	7
Franschhoek	4,854	3
Total	**22,175 518**	**20,633**

Source: DFPT Tree Census 2006

1.2 Origin of apple (*Malus domestica* Borkh.)

Central Asia is believed to be the centre of origin of most cultivated plants, including 38 important crops. About 20 percent of the world's cereals, more than 20 percent of the

vegetable and spice plants, and 90 percent of the major temperate fruit crops (such as apple, pear, apricot, blackberry and strawberry) are found in this region. Furthermore, wild fruits in this region possess genetic potential for resistance to biotic stress, diseases and pests (Dzhangaliev *et al.*, 2003).

The Turkistan region (Kazakhstan, Turkmenistan, Uzbekistan, Kyrgystan) is believed to be the place of origin of the domesticated apple because of the enormous diversity in this region (Janick *et al.*, 1996). *Malus sieversii*, a wild apple from the Heavenly Mountains (Almaty) (figure 1.3), is considered to be the main progenitor of *Malus domestica*, with *M. sylvestris*, *M. orientalis*, *M. baccata*, *M. mandshurica*, *M. prunifolia* contributing to its genetic makeup (Janick *et al.*, 1996; Forsline *et al.*, 2003). Germplasm collections from Central Asia (mostly Kazakhstan) confirm that all the traits in *M. sieversii* are also present in *M. domestica* (Forsline, 1995; Forsline *et al.*, 2003).

1.2.1 Taxonomy of apple

The apple, along with pear, quince, loquat, and medlar – belong to the *Maloideae* subfamily of *Rosaceae* (rose) family. It has been largely agreed that the genus *Malus* has 25 to 30 species and several sub-species of so called crabapples (Janick *et al.*, 1996; Jackson, 2003). The binomial *Malus* x *domestica* was first proposed in 1984 and is generally accepted as an appropriate scientific name for domesticated apple. This name was proposed to emphasize the hypothesis that the domesticated apple is a result of interspecific hybridization (denoted by 'x') among the wild *Malus* species (Janick *et al.*, 1996; Robinson *et al.*, 2001).

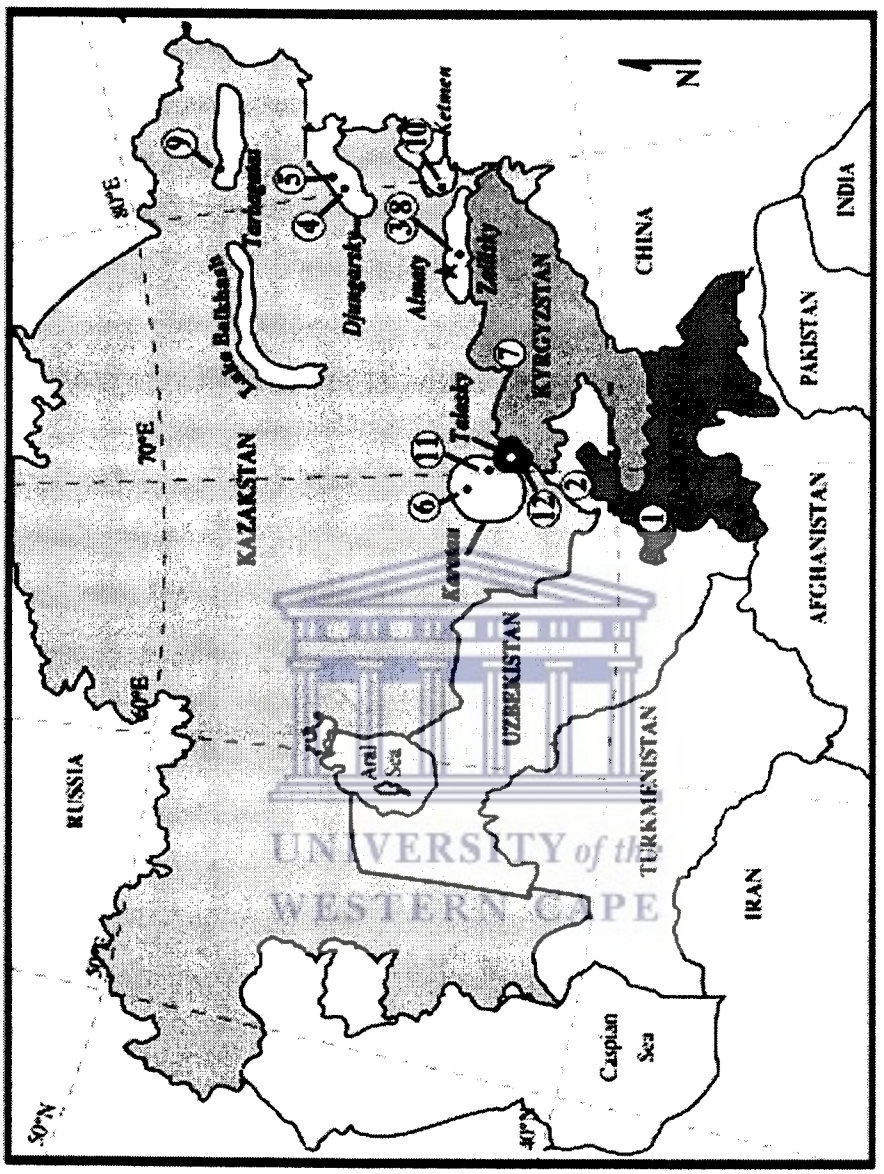


Figure 1.3. Map of regions in Central Asia where *Malus sieversii* (Lebed) was collected in 1989, 1993, 1995 and 1996 (Forsline *et al.*, 2003).

1.2.2 Phylogenetics of apple

The nuclear DNA (nDNA) and cytoplasmic DNA [chloroplast DNA (cpDNA) and mitochondrial DNA (mtDNA)] provide important clues about the origins of domesticated plants. The chloroplast DNA provides data about evolutionary relationships of the maternal line, whilst the bi-parentally inherited nuclear DNA provides independent data, which, combined with cpDNA, could help to determine the origin of the domesticated apple (Juniper and Mabberley, 2006; Harris *et al.*, 2002). In a survey of apples across the *Malus* genus by Harris *et al.* (2002), *mat*K, an 1800-bp long cpDNA-encoded region, of which 1341 bp was sequenced, showed only 16 phylogenetically informative characters. This resulted in poor resolution in the phylogenetic tree. However, two duplications were found 39 bp from the 3′ end of the *mat*K coding region. These duplications are: Duplication I (imperfect 8-bp duplication) and Duplication II (perfect 18-bp duplication) (see figure 1.4). The 8-bp duplication (Duplication I) was found in most of the species from *Malus* and *Sorbmalus* sections, while the 18-bp duplication occurred only on the Central Asian wild apple (*M. sieversii*) and the domesticated apple (*M. domestica*). This suggests that there is a close relationship between the two, and that *M. sieversii* may be the maternal contributor to the domesticated apple. The occurrence of 8-bp duplication in the domesticated apple and *M. sylvestris* attest to the assertion by Janick *et al.* (1996) that *M. sylvestris* might have contributed to the genetic makeup of *M. domestica*. However, the exact origin of *M. domestica* is still unclear. Modern molecular techniques such as microsatellite markers (known as SSRs) have the potential of providing more information on the origin and parentage of today's commercial apple since they are capable of

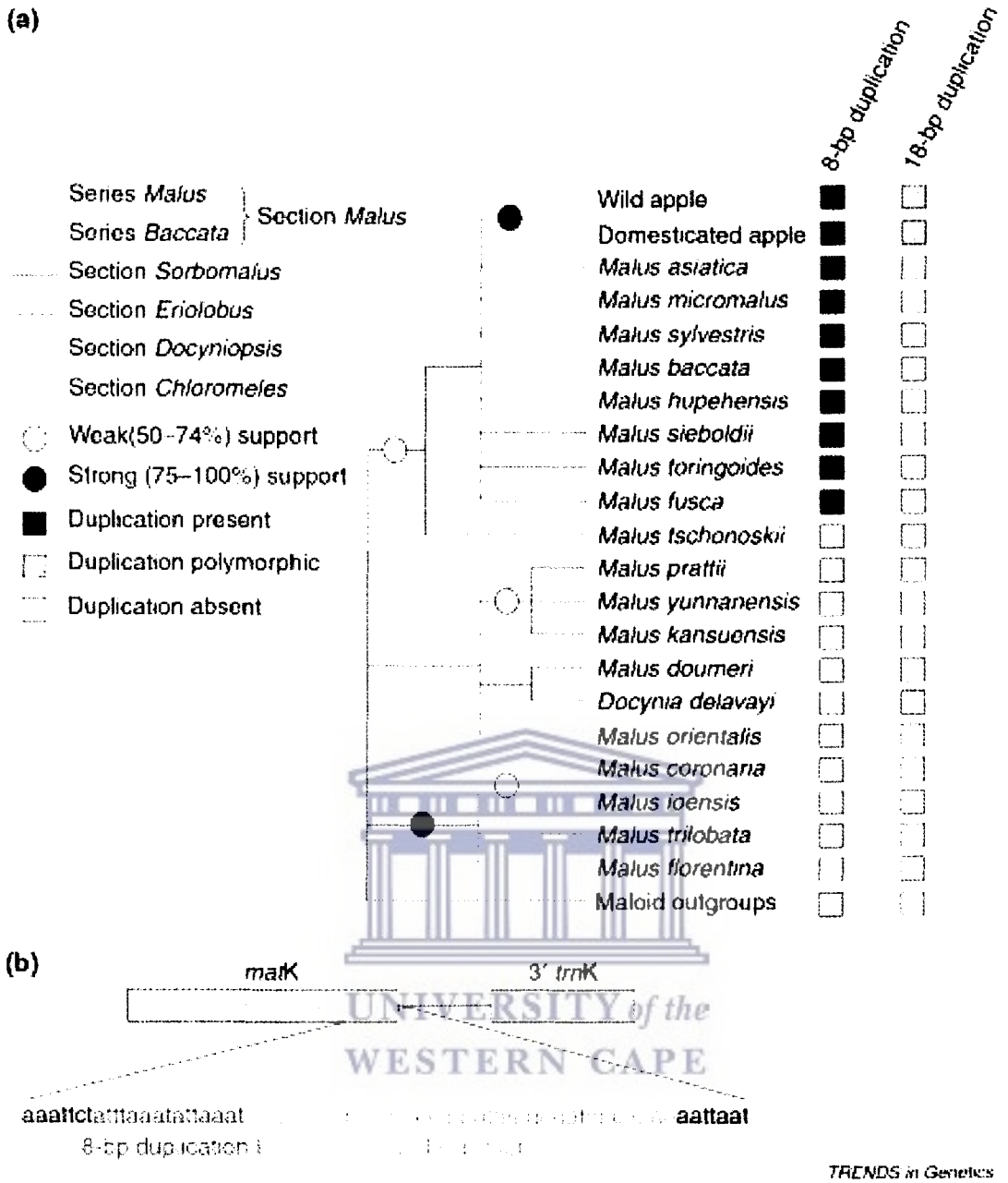


Figure 1.4. Phylogeny of the chloroplast DNA-encoded gene *mat*K in the genus *Malus* (a). A cartoon of *mat*K indicating the positions of the two duplications (8-bp and 18-bp duplications) is shown in (b). Wild apple refers to the Central Asian wild apple, *M. sieversii*. Circles indicate the level of statistical support for particular groups. The distribution of the two *mat*K duplications is shown on the tree (Harris et al., 2002).

distinguishing closely related species. Section 1.5.1 provides a detailed discussion of microsatellite markers.

1.2.3 Genome organization

Most cultivated apples are functional diploids ($2n = 34$) (Janick *et al.*, 1996). Lespinasse *et al.* (1999a) argues that the apple is an allopolyploid derived from a hybrid between two ancestors Spiraeoidae ($x = 9$) and Prunoidae ($x = 8$). This resulted in a basic haploid number $x = 17$. About 10% of the commonly known cultivars (e.g. Jonagold), are triploids ($2n = 51$) (Gianfranceschi *et al.*, 1998). Triploids have arisen naturally from the fertilization of unreduced gametes. They are more vigorous than diploids and tend to have larger fruits. Triploids, however, produce poor pollen, leading to pollination problems in the orchard. They require diploids as pollinators. Tetraploids ($2n = 68$) have also been discovered. They usually occur as chimeras with one or more layers of diploid tissue. Shoots of tetraploids are typically thick and sparsely branched with short internodes, and their fruits are large, and often flattened (Janick *et al.*, 1996).

There is not much information available for the karyotype of a cultivated apple. Bouvier, *et al.*, (2000) proposed that the length of the seventeen chromosomes of a haploid apple range from 1.5 to 3.5μm. With respect to the position of the centromere, about eleven of those chromosomes are sub-metacentric and six are metacentric, with the chromosome bearing a satellite being the longest one.

1.3 Apple powdery mildew

Apple powdery mildew, caused by *Podosphaera leuchotricha* (Ellis et and Everh.) Salm., is one of the most common diseases of the cultivated apple in the Western Cape (South Africa). The high infection rate of apples in the orchards by this pathogen is a major threat to the deciduous fruit industry. Furthermore, the damage caused by this pathogen leads to severe losses in fruit yield as most of the affected fruit becomes unattractive. This greatly affects the agricultural sector as more costs are spent on the management of this disease in order to control the damage of crops.

1.3.1 Disease cycle of powdery mildew

Powdery mildew affects both the leaves and stem of the apple tree, resulting in leaf drop and shoot stunting (Korban and Riemer, 1990). It overwinters as mycelia in dormant leaf buds (see figure 1.5). When the buds break in spring, the fungus infects the leaves emerging from those buds. The fungal spores (conidia) from these infected leaves are responsible for the secondary mildew infections on the shoots. As the infection develops, the disease spreads to the flowers, and ultimately to the fruit. The leaves curl upwards (figure 1.6a), and in severe cases, the infected fruit develops a web-like russet on the skin (figure 1.6b).

1.3.2 Impact of the environment to mildew infection

The degree of infection refers to both the incidence and severity of the disease. Disease incidence refers to the proportion of the plant that is visibly diseased, while severity is the area of the plant tissue that is infected relative to the total area (Xu and Madden, 2002).

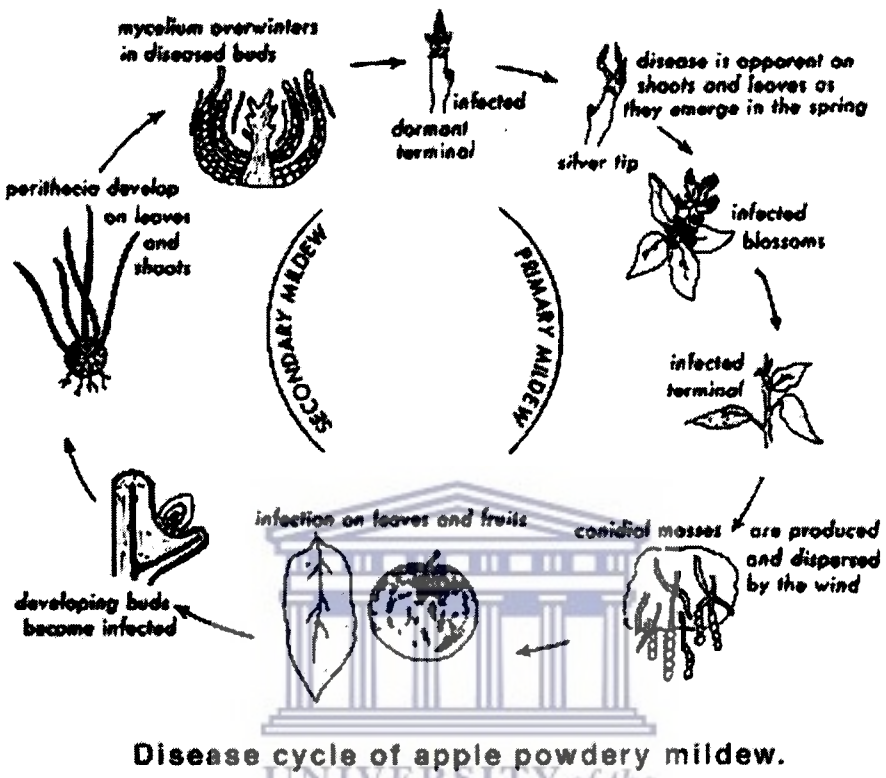


Figure 1.5. Disease life cycle of powdery mildew (West Virginia University).

(a)

(b)

Figure 1.6 Represent apple powdery mildew symptoms on both apple foliage and apple fruit. **(a)** Right: infected foliage - notice the upward curling of leaves and white appearance, left: Healthy foliage. **(b)** Affected fruit (left) and clean apple fruit (right) (Utah State University Co-orporative Extension, 2000).

The degree of infection is influenced by several factors. Physiological factors such as plant age and turgidity are believed to influence the degree of infection by the pathogen (Korban and Riemer, 1990). Environmental conditions are known to be the most influential of all factors and have been implicated to the varying degrees of infection of plants by *P. leucotricha* (Xu and Butt, 1998). High humidity and shady conditions are the most important factors influencing the germination of the fungal spores (conidia) of *P. leucotricha*. The spores germinate at relatively high humidity, but will not germinate in free water. The high amount of water in the plant surface does not encourage the germination of fungal spores (Pest Notes, 2001). It has been demonstrated that heavy rainfall decreases the level of powdery mildew infection (Sestra, 2003). This explains why at times greenhouse infections tend to be more severe than field infections. Sometimes field evaluations reveal a higher degree of infection than the greenhouse evaluations. This normally happens when the trees are under the nets, as shady conditions favor the development of *P. leucotricha* spores (Pest Notes, 2001).

1.4 Apple genetics

Traditional breeding methods have been used in apple breeding. Unlike cereals, vegetables and other crops, apples are known for their lengthy juvenile phase and a high level of heterozygosity (Janick *et al.*, 1996). This makes apple breeding using conventional methods a difficult task. Furthermore, conventional breeding is known to be laborious, costly, and time-consuming (Guilford *et al.*, 1997). Besides the labour, costs and time, the screening of plants for disease resistance by infecting them with different pathogens under controlled environments has its shortcomings. For example, other

susceptible plants can escape pathogen attack (Mohan *et al.*, 1997). This problem could lead to false breeding lines.

The use of molecular-based techniques for the selection of seedlings carrying valuable traits has so far produced promising results in overcoming the shortcomings of conventional breeding. These techniques hold great potential of enabling breeders to pre-screen seedlings to select for those that carry valuable traits prior to field trials. Early selection for important agronomic traits such as resistance to apple powdery mildew; apple scab; woolly apple aphid and fruit quality are difficult to evaluate using conventional methods. Molecular-based techniques are more precise, cost-effective and less time-consuming as compared to phenotypic methods like conventional breeding (Mohan *et al.*, 1997), and can help reveal such complicated traits (Stankiewicz *et al.*, 2002).

1.4.1 Marker-assisted selection in apple breeding

Marker-assisted selection (MAS) is a molecular-based crop selection technique. It involves the use of molecular markers as diagnostic tools to trace the inheritance of genes of agronomic importance. This technique greatly improves the efficiency of conventional breeding by carrying out selection using molecular markers linked to that trait. It provides opportunities to select plants at the seedling stage. To understand how the selection process works, it is important to understand the basic principle of this technique (MAS). The inheritance of any agronomically important trait is inversely proportional to the distance between that trait and the marker (Kumar, 1999). This simply means that as the

distance between the molecular marker and the trait of interest increases, the chance of recombination between the gene and the marker also increases. During the selection process, two markers flanking the gene of interest can be used if the linkage distance between the gene and each marker is close. The tight linkage between the molecular marker and the gene of interest is therefore a prerequisite for the efficiency of marker-assisted selection.

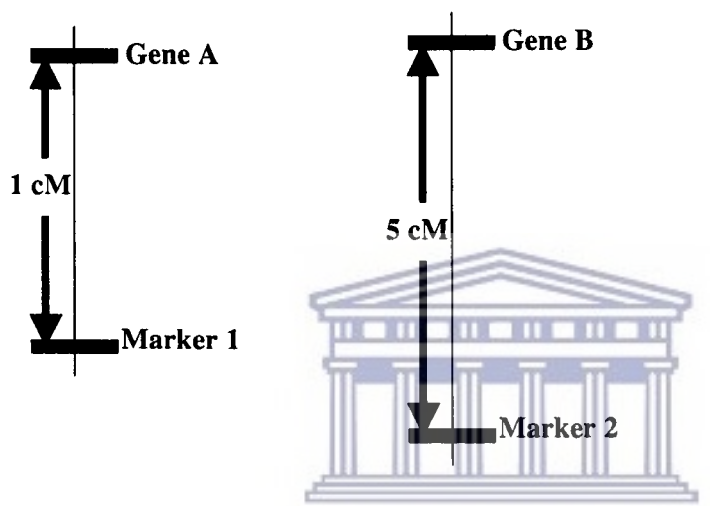


Figure 1.7: An illustration of the relationship between markers and genes.

With marker 1, there's only 1% chance that crossover recombination will occur. The likelihood of marker 1 being transferred with the gene is 99% while the likelihood of the transference of marker 2 is 95%.

1.4.2 Breeding for durable disease resistance in apple

Powdery mildew, caused by *Podosphaera leucotricha*, scab caused by *Venturia inaequalis* and woolly apple aphid caused by *Eriosoma lanigerum*, are the most common

diseases of the cultivated apples in South Africa. The high infection rate of apples in the orchards by these pathogens is a major threat to the deciduous fruit industry.

In the early years of apple breeding, single resistance genes were selected for resistance against scab and powdery mildew, using wild species such as *M. floribunda* 821, *M. zumi* and *M. robusta*. Until the early 1990s, single resistance genes seemed to offer durable resistance. The V_f gene has been a major resistance gene against apple scab. However, race 6 of *Venturia inaequalis* has been demonstrated to overcome the V_f barrier. Scab lesions were observed on apple selections carrying V_f (Parisi *et al.*, 1993). Moreover, race 7 showed virulence towards *M. floribunda* 821 (Benaouf and Parisi, 2000). The major resistance gene Pl-2 in apple has recently been shown to be overcome by a virulent population of powdery mildew (Caffier and Laurens, 2005; Caffier and Parisi, 2007). Clearly, single resistance genes can no longer be relied upon for durability due to the occurrence of virulent races of apple pathogens. Today, most apple breeding programmes aim towards solving this problem by developing new varieties that exhibit durable resistance to a wide range of pests and fungal pathogens. Pyramiding (combination) of different major resistance genes in one cultivar is a promising way to achieve durable resistance. However, the feasibility of pyramiding is directly dependent on the availability of molecular markers tightly linked to the genes of interest. These molecular markers help to detect and track the desired genetic combinations in the seedlings prior to field trials.

1.5 Molecular markers used in apple breeding

Molecular markers are known as 'genetic tags' or 'landmarks' that can be used to identify specific genes in relation to other genes. They are not genes but 'signposts' for genome analysis. The development of these diagnostic tools has greatly improved breeding in pome fruit by increasing selection efficiency (i.e. speed and accuracy). To date, various molecular markers have been used in marker-assisted selection depending on their technical requirements (labour and costs). Restriction fragment length polymorphism (RFLP) markers have been reliably used for cultivar identification and parentage analysis in apple (Nybom *et al.*, 1992; Nybom, 1990; Nybom and Schaal, 1990). Despite their co-dominant nature and usefulness in comparative studies, the laborious nature of these markers has led to their relegation in marker-assisted breeding (Mohan *et al.*, 1997). Randomly amplified polymorphic DNA (RAPD) markers have surpassed RFLPs (Koller *et al.*, 1993; Mulcahy *et al.*, 1993). Although the RAPD technique is time and cost effective but it is not reproducible, and difficulties can be experienced when interpreting. This is due to their dominant mode of inheritance (Lavi *et al.*, 1994). These markers were replaced by amplified fragment length polymorphism markers (Vos *et al.*, 1995). This technique yields up to thirty polymorphic dominant primer combinations, and therefore can be used for the saturation of existing genetic maps (Liebhard *et al.*, 2002). Despite their wide usage, AFLPs are not transferable between breeding progenies (James *et al.*, 2004). Microsatellites, also known as simple sequence repeats (SSRs) have become markers of choice for QTL mapping in apple. This is due to their co-dominant, multi-allelic nature. Furthermore, they are highly informative and transferable between cultivars (Liebhard *et al.*, 2002). Microsatellites are tandemly repeated DNA stretches of

1-5 nucleotides. They are abundant and randomly distributed throughout the genome. In the last decade, these markers have gained popularity in most plant systems. This is due to their high level of polymorphism, reproducibility and transportability between cultivars. The simple banding patterns and co-dominant mode of inheritance has made microsatellites to be markers of choice for DNA fingerprinting in apples (Guilford *et al.*, 1997). They are also useful in linkage analysis studies; agronomic trait selection; construction of genetic maps; cultivar identification; population and evolutionary studies (Maguire, 2001; Scott, 2001). The suitability of microsatellites for multiplexing (simultaneous amplification of more than one locus in a single reaction) is an additional advantage over other markers. This quality is useful for high throughput screening and has been used with great success in apple (Liebhard *et al.*, 2002). These markers are flanked by highly conserved region. Primers for the amplification of microsatellite loci are designed from these unique regions. The primers developed from one species can be used to amplify microsatellite loci in other related species. For example, primers developed from apple have been successfully used in pear (van Dyk *et al.*, 2005; Yamamoto *et al.*, 2001; Pierantoni *et al.*, 2004). Liebhard *et al.*, (2002) tested the transferability of SSR markers (15 primer pairs) in 43 *Rosaceae* species. All the fifteen primer pairs amplified scorable bands in *Maloideae* while only one primer pair amplified scorable bands in *Amygdaloideae*. This affirms the fact that SSRs can be transferred to other species of the same genus (Ellegren *et al.*, 1997). Microsatellites have been developed in most fruit tree species such as apple (Guilford *et al.*, 1997; Hokanson *et al.*, 1998; Liebhard *et al.*, 2002; Vinatzer *et al.*, 2004) and pear (Yamamoto *et al.*, 2002a, 2002b; van Dyk *et al.*, 2005).

1.5.1 Microsatellites derived from libraries

1.5.1.1 Genomic libraries

Screening small insert genomic libraries with labeled oligonucleotide probes is often used to generate microsatellite markers. In figure 1.8, the standard methods for the isolation of microsatellites from small insert libraries is clearly outlined, and it involves the following steps:

- ❖ creation of small insert library,
- ❖ screening of the library by hybridization with labelled oligonucleotides probe,
- ❖ identification and sequence of positive clones for verification of microsatellite presence.
- ❖ primer design from the regions flanking the microsatellite for locus-specific PCR amplification, and
- ❖ identification of polymorphism.

Liebhard *et al.*, (2003a) and Silfverberg-Dilworth *et al.*, (2006) have used this approach with great success in apple. Technically, this approach is simple and straightforward because it requires basic molecular biology techniques. However, when this method is compared to other methods of isolating microsatellites, it is the most expensive, laborious and time-consuming method (Holton, 2001). Various enrichment methods have been developed to overcome these limitations. Edward *et al.*, (1996) describes one of these methods as follows:

- ❖ digestion of genomic DNA,
- ❖ ligation of adaptors to the ends of the restricted fragments,

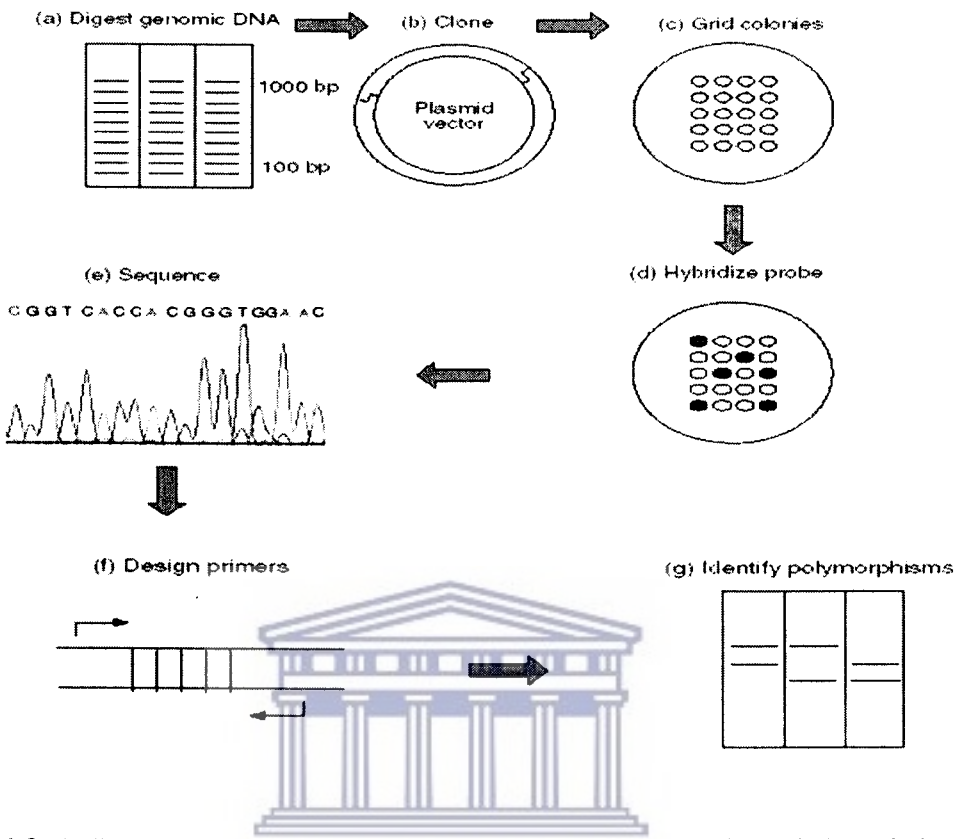


Figure 1.8. A diagrammatic representation of standard methods for the isolation of plant microsatellite loci: (a) digestion of genomic DNA, (b) ligation into plasmid vector, (c) transformation into E.coli and grid colonies, (d) hybridization with a labeled microsatellite oligonucleotides probe, (e) DNA sequencing of positive clones, (f) primer design and locus-specific PCR amplification, and, (g) identification of polymorphism, (Maguire, 2001)

- ❖ microsatellite enrichment through hybridization of the fragments to a Nylon filter membrane with many bound microsatellite oligonucleotides,
- ❖ after washing, the eluted fragments are amplified using adaptor sequences as PCR primers,
- ❖ the resulting colonies are isolated and digested, size-selected and ligated into a plasmid vector, and transformed into cells.

This method greatly increases the efficiency of microsatellite isolation from genomic libraries by reducing the time and costs spent in sequencing clones that do not contain the repeats (Maguire, 2001).

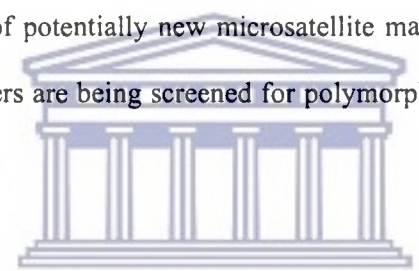
1.5.1.2 BAC libraries

Screening bacterial artificial chromosome (BAC) is another method of isolating microsatellite markers. This method is used to isolate microsatellites in specific regions of a genome. In other words, it is a targeted-approach to isolate microsatellite markers. This method has been used with great success in soybean (Cregan *et al.*, 1999) and apple (Vinatzer *et al.*, 2004). The screening of cDNA libraries is another method of isolating microsatellites. However, this method produces microsatellites identical to those derived from EST databases (Scott, 2001).

1.5.2 Database-derived microsatellites

Microsatellite markers can also be generated through electronic searching of public databases such as EMBL/GenBank, and EST databases. The high costs involved in creating and screening genomic libraries is a good reason to search for alternative

methods of developing microsatellites. The availability of sequences in the public databases such as EMBL or GenBank or EST databases, promises to be an alternative method of developing new microsatellite markers. As research on commercial plants expands, large numbers of sequences are deposited in these public databases. This database approach is simple and direct, and only requires electronic sorting of microsatellite-containing sequences and primer design from the flanking regions for PCR amplification. This database-approach of developing new microsatellites relies on previous research and it is time and cost effective. This approach has been used in most commercial crops such as sugarcane (Da Silva, 2001), grapes (Scott *et al.*, 2000) and rice (Cho *et al.*, 2000). The UWC marker-assisted breeding group (UWC-MAB) has identified a large number of potentially new microsatellite markers from the apple EST sequences, and these markers are being screened for polymorphism and used in different mapping populations.



Microsatellites derived from expressed sequence tags (ESTs) are highly transferable between closely related species and are representative of all repeat motifs (Cho *et al.*, 2000; Scott *et al.*, 2000; Da Silva, 2001). Since EST-SSRs represent real functional genes, they are useful for genetic mapping. These EST-derived markers provide a wealth of tri-nucleotides and higher repeats, which are easily automated owing to the absence of artefacts such as stutters (Holton, 2001). Though EST-derived microsatellites have some intrinsic advantages, they are slightly less polymorphic in comparison to genomic-derived microsatellites. This was revealed in a comparison of allelic variability between EST- and genomic-derived markers. Microsatellites derived from genomic libraries

detected a higher level of polymorphism than the EST-derived ones (Cho *et al.* 2000). The reduced level of polymorphism from the EST-derived microsatellites is believed to be due to the pressure for sequence conservation in gene regions (Scott, 2001). However, the degree of polymorphism in these EST-derived markers is still useful for mapping and identity applications (Scott *et al.*, 2000).

1.5.3 Disadvantages of microsatellites

Though microsatellites are valuable markers, they have their own shortcomings. These shortcomings can lead to genotyping and mapping errors. These shortcomings are discussed below.

1.5.3.1 Artefacts - Stutter bands/peaks.

During the PCR amplification of a SSR (mostly di-nucleotides), numerous bands/peaks, referred to as 'stutters', are produced. These stutter peaks are characterized by a 2bp difference and are usually smaller than the true allele. The slippage of the DNA polymerase during repeat amplification is believed to be the cause of these artefacts (Holton, 2001). The presence of stutter peaks causes serious difficulties as far as allele scoring is concerned, hence it can lead to the identification of false polymorphisms. For example, if two different alleles differ by 2bp (see figure 1.9), serious difficulties in discriminating between the two alleles will be experienced. Inconsistent allele scoring in di-nucleotide SSRs is one of the serious drawbacks of SSRs. However, this problem can be overcome or reduced by utilizing tri-nucleotides or repeats of higher order (Bryan *et al.*, 1997). Modifying the thermal cycling conditions (e.g. longer PCR extension time)

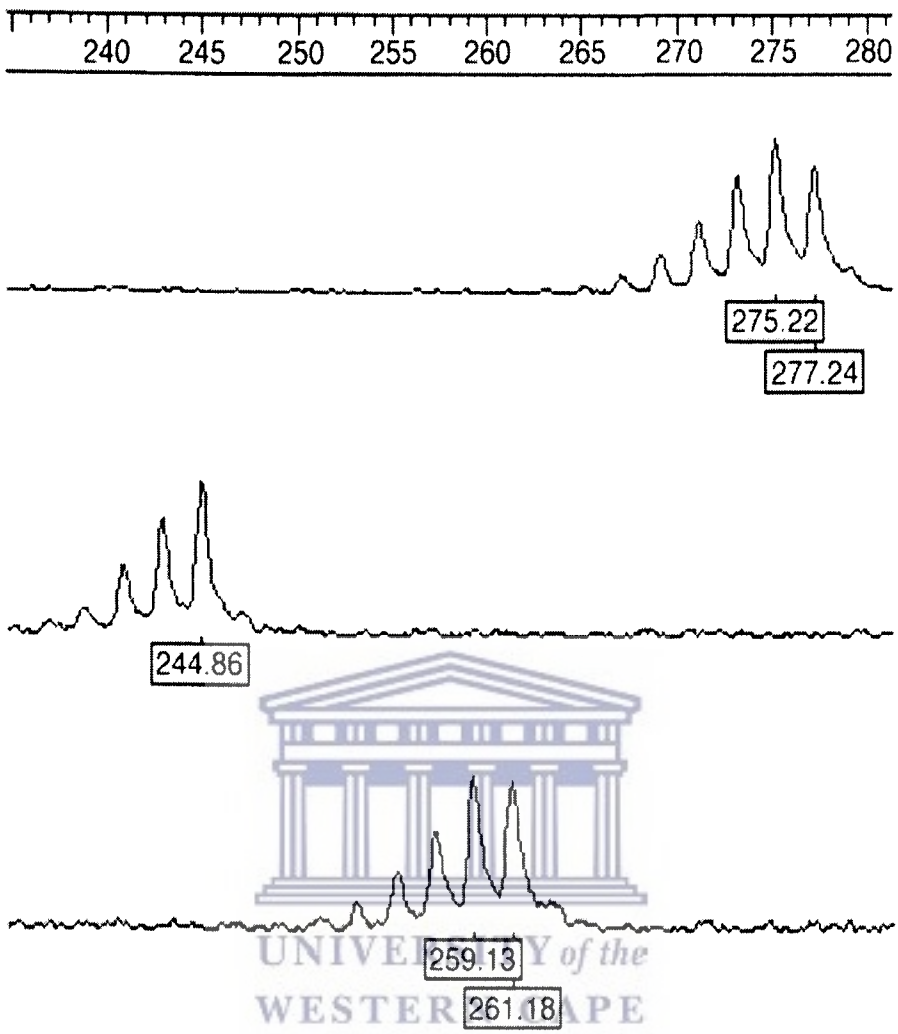


Figure 1.9. Di-nucleotide repeat demonstrating how stutter peaks can complicate the process of allele scoring (Harker, 2001).

can also reduce a number of stutter peaks.

1.5.3.2 Artefact – Non-template 'A' addition

The addition of a non-template nucleotide (adenosine) to the 3' end of the PCR products is another artefact experienced during microsatellite analysis (see figure 1.10), and *Taq* DNA polymerase catalyses it. This addition often leads to the mislabeling of alleles and presents a potential genotyping error. Fishback *et al.* (1999) has shown that the inconsistent addition of the non-template 'A' is a result of an increase in the amount of *Taq* DNA polymerase with a decrease in the amount of primer. Different approaches have been developed to try to deal with the effect of the non-template 'A' addition in genotyping studies. A labour-intensive manual editing of errors is one approach. Post-PCR treatment with T4 polymerase is another approach. It has been reported that the addition of T4 polymerase to the PCR products does remove the non-template adenosine (Ginot *et al.*, 1996). However, the costs involved in this approach should be weighed with the costs of genotyping errors versus the costs of manual editing. Manual editing of data is still a necessity to ensure that there is consistency in allele scoring.

1.5.4 Apple genome mapping

The earliest genetic map was developed late in the 20th century. This map was developed for a cross between 'Rome Beauty' and 'White Angel' (Hemmat *et al.*, (1994). This map consisted of over 400 markers (367 RAPDs, 34 isozymes and 8 AFLPs). Conner *et al.*, (1995) also developed genetic maps for accessions Wijcik McIntosh, NY 75441-67 and NY 75441-58. Since these maps were mainly based on RAPD markers, they were not

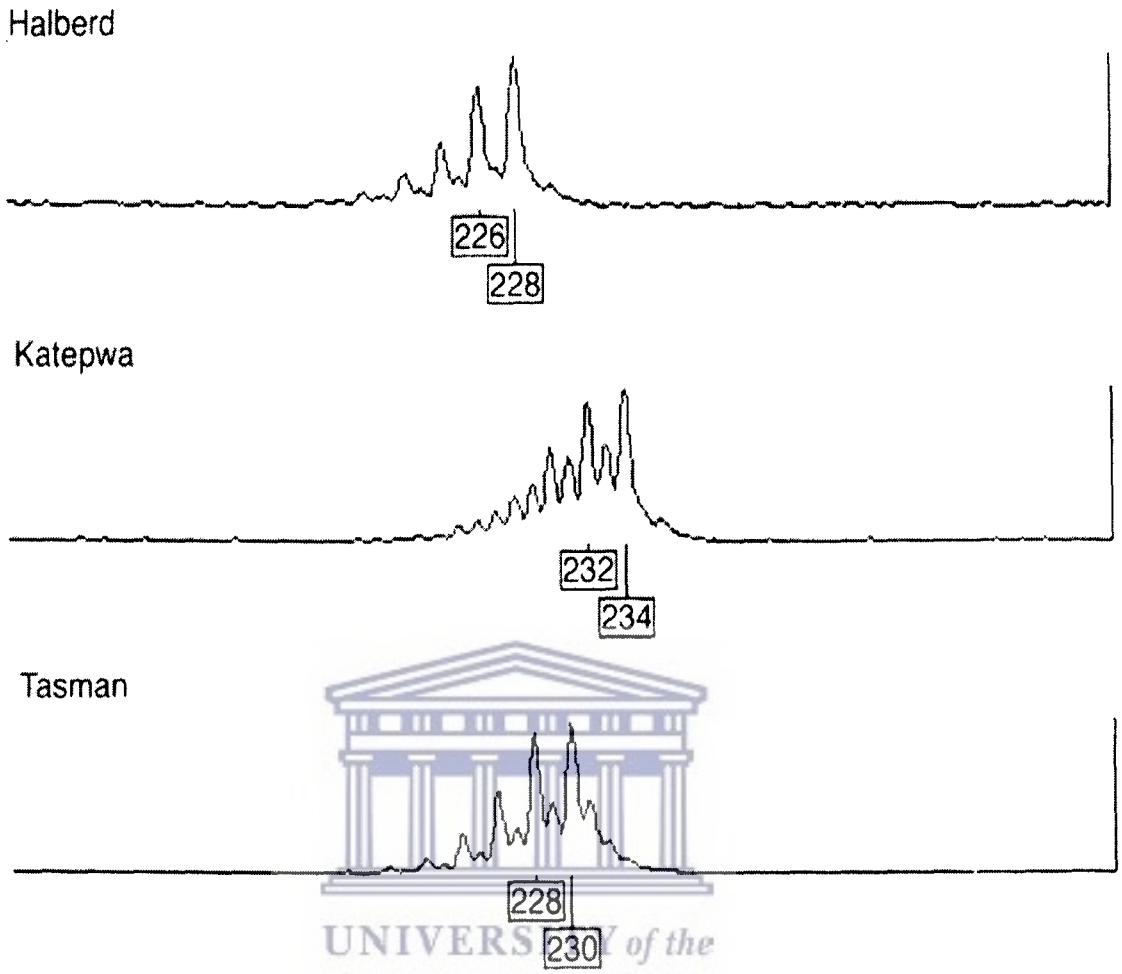


Figure 1.10. Electropherogram by 373 automated DNA sequencer. Amplification of wmc 112 from wheat varieties 'Halberd', 'Katepwa' and 'Tasman' show stutter peaks, which differ by increments of 2bp (e.g. 226 and 228). Additional peaks between the stutter peaks are due non-template 'A' addition by the *Taq* DNA polymerase (Holton, 2001).

easily transportable to other progenies. This necessitated maps of saturated by molecular markers that can be transported between laboratories. Markers such as microsatellites have become markers of choice for the construction of genetic maps. Ten microsatellite markers were used in a 'Prima' x 'Fiesta' progeny of 152 seedlings to construct a first integrated linkage map (Maliepaard *et al.*, 1998). This map included four markers developed by Horticultural Research International, five markers by Guilford *et al.*, (1997) and one marker obtained from Hemmat *et al.*, (1997). Seventeen linkage groups, putatively corresponding to the seventeen haploid chromosomes, were aligned using 67 multi-allelic markers that were heterozygous for both 'Prima' and 'Fiesta'. Few RAPD and AFLP markers were used to saturate the map. This map is a resulted of the European Apple Genome Mapping Project (EAGMP) (King *et al.*, 1991) and map has been used as a reference map in other apple mapping projects in Europe such as Durable Apple Resistance (DARE) (Lespinasse and Durel, 1999; Lespinasse *et al.*, 2000) and High-Quality Disease Resistant Apples for a Sustainable Agriculture (HiDRAS) (Gianfranceschi and Soglio, 2004). Silfverberg-Dilworth *et al.*, (2006) mapped three hundred microsatellite markers. Hundred and forty eight of those were developed within the framework of the HiDRAS European project (Gianfranceschi and Soglios, 2004) and hundred and fifty-two were developed by Guilford *et al.*, (1997); Gianfranceschi *et al.*, (1998); Hokanson *et al.*, (1998); Liebhard *et al.*, (2002) and Vinatzer *et al.*, (2004), respectively. This is the most saturated map to date.

1.5.5 Apple powdery mildew resistance genes

Most of the major genes conferring resistance to mildew originate from wild *Malus* species. These genes have varying levels of resistance to powdery mildew.

1.5.5.1 *Pl-w*

The *Pl-w* gene is one of the major resistance genes for powdery mildew derived from the ornamental crab apple 'White Angel'. Isozymes *Acp-3, Aat-p* and *Lap-2* were the first reported markers linked to the *Pl-w* gene, with *Lap-2* being the closest (Hemmat *et al.* 1994; Janick *et al.* 1996; Gardiner *et al*, 2007). Maliepaard *et al.* (1998) mapped *Lap-2* on linkage group 8, suggesting the assignment of *Pl-w* to this linkage group (LG 8). It was demonstrated that *Pl-w* gene is flanked by microsatellites CH01e12 and CH05a02y. These markers map at positions 10 and 13 cM, respectively from *Pl-w* (James and Evans, 2004). Liebhard *et al.* (2002) assign both these microsatellites to linkage group 8 (LG 8). A bulked segregant analysis done by Evans and James (2003) identified two SCAR markers (EM M01 and EM M02). These markers map at 4.6 and 6.4 recombination units from *Pl-w*, respectively (see figure 1.11). Three NBS markers (NBS3M7; NBS2R1 and NBS2R14) displaying homologies with putative disease resistance proteins have also been mapped on linkage group 8 of the Discovery x TN10-8 population. These markers map at 1, 4 and 6 recombination units to the *Pl-w* gene, respectively (Calenge *et al.*, 2005a). Another disease resistance gene *Vfh* has been mapped on this linkage group, close to *Pl-w* gene (Durel *et al.*, 2006).

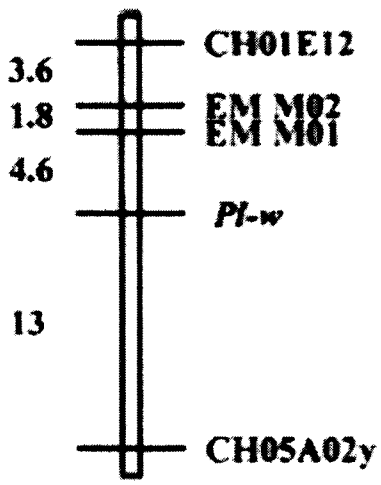


Figure 1.11: Map of a section of linkage group 8 (LG 8) showing recombination distances between EM M01, EM M02, the closest microsatellite markers and *Pl-w* (Evans and James, 2003).

1.5.5.2 *Pl-d*

The *Pl-d* gene is derived from *Malus* selection 'D12' (James *et al.*, 2004). The first markers linked to *Pl-d* gene were identified using bulked segregant analysis strategy (James *et al*, 2003). This consists of one RAPD and two AFLPs, of which one was successfully converted to a SCAR marker. The microsatellite markers CH03c02 and CH01d03 were flanking the *Pl-d* gene at 7 and 11 recombination units, respectively (see figure 1.12). The two more distant microsatellites (i.e. CH01d09 and CH01g12) placed *Pl-d* gene on the bottom of linkage group 12 in the published apple maps (Liebhard *et al.*, 2002; Silfverberg-Dilworth *et al.*, 2006). Other disease resistance genes such as *Vg* (Durel *et al.* 2000) and *Pl-1* (Dunemann *et al.*, 2007) have been mapped in this region. Two NBS markers (NBS2M14 and NBS3M11) have been mapped in this region in the

"Discovery" x TN10-8 mapping population (Calenge *et al.*, 2005).

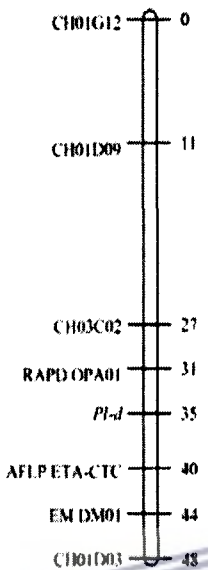


Figure 1.12: Linkage map of the *Pl-d* region on linkage group 12 (LG 12) in 'Fiesta' x A871-14 cross, showing the order of markers. Distances in centimorgans are indicated on the right (James *et al.*, 2004).

1.5.5.3 *Pl-1*

The *Pl-1* gene is derived from *M. robusta* (Evans and James, 2003). Markussen *et al.*, (1995) reported the $OPAT20_{450}$ SCAR marker to be closely linked to *Pl-1* gene. In a population derived from a cross X3191 x 'Novosibirski Sweet' OP, Dunemann *et al.*, (2004) verified that this marker maps at about 7 cM from the gene, and that SCAR marker AU-SCAR and CAPs marker AU-CAPs are more closely linked to *Pl-1* than the old SCAR marker ($OPAT20_{450}$). These markers map at 3-4 cM from *Pl-1*. Dunemann *et*

al., (2007) placed *Pl-1* at the bottom of linkage group 12, a region where other disease resistance genes, such as *Pl-d* (James *et al.*, 2004) and *Vg* (Durel *et al.*, 2000), have been mapped. This position was verified by the co-segregation of AT20-SCAR with the SSR Hi07f01, which is located at the distal end of LG 12 (Dunemann *et al.*, 2007).

1.5.5.4 *Pl-2*

The *Pl-2*, originating from *M. zumi*, is another major powdery mildew resistance gene mainly used in apple breeding programmes. Two major genomic regions containing genes controlling the resistance conferred by *Pl-2* in the Iduna x AA679-2 were detected through the use of the Quantitative Trait Loci (QTL) mapping approach (Seglias and Gessler, 1997). Dunemann *et al.* (1999) suggested a putative linkage between *Pl-2* and the *Pl-1* resistance because two primers that produced markers linked to the *Pl-1* gene also generated markers linked to the *Pl-1* gene. Two SCAR markers, N18-SCAR and U02-SCAR, flanked *Pl-2* at 8 and 7 cMs respectively (Gardiner *et al.*, 1999).

1.5.6 QTL mapping for mildew resistance in apple

Quantitatively inherited traits that are controlled by multiple loci are referred to as quantitative trait loci (QTL) (Young, 1996; Tanksley, 1993). QTL mapping is an approach used to identify chromosomal regions controlling these complex traits. The first QTL mapping involved fruit size, pH, and soluble solids in tomato (Paterson *et al.*, 1988), and has been subsequently used for disease resistance in tomato (Goldman *et al.*, 1995; Mangin *et al.*, 1999), maize (Krakowsky *et al.* 2004), rice (Zenbayashi *et al.*, 2002) and wheat (Otto *et al.*, 2002). The success of this QTL mapping depends on the DNA

markers, and the saturation of molecular maps with transportable markers will improve the power and accuracy of QTL mapping (Gardiner *et al.*, 2007, Xu, 1997). QTLs for different traits have been identified in apple. Using the reference genetic maps by Maliepaard *et al* (1998) and Liebhard *et al.*, (2003b), which are an essential foundation for QTL mapping, QTLs have been identified for several quantitative traits in apple. Durel *et al.*, (2003) mapped QTLs for resistance to apple scab, while Liebhard *et al.*, (2003a) identified quantitative physiological traits, such as stem diameter, height, leaf size, number of flowers, sugar content of fruit and fruit acidity. QTLs have also been found for fire blight resistance (Calenge *et al.*, 2004a, 2005b; Khan *et al.*, 2006). Stoeckli *et al.*, (2008) identified QTLs for resistance to *D. plantaginea* (rosy apple aphid) and *D. cf. devecta* (leaf-curling aphid) at linkage groups 17 and 7, respectively. Recently, QTL mapping has been used to analyze the inheritance of fruit quality traits within a population derived from a cross between the apple cultivars 'Telamon' and 'Braeburn' over two successive seasons. In this study, a total of 74 different QTLs were identified for all the major fruit physiological traits, which included fruit height, diameter, weight and stiffness, flesh firmness, rate of flesh browning, acidity, °Brix content and harvest date (Kenis *et al.*, 2008). Stankiewicz-Kosyl *et al.*, (2005) identified ten quantitative trait loci for powdery mildew resistance in 'U 211' and 'Idared' (figure 1.13). Five of the QTLs were associated with powdery mildew resistance in only one year. Locus U7, was associated with powdery mildew resistance, and explained 48 to 72% of the phenotypic variation. The effect of this locus (i.e. U7) was stable over three years in the nursery and a year in the orchard. This locus was localized on the linkage group G4 of the clone U

211 which corresponds to the linkage group 12 on the genetic map constructed on the 'Prima x Fiesta' progeny by Maliepaard *et al.*, (1998).

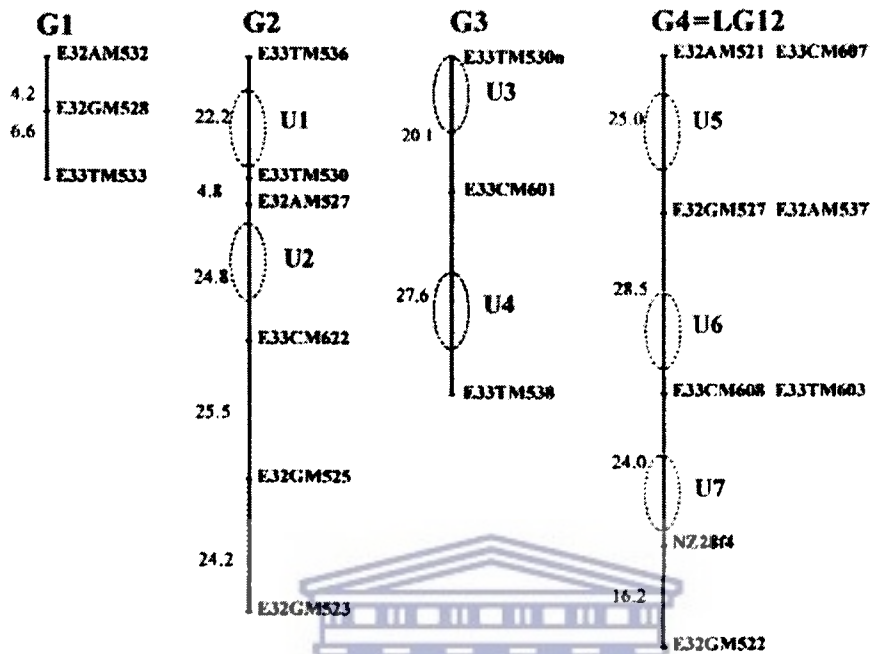


Figure 1.13: Genetic map of the clone U 211 genome sector. The numbers on the left of each linkage group indicate the distance in centimorgans. The ellipses indicate QTL localization (Stankiewicz-Kosyl *et al.*, 2005).

Both the apple powdery mildew resistance gene *Pl-d* and the gene *Vg* gene, which confers resistance to race 7 of *V. inaequalis*, were localized on this linkage group, suggesting the presence of resistance gene/QTL clusters (Gardiner *et al.*, 2007). A progeny of 149 seedlings from a 'Discover x TN10-8' cross was assessed over five seasons (Calenge and Durel, 2006), and the authors consistently identified two QTLs on linkage groups (LGs) 2 and 13. QTLs on LGs 2 and 8 mapped close to clusters of

resistance gene analogs (RGAs) and major genes for resistance to mildew or apple scab previously identified. These QTLs are of special interest for marker-assisted selection purposes. Other QTLs were identified on linkage groups 1 and 14 during one season, LG 10 for two seasons, and LGs 8, 17 for three seasons. Calenge and Durel, (2006) hypothesized that the instability of the QTLs on LGs 1, 8, 10, 14 and 17 could be appropriated to varying environmental conditions over the seasons. QTL-environment interactions have a significant impact during the analysis of polygenic traits (Mackay, 2004). Morpho-physiological status of the trees was hypothesized to be the cause of the QTLs instability. According to the authors, the instability of other QTLs over four seasons emphasizes the need to assess the powdery mildew incidence over more years than they did.

1.6 Objectives of the study

Recent advancements in fruit tree genomics have seen large numbers of expressed sequence tags (EST) sequences deposited on the public databases (Newcomb *et al.*, 2006). This offers opportunities for the identification and development of new SSR markers. In this study we report new co-dominant SSR markers developed by the Marker-Assisted Breeding group at the University of the Western Cape, South Africa (UWC-MAB). These markers were developed from the publicly available EST sequences. SSR markers from Guilford *et al.*, (1997), Gianfranceschi *et al.*, (1998), Liebhard *et al.*, (2002) and those developed within the framework of the European HiDRAS project (Silfverberg-Dilworth *et al.* 2006), were also used in the genetic analysis of a hybrid between 'African Carmine' and 'Simpson' provided by Dr

Labuschagné from ARC, Stellenbosch. The level of polymorphism detected by these SSR markers has been evaluated in the study. This study also seeks to demonstrate the significant role the multiplex PCR technique can play in large-scale SSR analysis in apple. This technique has been shown to play an important role as it helped to maximize throughput and reduce the costs involved in large-scale SSR analysis.

Specific aims of this study were:

- To develop new SSR markers
- To construct multiplex sets for high throughput genome analysis in apple
- To evaluate the potential of multiplex PCR technique to enhance efficiency in mass genotyping for crops of agronomic importance
- To test the SSR markers linked to apple powdery mildew resistance genes on the 'Carmine' x 'Simpson' hybrid.
- To construct a linkage map for linkage group 12 and for the entire genome.

2 Materials and Methods

2.1 List of chemicals and Suppliers

Agarose	Sigma
Ammonium persulphate (APS)	Merck
Bis-acrylamide (40% 19:1)	Promega
Boric acid	Saarchem
Bromophenol blue	Sigma
Buffer saturated phenol	Invitrogen
Chloroform	BDH
CTAB (N-acetyl-N-N-N-trimethyl ammonium bromide)	Saarchem
DTT (Dithiothreitol)	Roche
EDTA (Ethylene diamine tetra-acetic acid)	Merck
Ethanol	BDH
Ethidium bromide	Sigma
Formaldehyde (38%)	BDH
GeneScan™ 500 LIZ size standard	Applied Biosystems
Glycerol	Merck
Hi-Di™ Formamide	Applied Biosystems
Isoamyl alcohol	Merck
Isopropyl alcohol [isopropanol]	BDH
Magnesium chloride	Promega

Polyvinyl pyrolidone 40 (PVP-40)	Sigma
Performance OptimizedPolymer 4 (POP-4)	Applied Biosystems
Proteinase K	Roche
RNase A	Roche
Silver nitrate	Saarchem
Sodium acetate	Merck
Sodium borohydride ($NaBH_4$)	Separations
Sodium chloride (NaCl)	Merck
Sodium hydroxide	BDH
Spermidine (98% GC)	Sigma
TEMED (N, N, N, N-tetra methylethylene-diamine)	Promega
Tris (hrdromethyl) aminomethane	Merck
Urea	Merck
Xylene cyanol	BDH

2.2 General stock solutions

Polyacrylamide gel (6%)	6% [v/v] 19:1 acrylamide:bis-acrylamide , 7M urea, 10% [v/v] APS, 0.04 % [v/v] TEMED.
Agarose gel (0.8%)	0.8% [w/v] agarose in 1X TBE.
10% ammonium persulphate	10% [w/v] APS in deionised water.
2 X CTAB	2% [w/v] CTAB, 1% [w/v] PVP-40, 1.4 M NaCl, 0.1 M Tris-HCl, 0.02 M EDTA, 0.5 M DTT.

Chloroform-isoamylalcohol (CIA)	24:1 [v/v]
Silver nitrate developing solution	1.5% [w/v] NaOH, 0.01% [w/v] $NaBH_4$, 0.4% [v/v] formaldehyde (38%) in deionised water
DNA loading buffer (agarose)	0.25% [w/v] bromophenol blue, 0.25% [w/v] xylene cyanol, 30% [v/v] glycerol in deionised water.
DTT	1M DTT in water
Ethanol (80%)	80% [v/v] ethanol in deionized water
Formamide denaturing dye (95%)	0.05% bromophenol blue [w/v], 0.05% xylene cyanol [w/v], 10mM NaOH, 95% formamide [v/v]
Phenol-chloroform	1:1 [v/v]
RNase A buffer	0.1 M Sodium acetate, 0.3 M EDTA, pH 4.8
RNase A (DNase free)	20 mg/ml RNase A in RNase buffer
Silver nitrate staining solution	0.1% [w/v] $AgNO_3$ in deionised water
Sodium acetate	3 M NaOAc in deionized water, pH 5.5
10 X TBE	0.9 M Tris, 0.89 M boric acid, 0.3 M EDTA.
10 X TE	0.01 M EDTA, 0.1 M Tris-HCl, pH 7.5

2.3 Plant material

Four hundred and twenty-one seedlings derived from a cross between "Simpson" and "African Carmine" were produced at Bien Donné experimental farm, Agricultural Research Council Infruitec - Nietvoorbij, Stellenbosch, South Africa. A subgroup of ninety-seven individuals and the two parents "Simpson" and "Carmine" were used in this

study. The material for the cross was collected at Bien Donné and the material for all the apple parents was collected at Drosternes farm, Grabouw, South Africa. Table 2.1 shows a list of cultivars used in this study.

2.4 DNA isolation

DNA was isolated using a CTAB method of extracting DNA from plants (Doyle and Doyle, 1990) with modifications adopted from Porebski *et al.* (1997). The method is as follows:

Young leaves (approximately 0.5 g) were harvested and immediately frozen in liquid nitrogen, and stored at –80 °C until used. The leaf material was ground to fine powder in the presence of liquid nitrogen using mortar and pestle. 2ml of CTAB extraction buffer, pre-warmed at 60 °C, was added to the powder and homogenized gently. 1 ml of the homogenate was transferred into 2 ml tube and incubated at 62 °C for 30 minutes. Proteinase K (at final concentration of 10 ug/ml) was added and incubated at 37 °C for 30 minutes. An equal volume of chloroform: isoamyl alcohol [24:1 (v/v)] was added. The sample was inverted several times and centrifuged at 10 000 x g for 15 minutes to separate the phases. The upper aqueous layer was carefully transferred to a fresh tube. The DNA was precipitated with 2/3 volume of ice-cold isopropanol, and stored at –20 °C for 20 minutes. The precipitated DNA was treated with RNase A (10 mg/ml - DNase free). An equal volume of phenol: chloroform [1:1 (v/v)] was added, mixed briefly and centrifuged at 10 000 x g for 15 minutes. 0.1 volumes of 3 M sodium acetate and 2.5 volumes of ice-cold absolute ethanol were added to the upper aqueous solution. The

Table 2.1: Apple cultivars

Designated number	Cultivar	Phenotypic rating to powdery mildew
8	Priscilla	Moderately resistant
11	Malus Spectabilis	-
25	Austin	-
26	Golden Hornet	Resistant
30	Co-op 22	-
31	Starking	-
33	Nothern Spy	Susceptible
43	Liberty	Resistant
44	Braeburn	Susceptible
54	Cox Orange	-
64	Mildew Resistant	Resistant
68	Golden Delicious	Highly susceptible
69	Russian Seedling	-
77	Prima	Resistant
80	Lady Williams	-
-	Sharps Early	-
83	Summerking Red	-
106	Simpson*	Presumably resistant
119	Anna	-
123	Pink Lady	Resistant
-	Carmine*	Susceptible

* Parents used to derive the mapping population 'Simpson' x 'Carmine'

mixture was left at –20 °C for more than an hour to precipitate the DNA. The samples were centrifuged at 10 000 x g for 15 minutes to recover the DNA. The pellet was washed twice with 80 % ethanol and air-dried at 37 °C. The pellet was then re-suspended in 100 µl of TE buffer and stored at -20 °C until used.

2.5 Genomic DNA quantification

DNA concentration was determined by spectrophotometric measurement at 260 nm using Nanodrop™ ND-1000 Spectrophotometer.

2.6 Gel electrophoresis

2.6.1 Agarose gel electrophoresis

The DNA samples were separated on 0.8 % agarose gel containing 0.5 µg/ml ethidium bromide. The DNA sample was mixed with BPB-loading buffer and eletrophoresed in 1 X TBE at 10 V/cm for 1 hour. The DNA was then visualized on a UV trans-illuminator and images were captured using UVP image capture system.

2.6.2 Polyacrylamide gel electrophoresis

PCR products were separated using the Hoefer Mighty Small II SE 250 Mini-Vertical Electrophoresis unit. The samples were mixed with 2.5 volume of the formamide loading dye and denatured at 95 °C for 3-5 minutes and chilled on ice. The samples were loaded on a 6 % polyacrylamide gel (see section 2.2) and then electrophoresed in 1 X TBE at 10 V/cm for an hour. Following the electrophoresis, the gels were stained in silver nitrate

for 10 minutes and de-stained in water. The gel was further stained in developing solution (prepared as in section 2.2) until the bands were clearly developed.

2.7 Primer design, synthesis and dye labeling

2.7.1 Microsatellite markers from the literature

Apple SSR markers with known map positions were selected from Liebhard *et al.* (2002) and Silfverberg-Dilworth *et al.* (2006). These were selected on the basis of the number of alleles detected and map position.

2.7.2 Microsatellites from public EST sequences

Apple EST sequences were mainly acquired from GenBank, which is maintained at the National Center for Biotechnology Information (Bethesda, Md.) and is available at http://www.ncbi.nlm.nih.gov. These sequences were analyzed for SSR repeats using the Tandem Repeat Finder software (Benson, 1999) at http://www.tandem.bu.edu. Primer sets flanking the SSR repeat were designed from the EST sequences that contained the SSR repeats. The flanking primers were selected on the basis that they were located in positions where it was possible to design both the forward and reverse PCR primers and the ideal annealing temperature was 60 °C.

Oligonucleotides were commercially synthesized and fluorescently labeled at the 5' end with 6-FAM, NED, PET and VIC fluorophores (Applied Biosystems, South Africa).

2.8 Classification or Nomenclature of markers

The markers prefixed with **CH**, **MS**, **NZ** and **COL**, were published by Liebhard *et al.* (2002), and markers prefixed with **Hi** were published by Silfverberg-Dilworth *et al.*

(2006). The prefixes **CO**, **CN**, **CV** coupled with the GenBank accession numbers, were used for new markers developed from the apple sequence expressed tags.

2.9 Microsatellite PCR amplification

2.9.1 Standard PCR Components

The standard PCR amplifications were performed in a 25 µl volume containing 1 X PCR buffer, 2 mM $MgCl_2$, 50 µM of each dNTP, 1 unit of *Taq* DNA polymerase (Biotech lab, UWC), 0.016 ρmole of each primer (forward and reverse), 0.25 mM spermidine and 20 to 50 ng of genomic DNA. The PCR amplifications were performed as described in sections 2.9.2 (page 35), 2.9.3 (page 36) and 2.9.4 (page 36). PCR amplicons were analyzed on a 6% polyacrylamide gel as described in section 2.6.2.

2.9.2 Gradient PCR

The gradient PCR was performed on the Master Cycler Gradient (Eppendorf). The cycling conditions for each heating block were as follows: an initial denaturation step of 94 °C for 5 minutes followed by a 35 cycles of: 94 °C for 40 seconds (denaturation), a final extension cycle of 72 °C for 10 minutes followed by a holding step of 4 °C. The primers were annealed for 35 cycles for 40 seconds at various temperatures as shown in the table below:

Table 2.2: Annealing temperature profile for Gradient PCR

Heating block	1	2	3	4	5
Temperature (°C)	50.6	55.8	58.5	61.4	64.2

2.9.3 Touchdown PCR

Fragments were amplified using the following two touchdown PCR programs (i.e. 60-50 °C and 65-55 °C). Both programs had the initial denaturation step of 94 °C for 5 minutes. This step was followed by a first stage characterized by a stepwise lowering (-1 °C/cycle) of the annealing temperature for 10 cycles. This stage was then followed by a 25-cycle stage in which the annealing temperature remained constant. A final extension step of 72°C for 10 minutes was performed after stage two. Amplifications were carried out using both the GeneAmp® 2700 and GeneAmp® 9700 cycler (Applied Biosystems).

2.9.4 Multiplex PCR

Multiplex PCR was performed as in section 2.9.1, except that the number of primers per reaction increased. The amplification profile was designed as follows:

Table 2.3: Multiplex PCR cycling conditions

Hold **Temp - Time**	**Cycling Conditions** **Temp - Time**	**Number of Cycles**
94°C – 5 min	-	-
-	94 °C – 40 sec	40
-	55 °C – 1 minute	40
-	65 °C – 2 minutes	40
65°C – 30 min	-	-
4°C ~	-	-

2.10 Fragment analysis and allele scoring

Fragment analysis for each SSR marker was performed on an ABI 310 PRISM Genetic Analyzer (Applied Biosystems). Prior to analysis, PCR products were mixed with 5 volumes of deionised formamide and 0.1 volumes of GENESCAN™ 500-LIZ size standard (Applied Biosystems), then denatured at 95°C for 10 minutes and snap-cooled on ice. The samples were then analyzed by capillary electrophoresis according to the manufactures instructions. The fragment sizing was performed with the GeneScan™ analysis software version 3.1.2, and alleles were scored using Genotyper™ software version 2.5 according to the users manual (Applied Biosystems).

2.11 Linkage analysis

Linkage analysis was performed on 87 seedlings of "Carmine" x "Simpson" population and genetic maps (i.e. for the whole genome and for LG 12) were constructed using

JoinMap 3.0 software (http://www.kyazma.nl) (Van Ooijen and Voorrips 2001), applying the Kosambi mapping function. LOD score of 4.0 was used to group markers belonging to the same linkage group.

2.12 Disease assessment

The seedlings were derived from a cross between two diploid apple varieties – "Simpson", which is a crab-apple and "African Carmine", which is a commercial variety. The assessment of mildew infection on the seedlings was done at Bien Donné experimental station of the Agricultural Research Council, Stellenbosch, South Africa. The progeny seedlings were subjected to artificial infection in seedling trays in the first year of growth (i.e. 2001). The seedlings were then planted in the bags and were exposed to natural infection for two seasons (i.e. 2002-2003). These seedlings were exposed to direct sunlight for one season (i.e. 2002) and under shade netting for another season (i.e. 2003). Seedlings were then budded in M25 rootstocks and planted for one season (i.e. 2004). Based on the severity of the disease, seedlings were classified and scored on a 1-4 scale rating (see Table 2.4) (Kruger (1994). The disease incidence, referred as the degree of infection, was quantified using the formula: Percentage infection = Number of infected leaves / Total number of leaves x 100. The leaves that had a disease incidence of 30% or more were classified as susceptible (with leaves over 70% classified as highly susceptible) and the leaves with disease incidence of 29% or less (with no leaves over 29%) were classified as resistant (classes 1 and 2).

Table 2.4: Scales at which powdery mildew was assessed in the greenhouse and nets

Class	Description	Definition	Infection Percentage
1	No infection	Resistant	Zero
2	Slight infection	Moderately resistant	Less than 30%
3	Moderate infection	Susceptible	Less than 70%
4	Severe infection	Highly susceptible	Above 70%

3 Phenotypic assessment for mildew infection

3.1 Introduction

Apple powdery mildew, caused by *Podosphaera leucotricha* is one of the major diseases of cultivated apple in South Africa. The successful management program for this disease depends on the accurate, reproducible disease assessment. This involves artificial inoculation of plants with the pathogen inoculum, followed by exposing plants to natural mildew infection. The plant's response to the disease is then assessed for both glasshouse and field evaluations. Usually, this is done by measuring disease incidence or severity. Disease incidence refers to the relative area of the diseased plant tissue, while disease severity refers to the proportion of the plant that is diseased relative to the total area (Xu and Madden, 2002).

The aim of this section was to evaluate the response of all progenies to mildew infection under different environments.

3.2 Disease assessment

Seedlings were artificially infected in the first season (i.e. 2001) and left to natural infection in the second year (i.e. 2002). Most of the seedlings were scored for all the four seasons; but some were scored for three or two or one, and others died before they were evaluated. The disease severity ranged from no visible symptoms (class 1) to complete coverage of leaves (class 4) (see Table 3.1).

Artificial infection in the greenhouse (i.e. 2001) led to high mildew infection, which yielded high proportion of susceptible seedlings (i.e. about 74% of seedlings belonged to

Table 3.1: Quantification of powdery mildew over four seasons.

Seedlings	Year 2001 (Greenhouse)		Year 2002 (Sunlight)		Year 2003 (Shade Nets)		Year 2004 (M25 Rootstocks)	
	Infection %	Class	Infection %	Class	Infection %	Class	Infection %	Class
C1-19	52	3	0	1	-	-	-	-
E12-19	0.00	1	-	-	37	3	57	3
A6-21	52	3	0.00	1	-	-	-	-
E13-18	45	3	100	4	58	3	100	4
C9-21	48	3	31	3	100	4	-	-
A3-10	0.00	1	-	-	31	3	58	3
F9-18	92	4	45	3	-	-	-	-
D8-20	100	4	8	2	40	3	-	-
F12-10	100	4	-	-	69	3	74	4
F6-20	33	3	0.00	1	11	2	-	-
G5-10	0.00	1	-	-	15	2	51	3
A10-20	66	3	100	4	70	4	-	-
E13-10	82	4	33	3	20	2	-	-
G4-18	75	4	100	4	84	4	-	-
G12-18	86	4	44	3	42	3	53	3
E1-10	73	4	45	3	100	4	-	-
F2-19	9	2	-	1	60	3	28	2
D5-20	60	3	34	3	44	3	65	3
G14-20	66	3	10	2	18	2	67	3
B3-10	25	2	-	-	75	4	72	4
B11-21	97	4	44	3	84	4	-	-
E1-19	8	2	0.00	1	26	2	38	3
F2-18	30	3	7	2	89	4	38	3
D2-19	-	-	-	-	52	3	48	3

Table 3.1 (Continued)

Seedlings	Year 2001 (Greenhouse)		Year 2002 (Sunlight)		Year 2003 (Shade Nets)		Year 2004 (M25 Rootstock)	
	Infection %	Class	Infection %	Class	Infection %	Class	Infection %	Class
A3-10	-	-	-	-	31	3	58	3
D7-18	46	3	-	-	-	-	-	-
E7-10	-	-	-	-	36	3	-	-
C5-20	41	3	0.00	1	77	4	-	-
D6-20	-	-	-	-	-	-	-	-
D3-10	32	3	0.00	1	30	3	31	3
A6-20	28	3	-	-	29	2	55	3
D14-18	-	-	-	-	28	2	-	-
B3-20	79	4	0.00	1	90	4	-	-
G3-21	45	3	6	2	65	3	-	-
G7-20	70	4	0.00	1	46	3	-	-
D3-20	100	4	0.00	1	36	3	47	3
D13-19	-	-	-	-	64	3	-	-
G11-10	100	4	23	2	38	3	76	4
C6-10	82	4	-	-	38	-	-	-
A9-10	29	2	0.00	1	34	3	-	-
A7-20	36	3	100	4	100	4	-	-
E10-10	40	3	-	-	44	3	61	3
A13-19	60	3	11	2	39	3	45	3
E8-21	100	4	-	-	46	3	76	4
A13-20	41	3	23	2	60	3	58	3
D9-18	-	-	-	-	33	3	37	3
D10-20	41	3	0.00	1	15	2	57	-
D14-19	34	3	0.00	1	64	3	61	3
G1-19	34	3	15	2	34	3	48	3
C14-20	56	3	0.00	1	30	3	38	3

Table 3.1 (Continued)

Seedlings	Year 2001 (Greenhouse)		Year 2002 (Sunlight)		Year 2003 (Shade Nets)		2004 (M25 Rootstock)	
	Infection %	Class	Infection %	Class	Infection %	Class	Infection %	Class
E6-20	37.50	3	70	4	51	3	66	3
C1-21	100	4	36	3	41	3	-	-
E12-19	-	-	-	-	37	3	57	3
B11-20	100	4	22	2	100	4	-	-
A1-20	64	3	0.00	1	32	3	-	-
G6-21	100	4	100	4	65	4	-	-
B12-20	100	4	25	2	5	2	-	-
B14-20	53	3	15	2	39	3	69	4
F12-21	57	3	100	4	72	4	-	-
D1-19	-	-	-	-	48	3	33	3
G6-19	15	2	0.00	1	18	2	5.00	2
C1-18	30	3	0.00	1	58	3	-	-
C11-21	50	3	31	3	54	3	100	4
B13-21	95	4	-	-	-	-	-	-
A10-19	66	4	0.00	1	21	2	100	4
E12-18	100	4	5	2	25	2	45	3
B11-18	65	4	100	4	67	4	-	-
E2-10	20	2	0.00	1	59	3	57	3
C2-10	53	3	100	4	100	4	92	4
B14-19	18	2	0.00	1	63	3	81	4
E10-21	81	4	28	2	3	2	61	3
B12-21	44	3	57	3	50	3	46	3
A5-19	60	3	20	2	54	3	71	4
D12-18	26	2	80	4	76	4	-	-
A5-19	60	3	20	2	54	3	71	4
B13-20	100	4	83	4	74	4	95	4

Table 3.1 (Continued)

Seedlings	Year 2001 (Greenhouse)		Year 2002 (Sunlight)		Year 2003 (Shade Nets)		Year 2004 (M25 Rootstock)	
	Infection %	Class	Infection %	Class	Infection %	Class	Infection %	Class
F12-19	40	3	-	-	67	3	48	3
E11-19	59	3	0.00	1	60	3	61	3
B4-21	45	3	0.00	1	24	2	71	4
A12-10	52	3	-	-	23	2	81	4
A11-10	13	2	0.00	1	70	4	-	-
G4-19	59	3	0.00	1	47	3	81	4
C8-18	-	-	-	-	23	2	100	4
C1-10	100	4	11	2	83	4	70	4
G4-21	30	3	10	2	44	3	62	3
A8-10	7	2	0.00	1	34	3	42	3
G12-19	80	4	0.00	1	50	3	77	4

Dashes represent seedlings that were not scored or dead seedlings.

Table 3.1: Quantification of powdery mildew over four seasons.

Seedlings	Year 2001 (Greenhouse)		Year 2002 (Sunlight)		Year 2003 (Shade Nets)		Year 2004 (M25 Rootstocks)	
	Infection %	Class	Infection %	Class	Infection %	Class	Infection %	Class
C1-19	52	3	0	1	-	-	-	-
E12-19	0.00	1	-	-	37	3	57	3
A6-21	52	3	0.00	1	-	-	-	-
E13-18	45	3	100	4	58	3	100	4
C9-21	48	3	31	3	100	4	-	-
A3-10	0.00	1	-	-	31	3	58	3
F9-18	92	4	45	3	-	-	-	-
D8-20	100	4	8	2	40	3	-	-
F12-10	100	4	-	-	69	3	74	4
F6-20	33	3	0.00	1	11	2	-	-
G5-10	0.00	1	-	-	15	2	51	3
A10-20	66	3	100	4	70	4	-	-
E13-10	82	4	33	3	20	2	-	-
G4-18	75	4	100	4	84	4	-	-
G12-18	86	4	44	3	42	3	53	3
E1-10	73	4	45	3	100	4	-	-
F2-19	9	2	-	1	60	3	28	2
D5-20	60	3	34	3	44	3	65	3
G14-20	66	3	10	2	18	2	67	3
B3-10	25	2	-	-	75	4	72	4
B11-21	97	4	44	3	84	4	-	-
E1-19	8	2	0.00	1	26	2	38	3
F2-18	30	3	7	2	89	4	38	3
D2-19	-	-	-	-	52	3	48	3

Table 3.1 (Continued)

Seedlings	Year 2001 (Greenhouse)		Year 2002 (Sunlight)		Year 2003 (Shade Nets)		Year 2004 (M25 Rootstock)	
	Infection %	Class	Infection %	Class	Infection %	Class	Infection %	Class
A3-10	-	-	-	-	31	3	58	3
D7-18	46	3	-	-	-	-	-	-
E7-10	-	-	-	-	36	3	-	-
C5-20	41	3	0.00	1	77	4	-	-
D6-20	-	-	-	-	-	-	-	-
D3-10	32	3	0.00	1	30	3	31	3
A6-20	28	3	-	-	29	2	55	3
D14-18	-	-	-	-	28	2	-	-
B3-20	79	4	0.00	1	90	4	-	-
G3-21	45	3	6	2	65	3	-	-
G7-20	70	4	0.00	1	46	3	-	-
D3-20	100	4	0.00	1	36	3	47	3
D13-19	-	-	-	-	64	3	-	-
G11-10	100	4	23	2	38	3	76	4
C6-10	82	4	-	-	38	-	-	-
A9-10	29	2	0.00	1	34	3	-	-
A7-20	36	3	100	4	100	4	-	-
E10-10	40	3	-	-	44	3	61	3
A13-19	60	3	11	2	39	3	45	3
E8-21	100	4	-	-	46	3	76	4
A13-20	41	3	23	2	60	3	58	3
D9-18	-	-	-	-	33	3	37	3
D10-20	41	3	0.00	1	15	2	57	-
D14-19	34	3	0.00	1	64	3	61	3
G1-19	34	3	15	2	34	3	48	3
C14-20	56	3	0.00	1	30	3	38	3

Table 3.1 (Continued)

Seedlings	Year 2001 (Greenhouse)		Year 2002 (Sunlight)		Year 2003 (Shade Nets)		2004 (M25 Rootstock)	
	Infection %	Class	Infection %	Class	Infection %	Class	Infection %	Class
E6-20	37.50	3	70	4	51	3	66	3
C1-21	100	4	36	3	41	3	-	-
E12-19	-	-	-	-	37	3	57	3
B11-20	100	4	22	2	100	4	-	-
A1-20	64	3	0.00	1	32	3	-	-
G6-21	100	4	100	4	65	4	-	-
B12-20	100	4	25	2	5	2	-	-
B14-20	53	3	15	2	39	3	69	4
F12-21	57	3	100	4	72	4	-	-
D1-19	-	-	-	-	48	3	33	3
G6-19	15	2	0.00	1	18	2	5.00	2
C1-18	30	3	0.00	1	58	3	-	-
C11-21	50	3	31	3	54	3	100	4
B13-21	95	4	-	-	-	-	-	-
A10-19	66	4	0.00	1	21	2	100	4
E12-18	100	4	5	2	25	2	45	3
B11-18	65	4	100	4	67	4	-	-
E2-10	20	2	0.00	1	59	3	57	3
C2-10	53	3	100	4	100	4	92	4
B14-19	18	2	0.00	1	63	3	81	4
E10-21	81	4	28	2	3	2	61	3
B12-21	44	3	57	3	50	3	46	3
A5-19	60	3	20	2	54	3	71	4
D12-18	26	2	80	4	76	4	-	-
A5-19	60	3	20	2	54	3	71	4
B13-20	100	4	83	4	74	4	95	4

Table 3.1 (Continued)

Seedlings	Year 2001 (Greenhouse)		Year 2002 (Sunlight)		Year 2003 (Shade Nets)		Year 2004 (M25 Rootstock)	
	Infection %	Class	Infection %	Class	Infection %	Class	Infection %	Class
F12-19	40	3	-	-	67	3	48	3
E11-19	59	3	0.00	1	60	3	61	3
B4-21	45	3	0.00	1	24	2	71	4
A12-10	52	3	-	-	23	2	81	4
A11-10	13	2	0.00	1	70	4	-	-
G4-19	59	3	0.00	1	47	3	81	4
C8-18	-	-	-	-	23	2	100	4
C1-10	100	4	11	2	83	4	70	4
G4-21	30	3	10	2	44	3	62	3
A8-10	7	2	0.00	1	34	3	42	3
G12-19	80	4	0.00	1	50	3	77	4

Dashes represent seedlings that were not scored or dead seedlings.

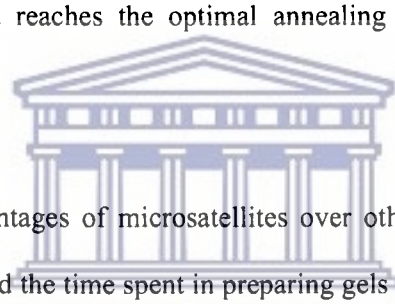
classes 3 and 4). In the 2002 season, seedlings that were susceptible (i.e. classes 3 and 4) in the previous season were free of symptoms or slightly infected. About 39% (i.e. 34) of the seedlings that were susceptible in the first year became resistant in the second year. Only one seedling changed the other way round. In 2003, only sixteen seedlings exhibited a slight immunity to powdery mildew. Sixty-four seedlings (~74%) were susceptible or highly susceptible to mildew, an increase from the previous year.

During the evaluation for the 2004 growing season, there was no significant change in the status of the seedlings from the previous year. Majority of seedlings were still susceptible to the disease. In this season, thirty-five (about 40%) seedlings, as indicated by the dashes in Table 3.1, died prior to evaluations.

4 PCR optimization and multiplexing of microsatellite loci

4.1 Introduction

Poor or non-specific amplification is one common problem encountered during microsatellite amplification. This necessitates the optimization of polymerase chain reaction (PCR) conditions to improve the quality of amplification of different loci. The "touchdown" thermo-cycling PCR program was designed to counter such non-specific amplifications (Don *et al.*, 1991; Senior *et al.*, 1996). This PCR program is a step-wise method of approaching optimal amplification. The annealing temperature (T_m) is selected to be slightly above the estimated temperature. As the reaction continues, the temperature gradually decreases until it reaches the optimal annealing temperature (Roux *et al.*, 1995).



Despite the numerous advantages of microsatellites over other markers, the high costs required for consumables and the time spent in preparing gels and scoring alleles for PCR reactions of an individual marker poses serious economic challenges, especially in large-scale SSR analysis. There is a need for approaches that will help maximize throughput and thereby reduce the costs and time spent in large-scale analysis. The simultaneous amplification of several markers in a single PCR (multiplexing) is an approach developed to counter these economic challenges (Masi *et al.*, 2003; James *et al.*, 2000). To avoid the overlapping of amplicons amplified by each marker, prior knowledge of allele size ranges for each marker is necessary. This knowledge is a prerequisite for successful application of multiplex PCR technique in large-scale SSR analysis.

The objectives of this section were: 1) to find optimal PCR conditions for the amplification of different loci, 2) to evaluate the level of polymorphism detected, and 3) to develop multiplex sets for large-scale genotyping in apple.

4.2 Simplex PCR optimization

4.2.1 Primer concentration

Primer concentration of 0.4 pmol/µl was used in most of the PCR reactions. This proved to be the optimal concentration for most of the markers. There were few instances of poor amplification or no amplification at all, and in such instances a primer concentration of 1 pmol/µl was used and it proved effective. This increment improved amplification significantly. However, at certain instances the improvement of amplification was accompanied by an increase in stutter bands.

4.2.2 Temperature gradient

Gradient PCR was used to determine optimal annealing temperature for each individual marker. Optimal annealing temperature was defined as the temperature, which resulted in strong intense bands (assumed to be the desired amplicons); with few or no stutter bands. Figure 4.1 shows the temperature gradient profile of marker DT0418361, using DNA sample from the cultivar *M. domestica* cv. "African Carmine". This primer pair was expected to amplify a 323 bp fragment. As shown in Figure 4.1, all the five temperature profiles amplified the target amplicon (indicated by arrow A). At lower annealing temperatures 50.6°C and 55.8°C (lanes 2 and 3), an extra band around 500 bp (indicated by the oval-shaped circles) was observed. At higher temperatures (i.e. 58.5°C, 61.4°C

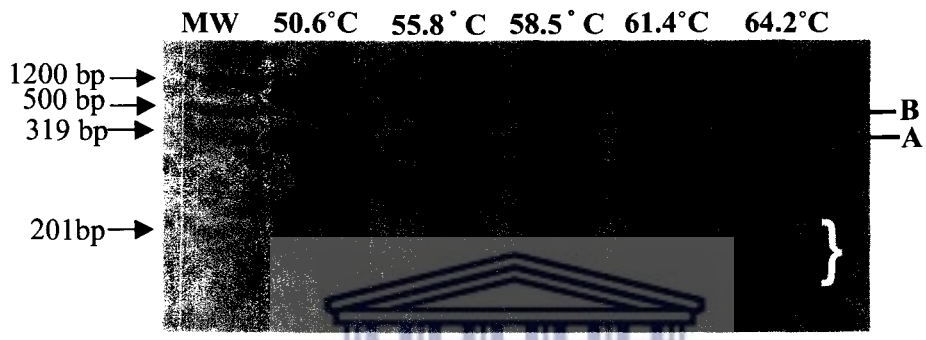


Figure 4.1 Gradient PCR profile of marker DT041836l separated by polyacrylamide gel electrophoresis 6% denaturing gel. The oval-shaped circles indicate the non-specific fragments. Arrows **A** and **B** are presumed to be the PCR products representing the two alleles amplified by this marker. The right brace indicates smearing beneath the target allele.

and 64.2°C (lanes 4-6)), this band disappeared, while a band just above the target amplicon(indicated by arrow B), presumed to be the second allele detected by this marker, intensified. At temperature 64.2°C (lane 6), this band became clearly visible, but the increased signal strength was accompanied by an increase of smear below the target amplicon (indicated by a right brace in the gel). Temperature 64.2°C was therefore considered to be the optimal for this particular marker. Overall, with few exceptions such as DT0418361, optimal annealing temperatures for most of the SSR primers pairs ranged from 50°C to 60°C.

4.2.3 Touchdown PCR

Since the annealing temperatures of the SSR primers ranged from 50°C to 60°C, touchdown 60-50°C program was used to achieve optimal amplifications. However, in some instances where there was excess smearing (as shown in figure 4.2), touchdown 65-55°C was used. Figure 4.2 shows the effects of different PCR amplification programs (i.e. touchdown 60-50°C and 65-55°C) during the analysis of SSR-CH01d03 across nine apple cultivars. This marker gave PCR products of high intensity. However, artefactual stutter fragments, below 130 bp (shown by the braces in figure 4.2A), accompanied the amplification of the alleles. As shown in figure 4.2B, touchdown program (65-55°C) reduced the stutter fragments for some of the cultivars, in particular Priscilla, Golden Hornet and Liberty.

Overall, ninety-nine primer pairs were examined in this study. Of the ninety-nine primer pairs, sixty-seven produced fragments of the expected size across selected apple cultivars, and sevnteen did not amplify any genomic DNA. Ten amplified products with complex

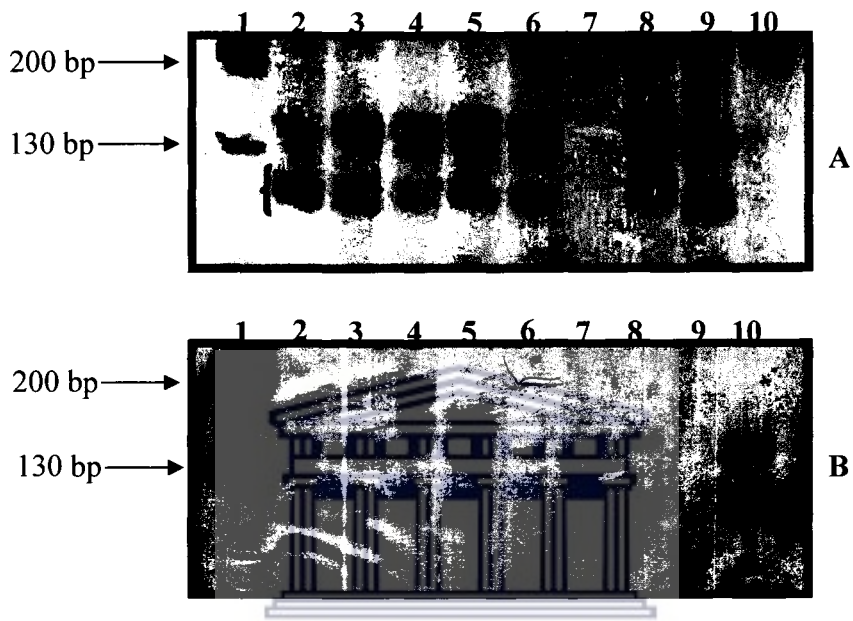


Figure 4.2 PCR fingerprints of marker CH01d03 on a 6% denaturing polyacrylamide gel stained in silver nitrate. **A:** Touchdown program (60-50°C) and **B:** Touchdown program (65-55°C). **Lanes 1:** Molecular weight marker; **Lane 2:** Priscilla; **Lane 3:** Golden Hornet; **Lane 4:** Co-op 22; **Lane 5:** Starking; **Lane 6:** Liberty; **Lane 7:** Cox; **Lane 8:** Golden Delicious; **Lane 9:** Carmine and **Lane 10:** Simpson, respectively. The braces indicate the stutter fragments (present in all the lanes).

banding patterns, and five primer pairs amplified fragments larger than the expected size. These primer pairs were excluded from the analysis because they were difficult or could not be analyzed on the ABI Genetic Analyzer.

4.2.4 Allele scoring and detection of polymorphism

PCR fragments for each marker were detected using the GeneScan™ analysis software version 3.1.2, and alleles were scored using Genotyper™ software version 2.5. Most domesticated apples are known to functional diploids ($2n$ = 34) (Janick *et al.*, 1996), therefore no more than two alleles would be expected to be identified in each of the selected cultivars. Most of the functional SSR markers showed two visible peaks for individual genotypes. These peak patterns represented two distinct alleles detected by the markers. Therefore, the detection of two distinct alleles in this study was in accordance with the amplification of a single locus from a species such as an apple. When only one peak was scored, it was assumed that the genotype was homozygous at that particular locus. An example illustrating the allelic status (i.e. homozygous or heterozygous) of SSR markers is shown in Figure 4.3. During fragment analysis, 'pull-up peaks' (peaks with intense fluorescent signals) would be mistakenly labeled as alleles, and 'weak peaks' (peaks with weak fluorescent signals) would get lost in the background signal. The presence of such artefactual stutter peaks was another drawback experienced during allele scoring. These artefacts made it difficult to discriminate between alleles that differed by two base pairs. This problem was especially common when SSR markers with di-nuleotide motifs were amplified as these stutter peaks were less prevalent when SSR repeats of higher order (i.e. tri- and tetra-nucleotides) were used.

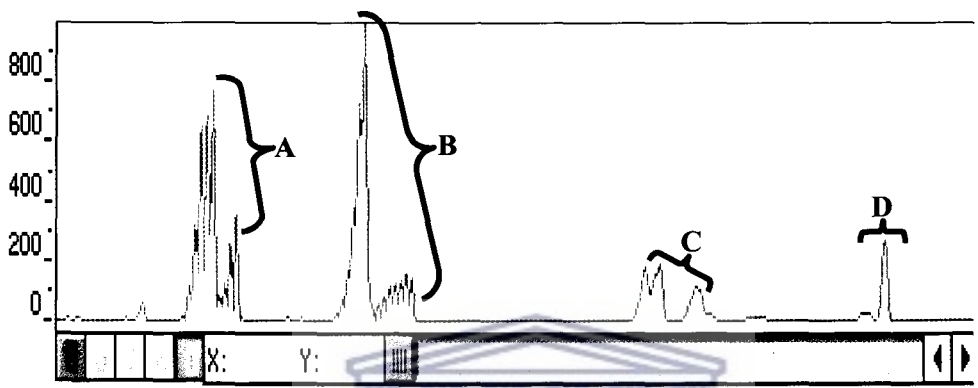


Figure 4.3 Electropherogram of SSR markers on apple cultivar "Simpson". **A-C**: represent heterozygous alleles at loci CH04e03, CH03d08, CN580620, respectively. **D:** indicates a homozygous allele at locus CV627191.

During SSR amplification, an extra non-template 'A' (adenosine) is added to the 3′ end of the PCR products. *Taq* DNA polymerase is known to catalyze this inconsistent addition (Smith *et al.*, 1995). This phenomenon also made it difficult to discriminate between alleles that differed by two base pairs and often led to inconsistent allele calling. Instead of employing the post-PCR treatment of amplicons with T4 polymerase, which is known to remove the non-template adenosine as suggested by Ginot *et al.*, (1996), PCR cycling conditions were modified. This approach proved to be effective in eliminating this problem for some primer pairs while others were refractory to such optimizations.

The level of polymorphism for each marker was determined by evaluating the allelic composition of nine apple cultivars differing in phenotypic characteristics and parentage to the mapping populations under study. Markers that showed polymorphism across the selected nine apple cultivars were further used to analyze the remaining apple cultivars. This was done to render a broad estimate of the allele size range for each marker. With the exception of CN870040, CN909118, CN880881, CN944444, AJ001681 and CN491050, all other markers were polymorphic, detecting two to fourteen distinguishable alleles per locus within the set of twenty-one cultivars. Figure 4.4 shows the allele patterns for marker CH01f03b in the seventeen apple cultivars. This marker amplified a total of fourteen distinguishable alleles (indicated by the arrows in the figure). Table 4.1 gives the summary of all the polymorphic markers optimized in this study.

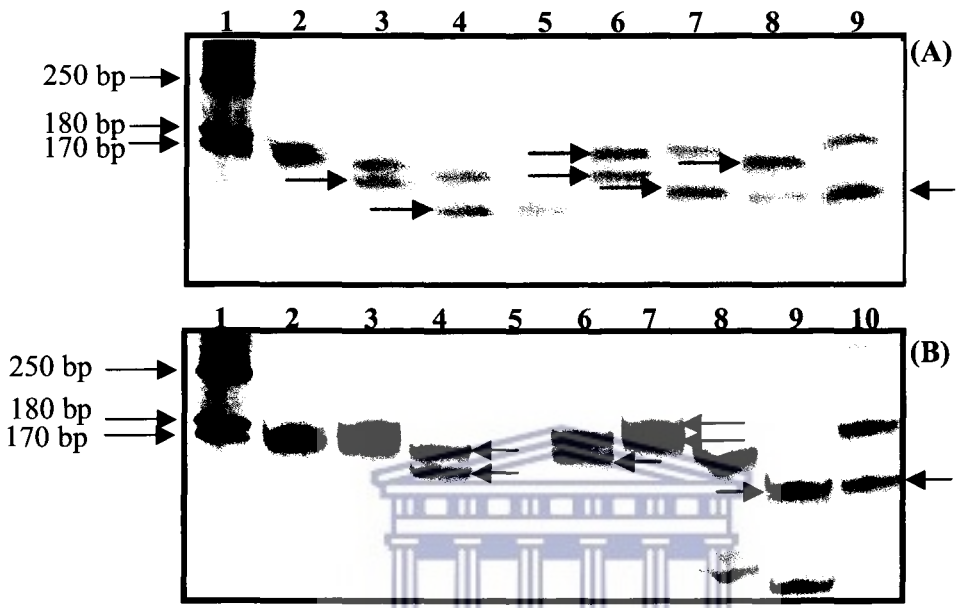


Figure 4.4 Allele patterns of the SSR marker CH01f03 across the seventeen apple genotypes on 6% polyacrylamide gels stained with silver nitrate. (A) **Lane 1: -** Molecular weight marker; **Lane 2:** Braeburn; **Lane 3:** Russian Seedling; **Lane 4:** Prima; **Lane 5:** Lady Williams; **Lane 6:** Sharps Early; **Lane 7:** Summerking; **Lane 8:** Anna; **Lane 9:** Pink Lady, respectively. (B) **Lane 1: -** Molecular weight marker; **Lane 2:** Priscilla; **Lane 3:** Austin; **Lane 4:** Golden Hornet; **Lane 5:** Co-op 22; **Lane 6:** Starking; **Lane 7:** Liberty; **Lane 8:** Cox Orange; **Lane 9:** Carmine; **Lane 10:** Golden Delicious, respectively. The arrows represent different alleles amplified by this marker.

Table 4.1 SSR name, primer sequences, marker type (perfect, imperfect and compound), repeat type (di-, tri- and tetra-nucleotide), allele size range, alleles detected, type of marker (presumed or confirmed single or multi locus) and linkage groups.

SSR marker	Primer sequence (5'- 3')	Marker type	Repeat Type	Size Range	No. of alleles	Type of marker	Linkage group
CH02d08	tcc aaa atg gcg tac ctc tc gca gac act cac tca cta tct ctc	Perfect	Dinucleotide	210-254	7	Single locus	11
CH02b03	ata agg ata caa aaa ccc tac aca g gac atg ttt ggt tga aaa ctt g	Perfect	Dinucleotide	77-109	8	Single locus	10
CH05g11	gca aac caa cct ctg gtg at aaa ctg ttc caa cga cgc ta	Imperfect	Dinucleotide	201-255	6	Single locus	14
CH04e02	ggc gat gac tac cag gaa aa atg tag cca agc cag cgt at	Imperfect	Dinucleotide	143-163	6	Single locus	4
CH02d12	aac cag att tgc ttg cca tc gct ggt ggt aaa cgt ggt g	Perfect	Dinucleotide	177-199	6	Single locus	11
CH03d08	cat cag tct ctt gca ctg gaa a tag ggc tag gga gag atg atg a	Perfect	Dinucleotide	129-161	5	Single locus	14
CH01h02	aga gct tcg agc ttc gtt tg atc ttt tgg tgc tcc cac ac	Imperfect	Dinucleotide	236-256	6	Single locus	9
CH05d03	tac ctg aaa gag gaa gcc ct tca ttc ctt ctc aca tcc act	Perfect	Dinucleotide	152-187	8	Single locus	14
CH05a04	gaa gcg aat ttt gca cga at gct ttt gtt tca ttg aat ccc c	Compound	Dinucleotide	159-189	8	Single locus	16
CH04e03	ttg aag atg ttt ggc tgt gc tgc atg tct gtc tcc tcc at	Perfect	Dinucleotide	179-222	11	Single locus	5
CH03b06	gca tcc ttg aat gag gtt cac t cca atc acc aaa tca atg tca c	Imperfect	Dinucleotide	111-131	5	Single locus	15
CH05g08	cca aga cca agg caa cat tt ccc ttc acc tca ttc tca cc	Imperfect	Dinucleotide	161-179	5	Single locus	1

Table 4.1 Continued.

SSR marker	Primer sequence (5'- 3')	Marker type	Repeat Type	Size Range	No. of alleles	Type of marker	Linkage group
CH01g12	ccc acc aat caa aaa tca cc tga agt atg gtg gtg cgt tc	Imperfect	Dinucleotide	112-186	6	Single locus	12
CH05a05	tgt atc agt ggt ttg cat gaa c gca act ccc aac tct tct ttc t	Imperfect	Dinucleotide	198-230	6	Single locus	6
CH03c02	tca cta ttt acg gga tca agc a gtg cag agt ctt tga caa ggc	Perfect	Dinucleotide	116-136	5	Single locus	12
CH05e04	ag gag aag acc gtg tga aat c cat gga taa ggc ata gtc agg a	Perfect	Dinucleotide	153-234	9	Presumed multi-locus	16
CH04d02	cgt acg ctg ctt ctt ttg ct cta tcc acc acc cgt caa ct	Perfect	Dinucleotide	118-146	3	Single locus	12
CH05e05	tcc tag cga tag ctt gtg aga g gaa acc acc aaa ccg tta caat	Perfect	Dinucleotide	138-160	7	Single locus	14
CH04f06	ggc tca gag tac ttg cag agg atc ctt aag cgc tct cca ca	Imperfect	Dinucleotide	159-179	7	Single locus	14
CH01h01	gaa aga ctt gca gtg gga gc gga gtg ggt ttg aga agg tt	Perfect	Dinucleotide	114-134	6	Single locus	17
CH03b10	ccc tcc aaa ata tct cct cct c cgt tgt cct gct cat cat act c	Perfect	Dinucleotide	99-121	5	Single locus	15
CH04c06	gct gct gct gct tct agg tt gct tgg aaa agg tca ctt gc	Imperfect	Dinucleotide	155-186	8	Single locus	12
CH01d08	ctc cgc cgc tat aac act tc tac tct gga ggg tat gtc aaa g	Perfect	Dinucleotide	238-290	6	Single locus	15
CH03e03	gca cat tct gcc tta tct tgg aaa acc cac aaa tag cgc c	Imperfect	Dinucleotide	106-216	6	Single locus	3
CH04g04	agt ggc tga tga gga tga gg gct agt tgc acc aag ttc aca	Perfect	Dinucleotide	170-186	5	Single locus	12

Table 4.1 Continued.

SSR marker	Primer sequence (5'- 3')	Marker type	Repeat Type	Size Range	No. of alleles	Type of marker	Linkage group
CH05d11	cac aac ctg ata tcc ggg ac gag aag gtc gta cat tcc tca a	Imperfect	Dinucleotide	171-211	5	Single locus	12
CH01d03	cca ctt ggc aat gac tcc tc acc tta ccg cca atg tga ag	Perfect	Dinucleotide	136-160	5	Single locus	4
COL	agg aga aag gcg ttt acc tg gac tca ttc ttc gtc gtc act g	Perfect	Dinucleotide	220-240	5	Single locus	10
CH01f03	gag aag caa atg caa aac cc ctc ccc ggc tcc tat tct ac	Imperfect	Dinucleotide	139-183	14	Single locus	9
Hi03a03	aca ctt ccg gat ttc tgc tc gtt tgt tgc tgt tgg att atg cc	Perfect	Dinucleotide	160-228	10	Confirmed multi-locus	6
Hi24f04	ccg acg gct caa aga caa c tga aaa gtg aag gga atg gaag	Imperfect	Trinucleotide	144-153	5	Single locus	2
Hi07h02	caa att ggc aac tgg gtc tg gtt tag gtg gag gtg aag gga tg	Perfect	Dinucleotide	246-276	10	Single locus	17
CH03a09	gcc agg tgt gac tcc ttc tc ctg cag ctg ctg aaa ctg g	Perfect	Dinucleotide	125-143	6	Single locus	5
Hi03a10	gga cct gct tcc cct tat tc gtt tca ggg aac ttg ttt gat gg	Imperfect	Dinucleotide	206-290	6	Single locus	7
Hi03g06	tgc caa tact cc ctc att tac c gtt taa aca gaa ctg cac cac atc c	Perfect	Dinucleotide	182-204	5	Single locus	15
AF527800[a]	ttg gtc aga cat aca ctg gg ggt ggt aaa tct cca cta cc	Perfect	Dinucleotide	168-194	5	Single locus	17
CH01d08	ctc cgc cgc tat aac act tc tac tct gga ggg tat gtc aaa g	Perfect	Dinucleotide	238-290	6	Single locus	15
MS06g03	cgg agg gtg tgc tgc cga ag gcc cag ccc ata tct gct	Perfect	Dinucleotide	154-190	9	Presumed multi-locus	10

Table 4.1 Continued.

SSR marker	Primer sequence (5'- 3')	Marker type	Repeat Type	Size Range	No. of alleles	Type of marker	Linkage group
CH01f02	acc aca tta gag cag ttg agg ctg gtt tgt ttt cct cca gc	Perfect	Dinucleotide	174-206		Single locus	12
CH03d10	ctc cct tac caa aaa cac caa a gtg att aag aga gtg atc ggg g	Compound	Dinucleotide	166-182	6	Single locus	2
CH02f06	ccc tct tca gac ctg cat atg act gtt tcc aag cga tca gg	Compound	Dinucleotide	135-158	7	Single locus	2
CH02c11	tga agg caa tca ctc tgt gc tga agg caa tca ctc tgt gc	Imperfect	Dinucleotide	219-239	6	Single locus	10
CH02b07	cca gac aag tca tca caa cac tc atg tcg atg tcg ctc tgt tg	Perfect	Dinucleotide	180-202	7	Single locus	10
CH01f12	ctc ctc caa gct tca acc ac ca aaa acc aca ggc ata ac	Perfect	Dinucleotide	145-162	6	Single locus	10
CH01f02	acc aca tta gag cag ttg agg ctg gtt tgt ttt cct cca gc	Perfect	Dinucleotide	174-206	7	Single locus	12
CH02c02	tgc atg cat gga aac gac tgg aaa aag tca cac tgc tcc	Perfect	Dinucleotide	78-126	5	Single locus	4
CH05c07	tga tgc att agg gct tgt act t ggg atg cat tgc taa ata gga t	Imperfect	Dinucleotide	111-149	7	Single locus	9
CH01f02	acc aca tta gag cag ttg agg ctg gtt tgt ttt cct cca gc	Perfect	Dinucleotide	174-206	7	Single locus	12
CH02c06	tga cga aat cca cta ctaatg ca gat tgc gcg ctt ttt aac at	Perfect	Dinucleotide	216-254	8	Single locus	2
CH03b10	ccc tcc aaa ata tct cct cct c cgt tgt cct gct cat cat act c	Perfect	Dinucleotide	99-121	5	Single locus	15
CH02c02	tgc atg cat gga aac gac tgg aaa aag tca cac tgc tcc	Perfect	Dinucleotide	78-126	5	Single locus	4

Table 4.1 Continued.

SSR marker	Primer sequence (5'- 3')	Marker type	Repeat Type	Size Range	No. of alleles	Type of marker	Linkage group
NZ05g08	cgg cca tcg att atc tta ctc tt gga tca atg cac tga aat aaa cg	Perfect	Dinucleotide	115-147	6	Single locus	4
CN581649[a]	agc cct gat ctt cct cta gc acg aac tac cac ctc aaa cc	Perfect	Trinucleotide	382	4	Single locus	n.d.
CH02c09	tta tgt acc aac ttt gct aac ctc aga agc agc aga gga gga tg	Perfect	Dinucleotide	233-257	6	Single locus	15
CN935817[a]	gcc ttc caa gcg tct tgg tta tca aca agc gcc gtt cc	Perfect	Trinucleotide	239	2	Single locus	n.d.
CN935817[a]	gcc ttc caa gcg tct tgg tta tca aca agc gcc gtt cc	Perfect	Trinucleotide	239	2	Single locus	n.d.
CN865016[a]	ttc ttc aca ccc ttc aat cc aaa gcg cct gcg att gcg	Imperfect	Trinucleotide	325-360	7	Single locus	n.d.
CN490349[a]	gta cta tca gca gaa act gg gat ttg agc aca aca tac gg	Perfect	Trinucleotide	200-207	4	Single locus	n.d.
CN579502[a]	tcg tga agt gcc aag tat cg tgg cgg act gct caa ttg c	Perfect	Trinucleotide	240-290	6	Single locus	n.d.
CN887525[a]	tag tag cta cac act ctt tcc gca ttg cct tga gct cca g	Perfect	Tetranucleotide	207-217	7	Single locus	n.d.
CN490740[a]	agg atc ctt cct cga ttt gc ggc att gag gtt ctt gat cc	Imperfect	Trinucleotide	213	8	Single locus	n.d.
NZ23g04	ttt ctc tct ctt tcc caa ctc agc cgc ctt gca tta aat ac	Perfect	dinucleotide	84-116	9	Single locus	6
NZ28f04	tgc ctc cct tat ata gct ac tga gga cgg tga gat ttg	Perfect	Dinucleotide	98-112	4	Single locus	12

Table 4.1 Continued.

SSR marker	Primer sequence (5'- 3')	Marker type	Repeat Type	Size Range	No. of alleles	Type of marker	Linkage group
CN580620[a]	tgc ggt caa cga tgt ctt cg aag gta caa gcc cgc aaa gg	Perfect	Trinucleotide	376-385	7	Single locus	n.d.
CO903298[a]	ttg aga agc aat gct gcc tc tgc cac agt tgg aag gtg g	Perfect	Trinucleotide	340-358	6	Single locus	n.d.
CN444794[a]	cat ggc agg tgc taa act tg gtt tgc aac tca cac aat gca ac	Perfect	Dinucleotide	250-280	7	Single locus	7
CV627191[a]	ctt aat cac cca tca ttc ccc ctc tgt cgg cta act aac cc	Imperfect	Trinucleotide	296-319	6	Single locus	n.d.

[a] SSRs derived from the publicly available EST sequences
All other SSRs are from Silferberg-Dilworth et al., 2006
n.d. = not determined

Table 4.1 SSR name, primer sequences, marker type (perfect, imperfect and compound), repeat type (di-, tri- and tetra-nucleotide), allele size range, alleles detected, type of marker (presumed or confirmed single or multi locus) and linkage groups.

SSR marker	Primer sequence (5'- 3')	Marker type	Repeat Type	Size Range	No. of alleles	Type of marker	Linkage group
CH02d08	tcc aaa atg gcg tac ctc tc gca gac act cac tca cta tct ctc	Perfect	Dinucleotide	210-254	7	Single locus	11
CH02b03	ata agg ata caa aaa ccc tac aca g gac atg ttt ggt tga aaa ctt g	Perfect	Dinucleotide	77-109	8	Single locus	10
CH05g11	gca aac caa cct ctg gtg at aaa ctg ttc caa cga cgc ta	Imperfect	Dinucleotide	201-255	6	Single locus	14
CH04e02	ggc gat gac tac cag gaa aa atg tag cca agc cag cgt at	Imperfect	Dinucleotide	143-163	6	Single locus	4
CH02d12	aac cag att tgc ttg cca tc gct ggt ggt aaa cgt ggt g	Perfect	Dinucleotide	177-199	6	Single locus	11
CH03d08	cat cag tct ctt gca ctg gaa a tag ggc tag gga gag atg atg a	Perfect	Dinucleotide	129-161	5	Single locus	14
CH01h02	aga gct tcg agc ttc gtt tg atc ttt tgg tgc tcc cac ac	Imperfect	Dinucleotide	236-256	6	Single locus	9
CH05d03	tac ctg aaa gag gaa gcc ct tca ttc ctt ctc aca tcc act	Perfect	Dinucleotide	152-187	8	Single locus	14
CH05a04	gaa gcg aat ttt gca cga at gct ttt gtt tca ttg aat ccc c	Compound	Dinucleotide	159-189	8	Single locus	16
CH04e03	ttg aag atg ttt ggc tgt gc tgc atg tct gtc tcc tcc at	Perfect	Dinucleotide	179-222	11	Single locus	5
CH03b06	gca tcc ttg aat gag gtt cac t cca atc acc aaa tca atg tca c	Imperfect	Dinucleotide	111-131	5	Single locus	15
CH05g08	cca aga cca agg caa cat tt ccc ttc acc tca ttc tca cc	Imperfect	Dinucleotide	161-179	5	Single locus	1

Table 4.1 Continued.

SSR marker	Primer sequence (5'- 3')	Marker type	Repeat Type	Size Range	No. of alleles	Type of marker	Linkage group
CH01g12	ccc acc aat caa aaa tca cc tga agt atg gtg gtg cgt tc	Imperfect	Dinucleotide	112-186	6	Single locus	12
CH05a05	tgt atc agt ggt ttg cat gaa c gca act ccc aac tct tct ttc t	Imperfect	Dinucleotide	198-230	6	Single locus	6
CH03c02	tca cta ttt acg gga tca agc a gtg cag agt ctt tga caa ggc	Perfect	Dinucleotide	116-136	5	Single locus	12
CH05e04	ag gag aag acc gtg tga aat c cat gga taa ggc ata gtc agg a	Perfect	Dinucleotide	153-234	9	Presumed multi-locus	16
CH04d02	cgt acg ctg ctt ctt ttg ct cta tcc acc acc cgt caa ct	Perfect	Dinucleotide	118-146	3	Single locus	12
CH05e05	tcc tag cga tag ctt gtg aga g gaa acc acc aaa ccg tta caat	Perfect	Dinucleotide	138-160	7	Single locus	14
CH04f06	ggc tca gag tac ttg cag agg atc ctt aag cgc tct cca ca	Imperfect	Dinucleotide	159-179	7	Single locus	14
CH01h01	gaa aga ctt gca gtg gga gc gga gtg ggt ttg aga agg tt	Perfect	Dinucleotide	114-134	6	Single locus	17
CH03b10	ccc tcc aaa ata tct cct cct c cgt tgt cct gct cat cat act c	Perfect	Dinucleotide	99-121	5	Single locus	15
CH04c06	gct gct gct gct tct agg tt gct tgg aaa agg tca ctt gc	Imperfect	Dinucleotide	155-186	8	Single locus	12
CH01d08	ctc cgc cgc tat aac act tc tac tct gga ggg tat gtc aaa g	Perfect	Dinucleotide	238-290	6	Single locus	15
CH03e03	gca cat tct gcc tta tct tgg aaa acc cac aaa tag cgc c	Imperfect	Dinucleotide	106-216	6	Single locus	3
CH04g04	agt ggc tga tga gga tga gg gct agt tgc acc aag ttc aca	Perfect	Dinucleotide	170-186	5	Single locus	12

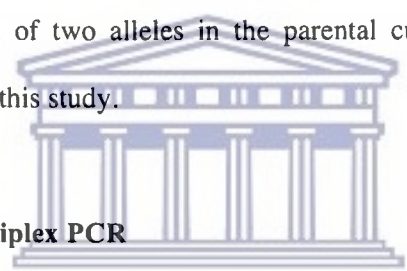
Table 4.1 Continued.

SSR marker	Primer sequence (5'- 3')	Marker type	Repeat Type	Size Range	No. of alleles	Type of marker	Linkage group
CH05d11	cac aac ctg ata tcc ggg ac gag aag gtc gta cat tcc tca a	Imperfect	Dinucleotide	171-211	5	Single locus	12
CH01d03	cca ctt ggc aat gac tcc tc acc tta ccg cca atg tga ag	Perfect	Dinucleotide	136-160	5	Single locus	4
COL	agg aga aag gcg ttt acc tg gac tca ttc ttc gtc gtc act g	Perfect	Dinucleotide	220-240	5	Single locus	10
CH01f03	gag aag caa atg caa aac cc ctc ccc ggc tcc tat tct ac	Imperfect	Dinucleotide	139-183	14	Single locus	9
Hi03a03	aca ctt ccg gat ttc tgc tc gtt tgt tgc tgt tgg att atg cc	Perfect	Dinucleotide	160-228	10	Confirmed multi-locus	6
Hi24f04	ccg acg gct caa aga caa c tga aaa gtg aag gga atg gaag	Imperfect	Trinucleotide	144-153	5	Single locus	2
Hi07h02	caa att ggc aac tgg gtc tg gtt tag gtg gag gtg aag gga tg	Perfect	Dinucleotide	246-276	10	Single locus	17
CH03a09	gcc agg tgt gac tcc ttc tc ctg cag ctg ctg aaa ctg g	Perfect	Dinucleotide	125-143	6	Single locus	5
Hi03a10	gga cct gct tcc cct tat tc gtt tca ggg aac ttg ttt gat gg	Imperfect	Dinucleotide	206-290	6	Single locus	7
Hi03g06	tgc caa tact cc ctc att tac c gtt taa aca gaa ctg cac cac atc c	Perfect	Dinucleotide	182-204	5	Single locus	15
AF527800[a]	ttg gtc aga cat aca ctg gg ggt ggt aaa tct cca cta cc	Perfect	Dinucleotide	168-194	5	Single locus	17
CH01d08	ctc cgc cgc tat aac act tc tac tct gga ggg tat gtc aaa g	Perfect	Dinucleotide	238-290	6	Single locus	15
MS06g03	cgg agg gtg tgc tgc cga ag gcc cag ccc ata tct gct	Perfect	Dinucleotide	154-190	9	Presumed multi-locus	10

Table 4.1 Continued.

SSR marker	Primer sequence (5'- 3')	Marker type	Repeat Type	Size Range	No. of alleles	Type of marker	Linkage group
CH01f02	acc aca tta gag cag ttg agg ctg gtt tgt ttt cct cca gc	Perfect	Dinucleotide	174-206		Single locus	12
CH03d10	ctc cct tac caa aaa cac caa a gtg att aag aga gtg atc ggg g	Compound	Dinucleotide	166-182	6	Single locus	2
CH02f06	ccc tct tca gac ctg cat atg act gtt tcc aag cga tca gg	Compound	Dinucleotide	135-158	7	Single locus	2
CH02c11	tga agg caa tca ctc tgt gc tga agg caa tca ctc tgt gc	Imperfect	Dinucleotide	219-239	6	Single locus	10
CH02b07	cca gac aag tca tca caa cac tc atg tcg atg tcg ctc tgt tg	Perfect	Dinucleotide	180-202	7	Single locus	10
CH01f12	ctc ctc caa gct tca acc ac ca aaa acc aca ggc ata ac	Perfect	Dinucleotide	145-162	6	Single locus	10
CH01f02	acc aca tta gag cag ttg agg ctg gtt tgt ttt cct cca gc	Perfect	Dinucleotide	174-206	7	Single locus	12
CH02c02	tgc atg cat gga aac gac tgg aaa aag tca cac tgc tcc	Perfect	Dinucleotide	78-126	5	Single locus	4
CH05c07	tga tgc att agg gct tgt act t ggg atg cat tgc taa ata gga t	Imperfect	Dinucleotide	111-149	7	Single locus	9
CH01f02	acc aca tta gag cag ttg agg ctg gtt tgt ttt cct cca gc	Perfect	Dinucleotide	174-206	7	Single locus	12
CH02c06	tga cga aat cca cta ctaatg ca gat tgc gcg ctt ttt aac at	Perfect	Dinucleotide	216-254	8	Single locus	2
CH03b10	ccc tcc aaa ata tct cct cct c cgt tgt cct gct cat cat act c	Perfect	Dinucleotide	99-121	5	Single locus	15
CH02c02	tgc atg cat gga aac gac tgg aaa aag tca cac tgc tcc	Perfect	Dinucleotide	78-126	5	Single locus	4

SSR-Hi03a03 amplified five different alleles and was therefore presumed to be multi-locus. The parental alleles for this marker were scored as '156:169' for both parentals (i.e. Carmine and Simpson), while '210:224' and '224:233' were scored for Carmine and Simpson, respectively. These presumably belong to two loci (i.e. locus *y* and *z*). To validate the possibility of two loci, this marker was further used to analyze the "Simpson x Carmine" mapping population. The segregation patterns in the mapping population confirmed that SSR-Hi03a03 was indeed a multi-locus marker, with alleles '210:224' for locus *y*; and '224:233' for locus *z* (see Table 5.1). This was in agreement with the findings by Silfverberg-Dilworth *et al.* (2006), who detected two loci for SSR-Hi03a03. SSR marker MS06g03 was reported to be multi-locus by Liebhard *et al.* (2002), but it only amplified a maximum of two alleles in the parental cultivars as well as in the mapping population used in this study.



4.3 Optimization of multiplex PCR

4.3.1 Multiplex set development

Avoiding overlapping of alleles between individual components of the multiplex is the key requirement in the development of multiplex sets. In this study, the occurrence of allele-overlapping was avoided by combining primer pairs labelled with the same fluorophore at the 5' ends. Furthermore, a 10-bp zone separating the allele size ranges of the SSR loci was maintained throughout. Based on these parameters, multiplex sets were developed by testing different primer combinations. SSR markers with allele size ranges of 90-400 bp were used in developing multiplex sets, while markers with allele sizes larger than 400 bp were eliminated because of problems in scoring by the ABI 310

PRISM Genetic Analyzer (Applied Biosystems).

Initially, combinations of two (duplex) or three (triplex) primer pairs, labelled with the same dye but differ by at least 10 bp, were constructed and tested on six randomly selected DNA samples. Due to the availability of SSR-containing sequences in the public domain, more primer pairs, depending on the expected allele size ranges, were incorporated into the already developed triplex sets. Up to five markers were simultaneously amplified in a single reaction (see Figure 4.5). Table 4.2 shows the alleles amplified for this multiplex set. As more primer pairs were incorporated into the triplex sets, uneven amplifications of the target loci were observed. For example, SSR markers NZ23g4 and CH01f12, in Figure 4.5, amplified poorly, while markers CN579502, CN865016 and CH03e03 generated strong intense products. It was postulated that this uneven amplification was a result of the interaction between primers. Therefore, a strategy of decreasing primer concentration of the primers generating strong products and increasing the concentration of those that amplified poorly was tried. This proved to be effective for some multiplex sets but for some (such as multiplex F5), it did not yield any positive results. It was later realized that those poorly amplified primers were still scorable on the Genotyper™ software version 2.5 (Applied Biosystems).

Reliability of the multiplex sets is an important aspect for mass genotyping, and SSR markers are known to be reliable and reproducible (Jones *et al.*, 1997; Liebhard *et al.*, 2002). To ensure reproducibility of the allele banding patterns, the best multiplex sets were tested on the twenty apple genotypes several times. All those sets that did not

Table 4.2 Allele sizes of the F5-multiplex markers.

Marker	Apple Cultivars									
	Priscilla	**Austin**	**G.Hornet**	**Co-op 22**	**Starking**	**N. Spy**	**Cox Orange**	**G.Delicious**	**Carmine**	**Simpson**
23g4	84/108	98/108	98/108	100/108	102/108	102/108	108	107	84/108	108
CH01f12	146	146	133	146/157	146/159	146	151	143/159	146/159	135/156
CH03e03	199	199/203	187/199	197	184/199	199	199	199	184/203	187/199
CN579502	281/288	281/288	281	281/288	281/288	281/288	281/288	281/288	281/288	281/288
CN865016	340/345	340/345	334/340	340/345	340	345	340/345	334/340	334/340	340/345

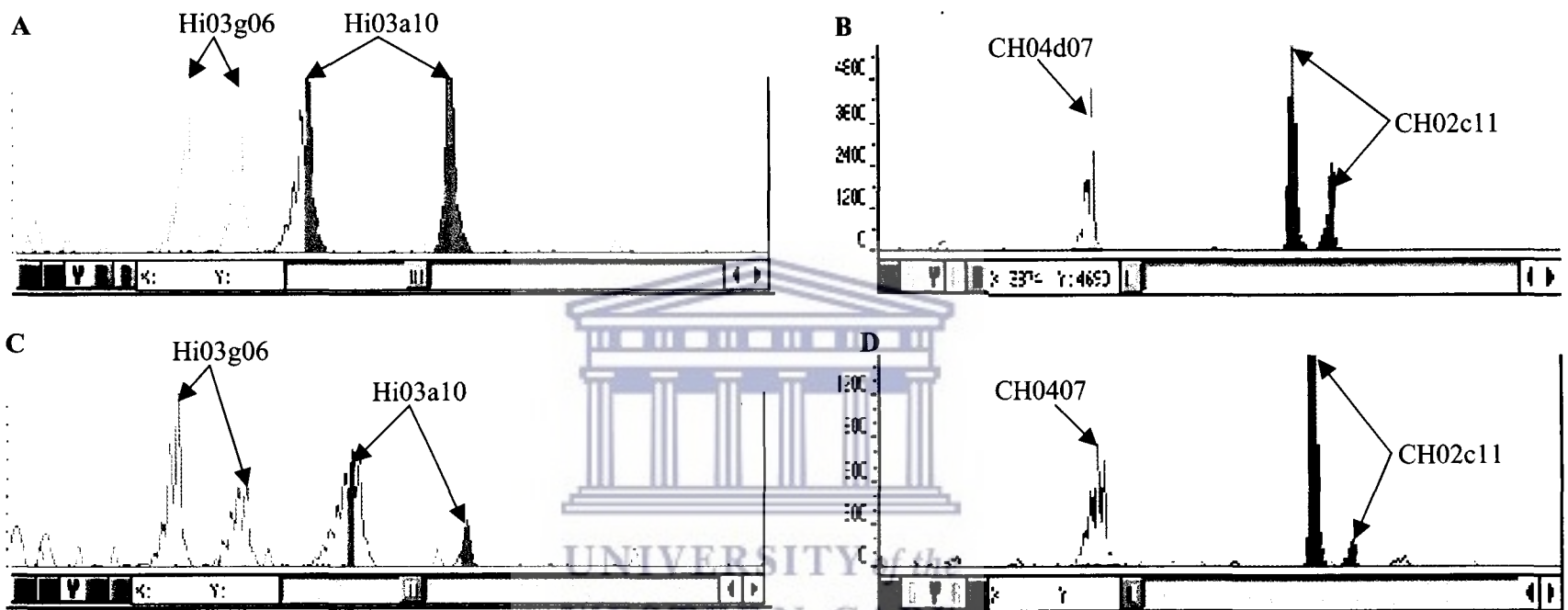


Figure 4.6. Co-electrophoresis of SSR markers on two apple cultivars. **A:** Hi03g06 and Hi03a10 on "Carmine", **B:** CH04d07 and CH02c11 on "Carmine", **C:** Hi03g06 and Hi03a10 on "Simpson" and **D:** CH04d07 and CH02c11 on "Simpson". The arrows indicate the distinguishable alleles for each marker.

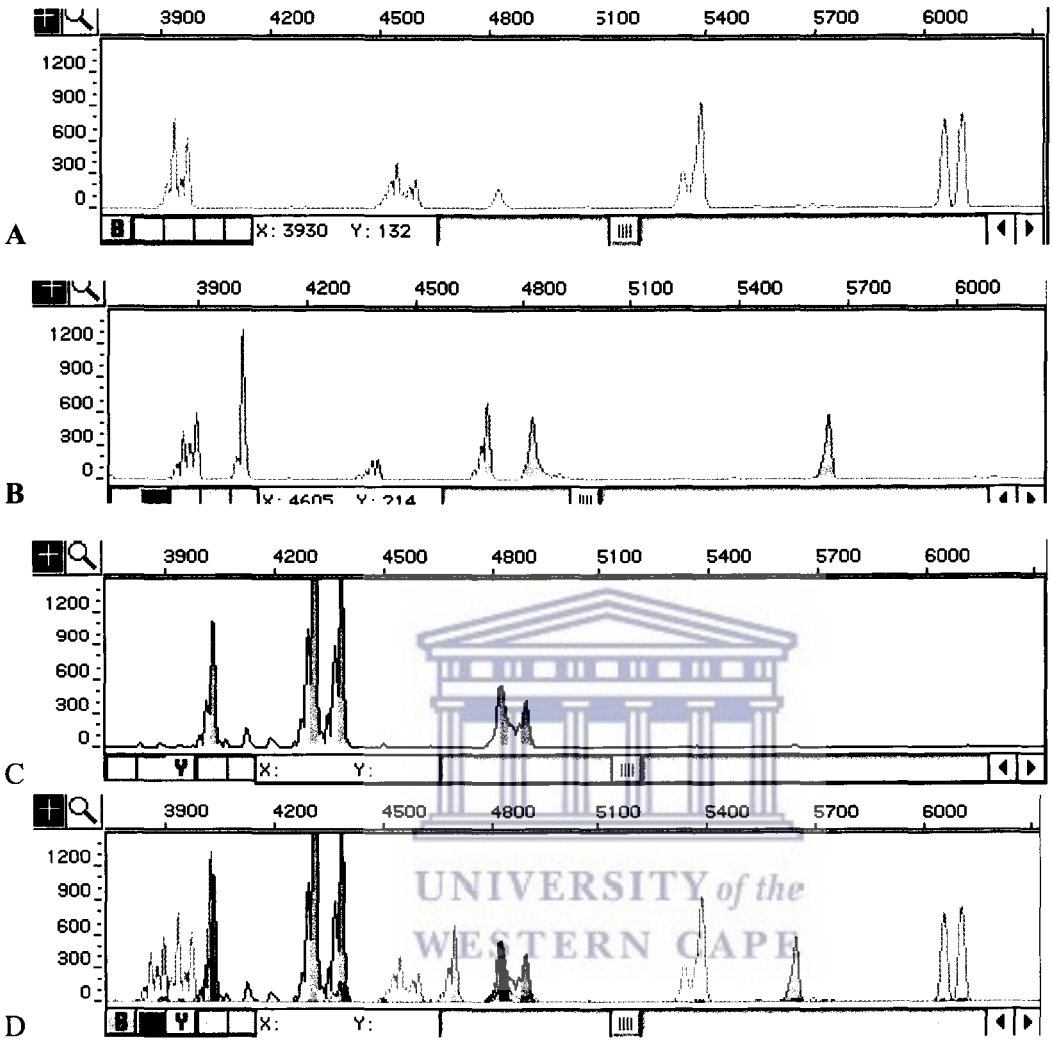


Figure 4.7 Co-electrophoresis of three multiplex sets. **A:** multiplex F8, **B:** multiplex V1, **C:** multiplex N7 and **D:** multiplexes F8:V1:N7 pooled together. The peaks represent the alleles scored.

Table 4.3 SSR markers included in each multiplex set used in this study.

Multiplex set designation	SSR markers	Size Range
F5[a]	A14	88-108
	A73	145-162
	A87	180-203
	A217	280-290
	A238	340-350
F6[a]	A106	116-136
	A63	152-187
	A99	210-223
	A380	240-250
F7[a]	A70	104-126
	A119	159-189
	A345	210-230
	A344	407-410
F8	A43	121-173
	A59	179-222
	A422	296-319
	A219	376-385
F10	A101	110-144
	A32	156-179
	A46	198-233
	A383	340-358

Table 4.3 Continued.

Multiplex set designation	SSR markers	Size Range
F11	A105	102-186
	A188	190-213
	A343	218-350
F3	A12	112-125
	A89	130-163
	A98	170-199
	A78	218-268
	A283	330-385
F8	A43	121-173
	A59	179-222
	A422	296-319
	A219	376-385
F10	A101	110-144
	A32	156-179
	A46	198-233
	A383	340-358
V1	A41	78-126
	A97	139-183
	A37	216-254
	A319	310-340

Table 4.3 Continued.

Multiplex set designation	SSR markers	Size Range
V6	A29	56-100
	A92	125-143
	A122	155-186
N4	A115	99-121
	A72	135-192
	A76	219-239
N6	A64	138-160
	A109	171-211
	A67	233-257
N8	A15	112
	A80	170-194
	A279	217

[a] Multiplex sets developed in this study

4.4 Summary

Due to the fact that most of the primer pairs had annealing temperature profiles ranging from 50-60°C, therefore, thermal cycling program touchdown (50-60°C) was routinely used for the simplex PCR analysis of most SSR markers. This program produced highly intense PCR products. However, in some instances this increased signal strength was accompanied by an increase of smear below the target amplicon. In such instances, touchdown (55-65°C) PCR program was used as an alternative. This was done in order to curb the smears. Overall, eighty-six SSR markers were successfully analyzed in this study using touchdown 60-50°C and 65-55°C.

Both the GeneScan™ analysis software version 3.1.2 and Genotyper™ software version 2.5 were successfully used for fragment and allele scoring, respectively. With the exception of CN870040, CN909118, CN880881, CN944444, AJ001681 and CN491050, all of the optimized markers were identified as polymorphic, detecting two to fourteen distinguishable alleles per locus within the set of twenty-one cultivars.

A total of twelve SSR markers were successfully integrated into three multiplex sets. These sets varied in the number of markers incorporated. Some sets had a minimum of three markers while others composed of up to five markers. These multiplex sets were pooled together during the fluorescent-based fragment analysis. This pooling strategy made it possible to analyze up to twenty SSR markers in a single capillary injection. This proved to be a cost and labor-effective strategy.

5 Genotyping and linkage mapping

5.1 Introduction

Saturated apple linkage maps with portable markers such as SSRs have been published (Liebhard *et al.*, 2003b, Silfverberg-Dilworth *et al.*, 2006). This has facilitated the assignment of major mildew resistance genes to their particular linkage groups. For example, James and Evans (2004) have located the *Pl-w* gene at the top of linkage group 8, while *Pl-d* was mapped at the bottom end of linkage group 12 (James *et al.* 2004). The *Pl-2* gene has been assigned to linkage group 11, and is flanked at 8 and 7 cMs by N18-SCAR and U02-SCAR, respectively (Gardiner *et al.*, 2003; Seglias and Gessler 1997; Gardiner *et al.*, 1999). This section aims at constructing linkage maps for the entire genome and the bottom end of linkage 12.

5.2 Segregation types

Segregation analysis was performed on a total of eighty-seven individual seedlings of the "Carmine" x "Simpson" family using fifty-six SSR markers. Of the fifty-six SSR markers used, thirty-one were bi-allelic and dominant for one parent (i.e. fifteen with *'lm'* x *'ll'* and sixteen with *'nn'* x *'np'*), three were bi-allelic but co-dominant (i.e. *'hk'* x *'hk'*), fifteen, including the second locus of SSR-Hi03a03, were tri-allelic (i.e. *'ef'* x *'eg'*) and seven were tetra-allelic (*'ab* x *cd'*). Table 5.1 gives a summary of the segregation types of all the markers.

Table 5.1 Segregation types for 'Carmine' and 'Simpson' and the mapping population

Designated name	SSR locus	Parental Alleles		Segregation Type	
		Carmine	Simpson	Parents	Seedlings
A10	NZ02b	218/242	242/222	*ef* x *eg*	*ee: ef:eg:fg*
A12	NZ05g8	118	105/109	*nn* x *np*	*nn:np*
A14	NZ23g04	84/108	108	*lm* x *ll*	*lm:ll*
A15	NZ28f4	108	93/100	*nn* x *np*	*nn:np*
A32	CH05g08	176	158/162	*nn* x *np*	*nn:np*
A35	CH01f02	170/179	174/179	*ef* x *eg*	*ee: ef:eg:fg*
A37	CH02c06	216/236	236/241	*ef* x *eg*	*ee: ef:eg:fg*
A43	CH04e03	197	186/203	*nn* x *np*	*nn:np*
A46	CH05a05	196/221	210	*lm* x *ll*	*lm:ll*
A48	CN444542	236	200/233	*nn* x *np*	*nn:np*
A49	CH05c07	107/121	121	*lm* x *ll*	*lm:ll*
A51	CH02b07	104	100/118	*nn* x *np*	*nn:np*
A52	CH02d08	211/225	225	*lm* x *ll*	*lm:ll*
A59	CH03d08	127	131/140	*nn* x *np*	*nn:np*
A62	CH04f06	160/178	160	*lm* x *ll*	*lm:ll*
A63	CH05d03	163/169	158/169	*ef* x *eg*	*ee: ef:eg:fg*
A64	CH05e05	138/150	143/148	*ef* x *eg*	*ee: ef:eg:fg*
A65	CH05g11	237/248	237	*lm* x *ll*	*lm:ll*
A67	CH02c09	237/241	216/228	*ab* x *cd*	*ac:ad:bc:bd*
A71	CH01h01	109/114	102	*lm* x *ll*	*lm:ll*
A73	CH01f12	146/159	135/156	*ab* x *cd*	*ac:ad:bc:bd*
A76	CH02c11	194/207	194/207	*hk* x *hk*	*hh:hk:kk*
A78	COL	233	214/233	*nn* x *np*	*nn:np*
A81	MS06g03	155/179	173	*lm* x *ll*	*lm:ll*
A84	CH02f06	142/149	136/149	*ef* x *eg*	*ee: ef:eg:fg*

Table 5.1 Continue.

Designated name	SSR locus	Parental Alleles		Segregation Type	
		Carmine	Simpson	Parents	Seedlings
A86	CH03d10	164/170	172	*lm* x *ll*	*lm:ll*
A87	CH03e03	184/203	189/199	*ab* x *cd*	*ac:ad:bc:bd*
A92	CH03a09	127/131	127/179	*ef* x *eg*	*ee: ef:eg:fg*
A97	CH01f03	137	137/150	*nn* x *np*	*nn:np*
A98	CH02d12	197/203	184/187	*ab* x *cd*	*ac:ad:bc:bd*
A105	CH01g12	105/142	128/147	*ab* x *cd*	*ac:ad:bc:bd*
A106	CH03C02	120	120/134	*nn* x *np*	*nn:np*
A107	CH04d02	119/130	128	*lm* x *ll*	*lm:ll*
A109	CH05d11	172	168/179	*nn* x *np*	*nn:np*
A113	CH01d08	242/253	240/248	*ab* x *cd*	*ac:ad:bc:bd*
A115	CH03b10	100	92/116	*nn* x *np*	*nn:np*
A119	CH05a04	158	170/183	*nn* x *np*	*nn:np*
A133	CH01d03	137/157	131/140	*ab* x *cd*	*ac:ad:bc:bd*
A186	CN490349	200/207	195/200	*ef* x *eg*	*ee: ef:eg:fg*
A219	CN580620	376/379	384	*lm* x *ll*	*lm:ll*
A222	CN581649	182/185	188	*lm* x *ll*	*lm:ll*
A227	CN493171	170	170/175	*nn* x *np*	*nn:np*
A238	CN865016	333/340	340/345	*ef* x *eg*	*ee: ef:eg:fg*
A260	CN935817	243	239/243	*nn* x *np*	*nn:np*
A279	CN887525	208/217	208	*lm* x *ll*	*lm:ll*
A319	AF527800	330	330/344	*nn* x *np*	*nn:np*
A345	CO755814	259/264	262	*lm* x *ll*	*lm:ll*
A383	CO903298	345/356	365	*lm* x *ll*	*lm:ll*
A422	CV627191	296/312	290/312	*ef* x *eg*	*ee: ef:eg:fg*
A542	Hi03g06	182/196	176/196	*ef* x *eg*	*ee: ef:eg:fg*

Table 5.2 Continue.

Designated name	SSR locus	Parental Alleles		Segregation Type	
		Carmine	Simpson	Parents	Seedlings
A547	Hi03a10	216/257	224/257	*ef* x *eg*	*ee: ef:eg:fg*
A552	CN444794	263/270	256/270	*ef* x *eg*	*ee: ef:eg:fg*
A553	Hi07h02	246/257	240/246	*ef* x *eg*	*ee: ef:eg:fg*
A564	Hi24f04	144/147	144/147	*hk* x *hk*	*hh:hk:kk*
*A567*y*	Hi03a03*y*	156/169	156/169	*hk* x *hk*	*hh:hk:kk*
*A567*z*	Hi03a03z	210/224	224/233	*ef* x *eg*	*ee: ef:eg:fg*

*Represent the two loci (i.e. loci *y* and *z*) for SSR-Hi03a03.

5.3 Screening of SSR markers located at LG 12

SSR markers MS14b04, CH05d04, CH04g04, located on the top half of linkage group 12 (LG 12) (Liebhard *et al.* 2002; Silfverberg-Dilworth *et al.* 2006), were homozygous for both parents, and therefore could not be used for the analysis of the progeny, while markers CH05d11 and NZ28f04, both segregated for two alleles, with genotypes *'nn'* x *'np'*.

Markers CH01g12 and CH01d03, which are located on the bottom half of linkage group 12 (LG 12) (Liebhard *et al.* 2002; Silfverberg-Dilworth *et al.* 2006), segregated for four alleles with genotypes *'ab'* and *'cd'* for "Carmine" and "Simpson", respectively (see Figure 5.1a). The individuals of the mapping population showed four segregation patterns (i.e. *'ac'*; *'ad'*; *'bc'* and *'bd'*) consistent with the parental genotypes (Figure 5.1b). These markers were regarded as highly informative. For marker CH01f02, three segregating alleles were obtained, with genotype *'ef'* being assigned to "Carmine" and *'eg'* to "Simpson", and the progeny segregated for: *'ef'*; *'ee'*; *'eg'* and *'fg'*. Like CH01g12 and CH01d03, this marker was also highly informative.

SSR-CH04d02 marker segregated for three alleles, 119-bp and 131-bp for Carmine and 128-bp for Simpson, with genotypes *'lm'* and *'ll'*. The 128-bp allele appeared in all the seedlings, while 119-bp and 131-bp alleles, occurred interchangeably with the 128-bp allele. Because this allele appeared in all individuals, the resistant parent (i.e. "Simpson") was therefore presumed to be homozygous for this marker, with genotype 128/128 (*'ll'*). Since the resistant parent is homozygous, the resistant and susceptible alleles could not be distinguished for this marker.

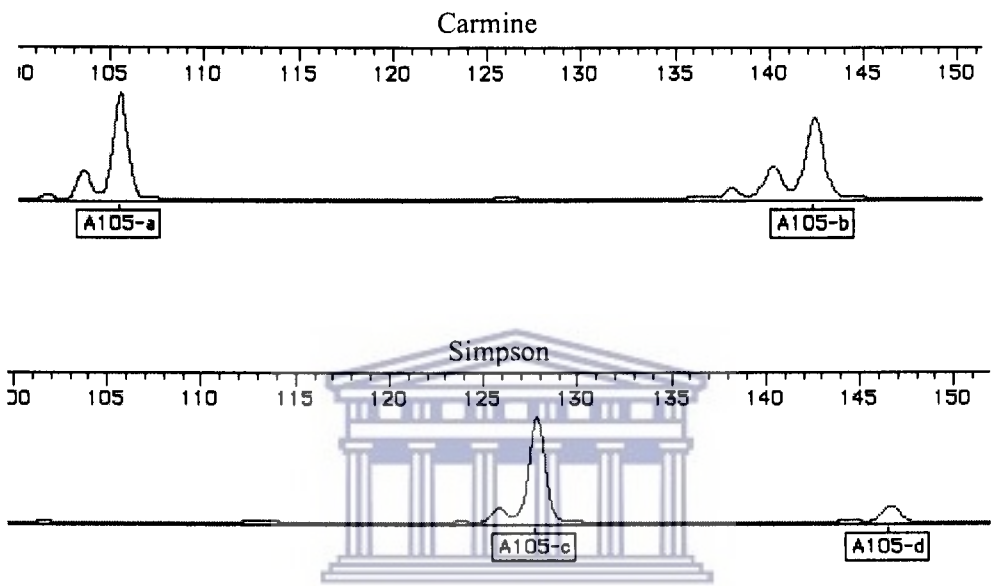


Figure 5.1a Electropherogram of parental genotypes for SSR-CH01g12. A105 is a designation given to marker CH01g12; while a, b, c and d are the genotypes indicating four alleles on "Carmine" and "Simpson", respectively.

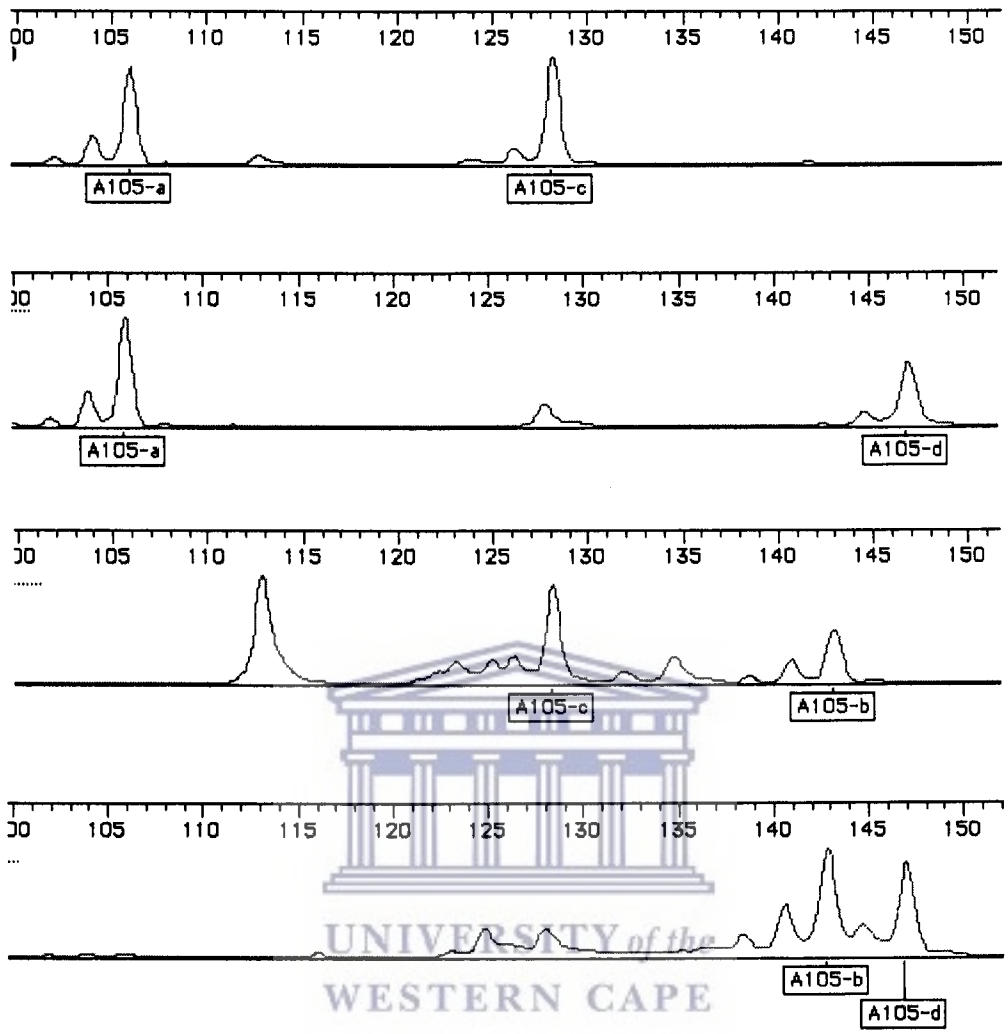


Figure 5.1b Electropherogram of four seedlings showing allelic inheritance. A105 is a designation given to marker CH01g12; while a, b, c and d are the four alleles inherited from the parents (i.e. "Carmine" and "Simpson").

For SSR-CH03c02 marker, mapping at 8 cM from the *Pl-d* gene (James *et al.*, 2004), 120-bp and 134-bp alleles were scored for the resistant parent (i.e. "Simpson"), while only the 124-bp allele was scored for the susceptible parent (i.e. "Carmine"). The 124-bp allele appeared in all screened seedlings, suggesting that "Carmine" is homozygous at this locus, with genotype 124/124.

Other markers (i.e. CH01b12*z* and Hi07f01) reported to be located at the bottom end of linkage group 12 and expected to be close to both *Pl-d* and *Pl-1* genes (Silfverberg-Dilworth *et al.*, 2006), were screened on both parents. SSR-Hi07f01 was homozygous for both parents, and therefore could not be used for the analysis of the progeny, while marker CH01b12*z* did not yield any amplification product for both parents (i.e. "Carmine" and "Simpson").

5.4 Linkage mapping

5.4.1 Linkage group 12 mapping

SSR markers CH03c02, CH01g12 and CH01d03*z*, known to be located around the *Pl-d* in the "Fiesta" x A871-14 progeny map (James *et al.*, 2004), together with CH01f02 and CH04d02 from Liebhard *et al.* (2002), Silfverberg-Dilworth *et al.* (2006), were chosen for mapping the bottom region of linkage group 12, a region surrounding the *Pl-d* gene. Markers CH01g12, CH01f02 and CH04d02 were successfully linked together in the same region (i.e. bottom region) of this linkage group (i.e. LG 12). Figure 5.2 shows the genetic map of this region (also referred to as a localized map). Genetic map of this region comprising amplified fragment polymorphic length polymorphism (AFLP),

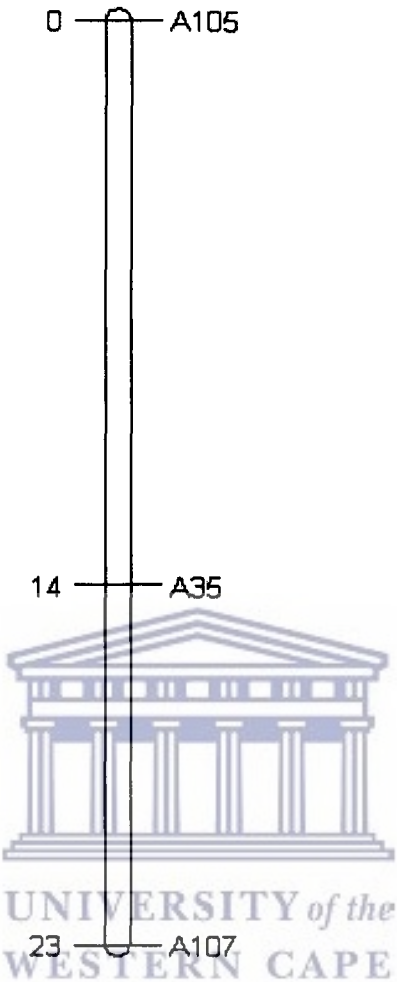


Figure 5.2 Genetic map of the bottom half of linkage group 12 (LG 12) created for 87 individuals of "Carmine" x "Simpson" population. Distances, in centimorgans, are indicated on the left. **A105:** CH01g12; **A35:** CH01f02 and **A107:** CH04d02.

random amplified polymorphic DNA (RAPD) and SSR markers has been constructed for "Fiesta" x A871-14" map by James *et al.*, (2004). This genetic map was compared with the localized genetic map for "Carmine x Simpson" mapping population. Comparison of these maps demonstrated that SSR-CH01g12 mapped at the top of the bottom region of LG 12 in the "Carmine x Simpson" family (see Figure 5.3). Further comparison of this localized map with that of "Idared" x "Robusta 5" and "Idared" x 78/18-4 (Dunemann *et al.*, 2007), provided further evidence that CH01g12 maps at the top of the bottom region of LG 12 (Figure 5.4). However, the order of markers CH01f02 and CH04d02 in the "Carmine x Simpson" localized map were not consistent with the order on the chromosomes in the other localized maps (i.e. *Pl* maps). For example, in the *Pl* maps, CH04d02 was placed on top of CH01f02, while in the "Carmine x Simpson" map the order of the markers was swapped (i.e. CH01f02 was placed on top of CH04d02).

Markers CH03c02 and CH01d03, which are also known to be located at the distal end of LG 12 in the published maps (Liebhard *et al.* 2002, Silfverberg-Dilworth *et al.* 2006), failed to link in the "Carmine x Simpson" mapping population.

5.4.2 Genome mapping

From the fifty-six genotyped SSR markers, forty-two (ten EST and thirty-two from the literature) were used to construct the genetic linkage map. Using LOD score of 4.0, about nineteen of those markers were grouped in six linkage groups (i.e. LG 02; 07; 10; 12; 14 and 15), which had 2-3 markers each. These markers were randomly distributed on the six linkage groups with linkage groups 02; 12; 14 and 15 consisting of three markers each, while linkage groups 07 and 10 consisted of two markers each (Figure 5.5). This

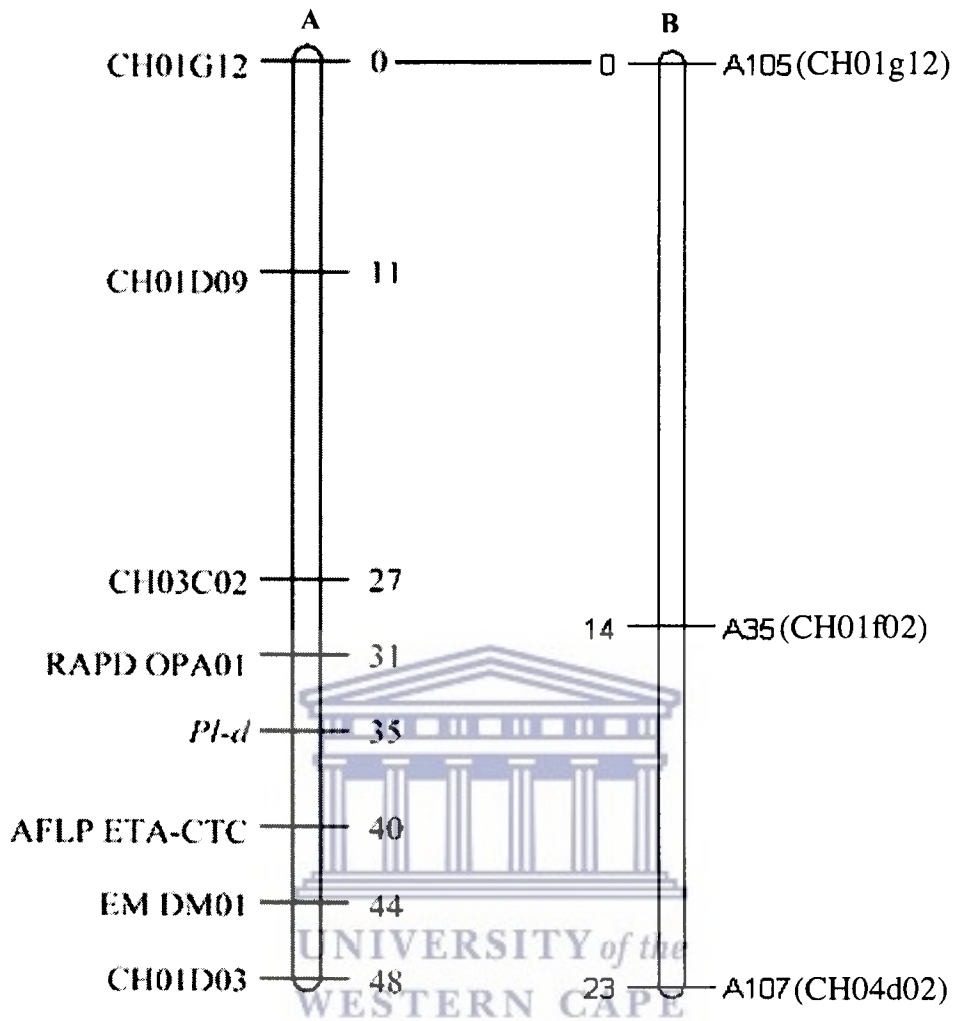


Figure 5.3 Genetic maps of the regions at the bottom half on linkage group 12 (LG 12). **A:** map of 86 seedlings of the "Fiesta" x A871-14 family (James *et al*, 2004), and **B:** map of 87 seedlings of the "Carmine" x "Simpson" family. Distances, in centimorgans, are indicated on the right and left, respectively.

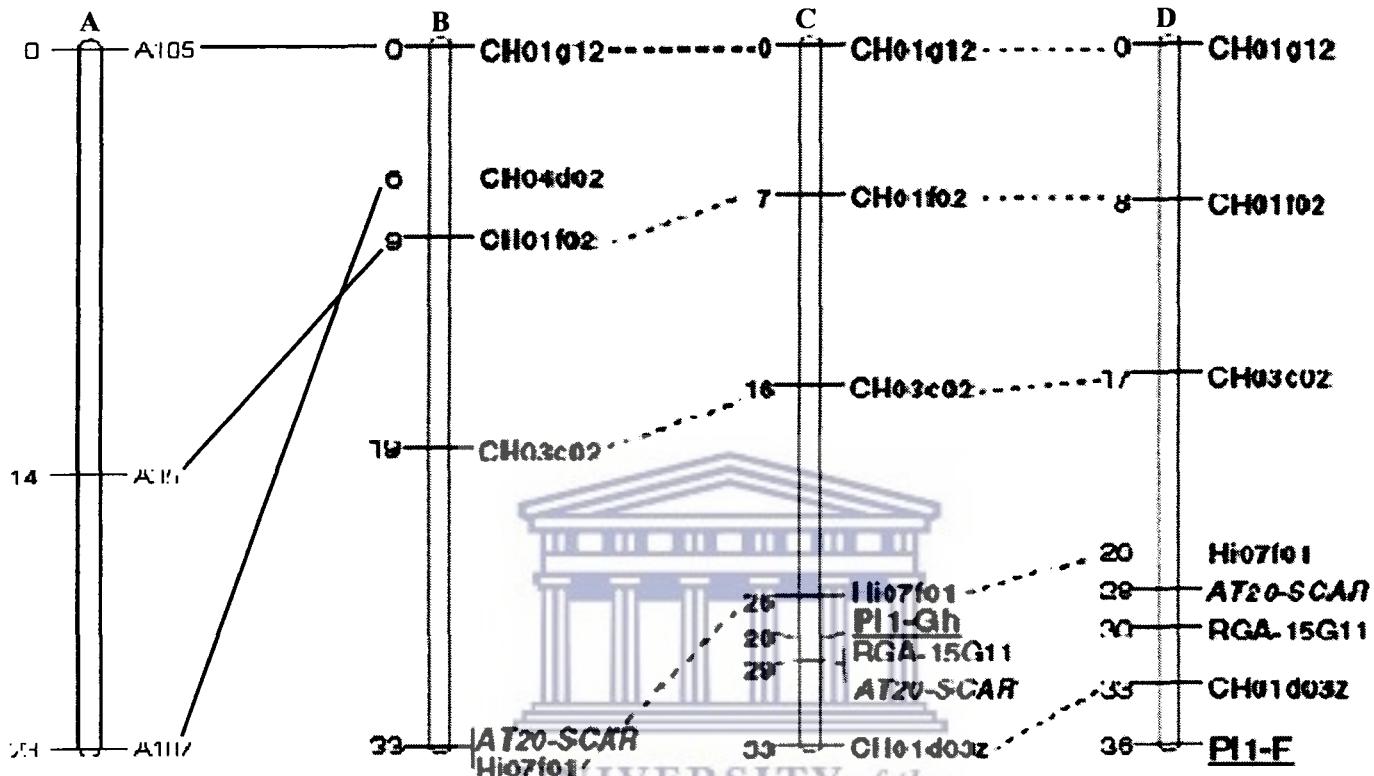


Figure 5.4 Genetic maps of the regions around the *Pl* genes on linkage group 12 (LG 12). **A:** 87 individuals of "Carmine" x "Simpson" population, **B:** 154 individuals of population 04/208, **C:** 161 individuals of population 99/2-Gh (mildew scored in a greenhouse, locus Pl1-Gh) and **D:** 125 individuals of population 99/2-F (mildew scored in the field, locus Pl1-F) (Dunemann *et al.*, 2007). Distances, in centimorgans are indicated on the left.

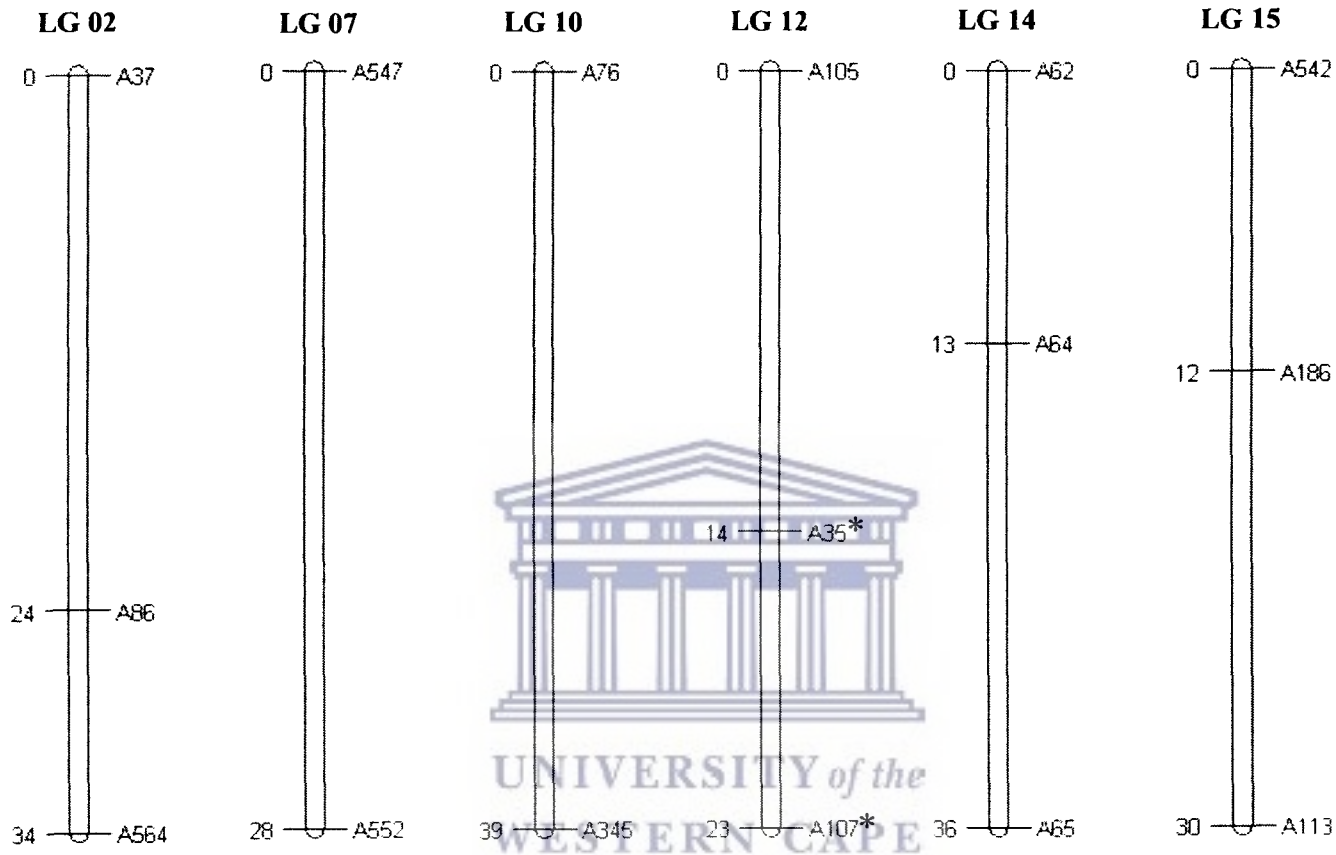


Figure 5.5 Graphical representation of genetic framework map for the "Carmine" x "Simpson" family. Distances, in centimorgans, and loci are indicated on the left and right, respectively. LG represents linkage groups, and *asterisks* (*) indicate markers with erroneous orders.

linkage map spans a total of 190 cM with sizes of the linkage groups ranging from 23 cM (LG 12) to 39 cM (LG 10). Table 5.2 summarizes the distribution of SSR markers on the linkage groups. The order of most markers on the linkage groups was according to the order on the chromosomes in those published apple maps (Liebhard *et al.*, 2002; Silfverberg-Dilworth *et al.*, 2006). However, in LG 12 a 9-cM inversion between the positions of SSR-CH01f02, designated A35 and SSR-CH04d02, designated A107 was observed. The order of these markers was not in agreement with those on the published maps.

5.5 Summary

A total of fifty-six SSR markers were used to genotype the mapping population of eighty-six individuals. SSR-Hi03a03 was confirmed to be multi-locus as it detected two different loci (i.e. loci *y* and *z*). Seven markers segregated for four alleles (i.e. *'ab* x *cd'*), fifteen showed a segregation of three alleles (i.e. *'ef'* x *eg'*), and thirty-four showed a simple segregation (i.e. sixteen *'nn* x *np'*; fifteen *'lm* x *ll'* and three *'hk* x *hk'*.

Of the fifty-six SSR markers genotyped, sixteen were successfully linked and grouped in six linkage groups in the genetic map constructed for this mapping population. In those linkage groups, two (i.e. CH04d02 and CH01f02) were inverted as far as order in the genetic map is concerned. About twelve SSR markers known to be located in linkage group 12 were screened on both parents. Seven of those markers were heterozygous in either one or both of the parents while three were homozygous in both parents and two did not yield any amplification product. From the seven of the heterozygous markers,

Table 5.2 Linkage group, size and number of SSR markers per linkage group of the 'Carmine' x 'Simpson' linkage map.

Linkage group	Size (cM)	No. of markers
02	34	3
07	28	2
10	39	2
12	23	3
14	36	3
15	30	3
Total	190	16

four segregated for two alleles (i.e. bi-allelic), one segregated for three alleles (i.e. tri-allelic) and two segregated for four alleles (i.e. tetra-allelic). The seven SSR markers (i.e. CH01f02, CH03c02, CH01g12, CH01d03*z*, CH04d02, CH05d11 together with NZ28f04, were used to construct a localized linkage map (i.e. the bottom region of linkage group 12). Only CH01g12, CH04d02 and CH01f02 were successfully linked together. The comparison of this localized map to other published maps of this region (i.e. bottom of LG 12) revealed that CH01g12 mapped at the top of the bottom region of LG 12. The order of markers CH04d02 and CH01f02 was inverted as compared to the order of SSRs in other published maps.

6 General discussion and Conclusion

6.1 Disease assessment

In the first year, about 74% of seedlings belonged to classes 3 and 4. This high mildew incidence can be attributed to the succulent conditions in the greenhouse at the time of inoculation. These conditions are known to be favorable for mildew infection (Korban and Riemer, 1990). It could be further reasoned that seedlings grown in the greenhouse had not fully expressed resistance to *P. leucotricha*, and this physiological status therefore made them prone to mildew infection. Contrary to the first year, infection declined (i.e. 24% of seedlings belonged to classes 3 and 4) in the second year as most of the seedlings that were infected in the previous year were free of symptoms. This decline in infection could be attributed to lower pathogen pressure. Furthermore, weather conditions not favorable for the growth of spores could have also contributed to this decline. Generally, powdery mildew spores are sensitive to extreme heat and direct sunlight (Pest Notes, 2001). This therefore explains why other seedlings which were observed to be severely infected during greenhouse evaluation were free of symptoms (class 1) or slightly infected (class 2) during the 2002 evaluation.

In 2003, only sixteen seedlings exhibited a slight immunity to powdery mildew (class 2), but none exhibited total immunity. A large portion (i.e. sixty-four) of the seedlings was susceptible to mildew, a significant increase from the previous year. During this growing season, seedlings were under shade netting, and these shady conditions are known to favour the development of *P. leucotricha* spores (Pest Notes, 2001). This explains the increase in mildew incidence. The same applies to the 2004 growing season, which also had high infection incidence. "Carmine", a commercial parent in this cross, is known to

be susceptible to powdery mildew (Dr. Labuschagne, personal communication). This explains the high proportion of susceptible seedlings in these consecutive years. In addition, the high proportion of the seedlings that died prior to evaluations could be attributed to their extreme vulnerability to the fungus, and therefore died.

In this study, the year-to-year variation in mildew incidence has been influenced by varying environmental conditions in which the seedlings were exposed to, and has complicated the selection for mildew resistance in the "Carmine x Simpson" cross.

6.2 Simplex PCR optimization

The ability of microsatellites to distinguish between genotypes and their potential in various applications such as genetic mapping is well documented in both apple and pear (Gianfranceschi *et al.*, 1998; Guilford *et al.*, 1997; Liebhard *et al.*, 2002; Yamamoto *et al.*, 2002c and Van Dyk *et al.*, 2005), but the technical problems associated with their amplifications limit their use. Due to the dynamic nature of PCR, minor changes, which results in non-specific amplifications, are introduced in each cycle. Therefore, the optimization of PCR conditions for individual markers becomes a necessity. In this study, both the gradient and touchdown PCR techniques were used to optimize the simplex PCR conditions prior to the large-scale applications of each locus. Gradient PCR technique was used to determine optimal temperature profiles for individual markers, while touchdown PCR was used as a step-wise method of approaching optimal amplification. The use of these techniques greatly improved the efficiency of microsatellite analysis because it minimized false calls by the Genotyper™ software (Applied Biosystems).

Touchdown (60-50°C), which was routinely used for the analysis of most SSR markers, produced specific and intense PCR products (amplicons). However, in some instances the increased intensity of the amplicons was accompanied by increased smearing below the target amplicon. The spurious dimeric PCR products resulting from annealing of primers could be the main cause of this smearing. Touchdown 65-55°C was used effectively in dealing with this problem of dimerization.

During SSR amplification, an extra non-template 'A' (adenosine) is added to the 3′ end of the PCR products. *Taq* DNA polymerase is known to catalyze this inconsistent addition (Smith *et al.*, 1995). This phenomenon also made it difficult to discriminate between alleles that differed by two base pairs and often led to inconsistent allele calling. The post-PCR treatment of amplicons with T4 polymerase is known to remove the non-template adenosine (Ginot *et al.*, 1996). However, this approach is time-consuming and must be evaluated in the light of the costs involved. Instead, other approaches such as the modifications of the cycling conditions (i.e. prolonged PCR extension time) should be tried as they are less-costly. This approach proved to be effective in eliminating this problem for some primer pairs while others were refractory to such optimizations. This is not a new phenomenon as it has also been observed by He *et al.*, (1994) and the reasons are not clear.

The presence of artefacts such as stutter fragments was another technical problem encountered during the SSR amplification. This is a technical problem especially common when di-nuleotide repeats are amplified (Brownstein *et al.*, 1996, Smeets *et al.*,

1989). The slippage of DNA polymerase over the template during replication is known to result in these low-molecular weight stutter fragments (Holton, 2001). The use of SSR repeats of higher order, such as tri- and tetra-nucleotides, as recommended by (Bryan *et al.*, 1997), proved to be effective in minimizing this problem.

The optimization of simplex PCR conditions, as presented in this study, was successful and can be of great assistance in minimizing, even eliminating, some of the technical problems experienced during SSR analysis. These optimized cycling conditions for single locus were used as a base of the subsequent optimization for multiplex PCR.

6.3 Multiplex PCR optimization

Multiplex PCR optimization involved the determination of primers to be grouped together to construct a set. This was the most challenging but critical step. It was challenging in that different primer pairs were to anneal under the same cycling conditions (see Table 2.3). In some instances, up to five SSR markers, labeled with the same dye, were successfully grouped together and amplified in one PCR reaction. Multiplex sets developed in this study, together with those developed by other members of the MAB group, were successfully implemented in the genotyping of the 'Carmine' x 'Simpson' mapping population. This increased throughput significantly. In contrast to the simplex procedure, multiplex was effective in reducing the time required for setting up PCR reactions for individual loci. For example, the average time required for the amplification of five primer pairs in five separate simplex PCR reactions was approximately 15 hours (i.e. 3 hours per reaction), but the simultaneous amplification of

those five primer pairs required only three hours. The operation time was significantly reduced by more than 75 percent when the multiplex PCR technique was employed. Furthermore, multiplexing several markers in a single reaction also helped to lower the costs as far as consumables are concerned as fewer reactions were performed and hence less reagents were used. It goes without saying that incorporating more markers into the existing sets would further reduce costs and labor for both PCR and gel electrophoresis.

The pooling and co-electrophoresis of multiplex PCR reactions in a single capillary injection also increased throughput for fluorescence-based genotyping in this study. This pooling strategy allowed up to twenty SSR loci to be analyzed in a single capillary injection. It took about eight hours to analyze these twenty SSR loci, but when the pooling strategy was employed the analysis of the same SSR loci it took only 24 minutes. This strategy was effective in reducing time by over 90%. Compared to conventional simplex procedure, multiplex PCR together with the co-electrophoresis proved to be time, labor and cost-effective procedures to be used in large-scale genotyping.

Reliability of data is the most important aspect of successful large-scale genotyping. To ensure reliability of data, analysis of the twenty one cultivars with multiplex sets was performed repeatedly. Multiplex sets developed in this study can be used to accelerate mass genotyping for pome fruit. This was demonstrated by their use in other apple mapping projects (Maharaj, 2007) and pear (S. Booi, personal communication).

6.4 Linkage analysis and genetic mapping

In this study, fifty-six SSR markers were used to construct a linkage map consisting of six linkage groups (LG 02, 07, 10, 12, 14 and 15). Only sixteen of those markers were successfully linked. A large percentage of the markers, which were previously mapped by Silfverberg-Dilworth *et al.* (2006), failed to link in our 'Carmine' x 'Simpson' linkage map. The genotyping errors highlighted in section 4.2.4 (page); the missing data observed during the genotyping of those markers; and the relatively small number of the seedlings could be the main reasons for the linkage failure. The sixteen markers that were successfully linked were randomly distributed across the six linkage groups (LG), and those linkage groups were shorter in length than those in other apple maps (Liebhard *et al.* 2002, Silfverberg-Dilworth *et al.*, 2006 and Naik *et al.*, 2006), with lengths ranging from 23 cM (LG 12) to 39 cM (LG 14). Initially, it was assumed that the variation in the number of mapped loci is the reason for the difference in linkage lengths. However, it was discovered that the size of the linkage group does not necessarily reflect the number of mapped loci. For example, LG 12 had a size of 23 cM, with 3 mapped loci, while LG 10 had only 2 markers but the size was 39 cM. Therefore, the shorter lengths of linkage groups in our map cannot be attributed to the variations in the mapped loci. The reasons for such variations is in linkage lengths is not yet clear, but we suspect that recombination events might have contributed to this.

The order of most markers for three linkage groups (LGs 10, 14 and 15) was consistent with that of the reference map of 'Fiesta' x 'Discovery', while the order in linkage groups 2, 7 and 12 were inverted. In the Silfverberg-Dilworth *et al.* (2006) reference map,

marker Hi24f04 (A564), is placed on top of both CH02c06 and CH03d10 (A86). In LG 7, the positions of markers Hi03a10 (A547) and CN444794 (A552) were also inverted. There was also a 9-cM inversion between their positions of markers CH01f02 (A35) and CH04d02 (A107), which mapped on linkage group 12 (see figure 5.5). These inversions could be attributed to the strongly skewed segregations observed for these markers. The skewed segregation may be due to the presence of gametophytic selection for sub-lethal genes - i.e. genes controlling the viability of pollen (Yan et al., 2005), and has been documented for a wide range of agriculturally important crops such as maize (Lu *et al.*, 2002), rice (Xu *et al.*, 1997), barley (Konishi *et al.*, 1992), oat (Pawlowshi *et al.*, 1998) and grain sorghum (Pereira *et al.*, 1994) including apple (Liebhard *et al.*, 2002). According to Vogl and Xu, (2000), chromosomal regions, referred to as segregation distortion loci (SDLs), cause systematic deviations from the expected segregation ratio. This normally happens in the early stages of life. Besides the presence of SDLs as mentioned above, genotyping errors may also give rise to segregation distortion because these errors cause systematic deviations from the expected segregation ratio (Sibov *et al.*, 2003). This phenomenon of segregation distortion is known to be the source of spurious linkage (i.e. inversions in the order or positions of markers) as demonstrated in studies by Lorieux *et al.*, (1995a,b). We therefore postulate that the inversions of the marker positions in our 'Carmine' x 'Simpson' map might have been partly caused by this phenomenon of skewed segregations. We further postulate that the relatively small number of the seedlings might have contributed to the inversion of the positions of those markers. This has been suggested in some simulation studies on the effect of population size and genome saturation with molecular markers on the reliability and accuracy of

genetic maps (Ferreira *et al.*, 2006). As much as the large number of markers is an essential factor to be considered for obtaining a reliable map, the population size is also an important factor. Maybe by genotyping a larger population size [i.e. n = 100-200], as suggested by Liu, (1998), we might have achieved a more reliable map – i.e. a map where markers are correctly positioned.

A point to note is that we did not opt for the elimination of those markers that show segregation distortions to solve our inversion challenges, as suggested in many reports. The reason was that the elimination of more markers would have exacerbated the situation by further reducing the saturation of the map. That might, in the future, affect any possibility of QTL identification as the saturation of the apple genome with transportable SSR markers is known to be the prerequisite for accurate QTL identification (Gardiner et al., 2007).

6.5 Conclusion

Poor or non-specific amplification is one common problem encountered during microsatellite amplification. The optimization of both simplex and multiplex PCR conditions, as presented in this study, provides the basis to solve this problem. In this study, we have demonstrated that: 1) SSR markers are multi-allelic, informative and transferable between cultivars and breeding progenies; 2) multiplex PCR technique enhances the efficiency of large-scale SSR genotyping; 3) multiplex PCR, together with fluorescence-based, semi-automated sizing technology have the advantage of reducing the unit cost of the SSR assay.

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