Genetic analysis for resistance to Woolly Apple Aphid in an apple rootstock breeding population

Mapurunyane Callies Selala

A thesis submitted in fulfilment of the requirements for the degree of

Masters in Science in the Department of Biotechnology, Faculty of Science,

University of the Western Cape

Supervisor: Prof DJG Rees

January 2007

ABSTRACT

Genetic analysis for resistance to Woolly Apple Aphid in apple rootstock breeding populations

MC Selala

MSc Thesis, Department of Biotechnology, Faculty of Science, University of the Western Cape.

The Woolly Apple Aphid (WAA) *Eriosoma lanigerum* (Hausm.) (Homoptera: Aphididae) is economically one of the most important pests in apple commercial production in the Western Cape province, South Africa. The apple cultivar Northern Spy possesses a single major gene (*Er*1) responsible for *E. lanigerum* resistance. This cultivar has been used as a commercial rootstock in apple breeding programmes. There are other genes also implicated in resistance to *E. lanigerum* from other cultivars. Manipulation and pyramiding of the *E. lanigerum* resistance genes (*Er*1, *Er*2 and *Er*3) might provide a necessary control for commercial apple production. The aim of this study was to construct a genetic linkage map for apple using microsatellite markers. The use of marker-assisted selection would greatly benefit local apple breeding programmes. Ninety six seedlings from a Northern Spy × Cox Orange Pippin mapping population were used for genetic linkage construction.

Phenotypic data collection and analysis were performed to determine the *E. lanigerum* infestation patterns and the levels of resistance conferred by the *Er*1 gene from Northern Spy using 52 *in vitro* propagated seedlings in the greenhouse. Classification and quantification

analysis showed association patterns between first assessments (30 days) to second assessment (60 days) in all replicate blocks. Roots and shoots data showed that it could be useful in quantitative trait loci (QTL) analysis, but may be used in different QTLs being identified due to the variations between roots and shoots data.

A preliminary linkage map was constructed using a mapping population from Northern Spy × Cox Orange Pippin (96 seedlings). Fluorescently labelled published and predicted microsatellite markers were used in map construction. Primers were optimised using single apple cultivar and the detection of polymorphisms using nine apple cultivars. Optimised markers were multiplexed for high throughput data generation using the Polymerase Chain Reaction (PCR) technique. Multiplexed PCR products were pooled and analysed on an ABI 310 PRISM™ Genetic Analyser to determine allele fragment sizes, and the inherited segregation types in the seedlings. Computer software GenoTyper® 2.5.2 and JoinMap® 3.0 was used in data analysis from ABI 310 PRISM™ Genetic Analyser and linkage map construction.

Seventy two markers were used in linkage map construction, which produced nine linkage groups with some segments from the same linkage group. Twenty-one markers were aligned on the map 20 published and one predicted. Only one linkage group consisted of five markers while other linkage groups had two markers each. This study has proved that the preliminary linkage map could be used as the basis of a complete linkage map of Northern Spy × Cox Orange Pippin.

Keywords:

Microsatellite markers

Woolly apple aphid

Optimisation

Multiplex

Genotype

Linkage analysis

DEDICATION: For my family.

DECLARATION.

I declare that *Genetic analysis for resistance to Woolly Apple Aphid in an apple rootstock breeding population* is my own work, that it 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.

Mapurunyane Callies Selala January 2007

Signed: ----------

TABLE OF CONTENTS

LIST OF FIGURES

Page

LIST OF TABLES

Page

ABBREVATIONS

AFLP	**A**mplified **f**ragment **l**ength **p**olymorphism
APS	**A**mmonium **p**er**s**ulphate
bp	**b**ase **p**air
BSA	**B**ovine **s**erum **a**lbumin
cM	**c**enti**M**organ
CP	**C**ross **p**ollinators
CTAB	N-**C**etyl-N, N, N-**t**ri-methyl-**a**mmonium-**b**romide
CIA	**C**hloroform-**i**soamyl**a**lcohol
Df	**D**egree of **f**reedom
DNA	**D**eoxyribo**n**ucleic **a**cid
dNTP	2'-**d**eoxy**n**ucleoside 5'-**t**ri**p**hosphate
DTT	1,4-**D**i**t**hio**t**hreitol
EDTA	**E**thylene **d**iamine **t**etra-**a**cetic acid
et al	et alibi
kb	**K**ilo **b**ase
LOD	**L**ogarithm of **od**ds
MAS	**M**arker **a**ssisted **s**election
ml	**M**illi**l**itre
mM	**M**illi**m**olar
PAGE	**P**olyacryl**a**mide **g**el **e**lectrophoresis
PCR	**P**olymerase **c**hain **r**eaction

QTL	**Q**uantitative **t**rait **l**oci
RAPD	**R**andom **a**mplified **p**olymorphic **D**NAs
RFLP	**R**estriction **f**ragment **l**ength **p**olymorphism
RNA	**R**ibo**n**ucleic **a**cid
SSR	**S**imple **s**equence **r**epeat
TBE	**T**ris, **b**oric acid, **E**DTA
TE	**T**ris, **E**DTA
TEMED	N, N, N', N'-**T**etra**m**ethyl**e**thylene**d**iamine
Tris	**T**ris [hydroxy] aminomethane
µl	**M**icro**l**itre
µM	**M**icro**m**olar
UV	**U**ltra **v**iolet
v/v	**V**olume per **v**olume
WAA	**W**oolly **a**pple **a**phid
w/v	**W**eight per **v**olume

ACKNOWLEDGEMENTS

I thank Prof. DJG Rees for encouraging me to fulfil my desire to complete this project. In his laboratory, I received training, support, and opportunities that I am grateful to have had. He continues to open pathways that offer the opportunity for greater accomplishment. My gratitude is due to all research assistants, Dr. N Leat, Dr. IF Labuschagné and Ms MM van Dyk for data collection, analysis and technical expertise on using equipments. Special thanks go to SETASA, DFPT, THRIP-dti and NRF for the funding during my studies.

THANKS.

CHAPTER 1. INTRODUCTION

1.1 Importance of apples

Apple (*Malus* × *domestica* Borkh) belongs to the family of *Rosaceae*, genus *Malus* and species *domestica*. It is one of the most important fruits and is consumed in many ways, for example processed or as fresh fruit due to its dietary values. The origin of cultivars of *Malus* is within the region of Asia Minor, the Caucasus, central Asia, Himalayan India and Pakistan and western China, in which at least 25 native species of *Malus* occurred. The domesticated apple migrated through Persia to Europe during the times of civilisation with necessary technological intervention. Then, it became a diverse crop for the last 2000 years (Harris *et al*., 2002). This crop has been affected by diseases and insects in many apple growing regions. The pests such as Woolly Apple Aphid (WAA), *Eriosoma lanigerum* (Hausm) have affected this fruit crop and caused huge economic loses in commercial apple production.

The production of several rootstocks highly resistant to *E. lanigerum* has been used to provide an alternative to the use of chemicals in agricultural product (Knight *et al*., 1962; Giliomee *et al*., 1968). The Northern Spy cultivar has been used commercially for various rootstocks production because it contains a single major resistance gene (*Er*1) for *E. lanigerum* (Knight *et al*., 1962). The aim of apple breeding programmes is to pyramid *Er*1 gene with other minor genes conferring resistance to *E. lanigerum* (Mohan *et al*., 1997; Patocchi *et al*., 2005; Gardiner *et al*., 2006). Gene pyramiding is defined as a process of combining more than two resistance genes against the pest or disease in breeding to increase

the durability of the resistance. The use of DNA techniques such as microsatellites or simple sequence repeats (SSR) markers to map genes associated with *E. lanigerum* resistance will assists apple breeding programmes with identification of the promising individual seedlings possessing the gene of interest a few weeks after germination. The main focus recently was the construction of a genetic linkage map using SSR markers and other techniques such as AFLPs, RFLPs and RAPDs to identify markers linked to gene of interest and other traits (Guilford *et al*., 1997; Liebhard *et al*., 2002; Yamamoto *et al*., 2002; Liebhard *et al*., 2003; Silfverberg-Dilworth *et al*., 2006).

1.1.1 An overview of apple production

The majority of apple fruits are produced in the Western Cape due to the favourable climatic conditions. The other apple fruits produced in other regions of South Africa are mostly sold on the domestic market while the apple fruit produced in the Western Cape region are exported to international markets. In the past four seasons, a large number of apples were exported from 2000-2001 to 2003-2004 seasons and declined in 2004-2005 season.

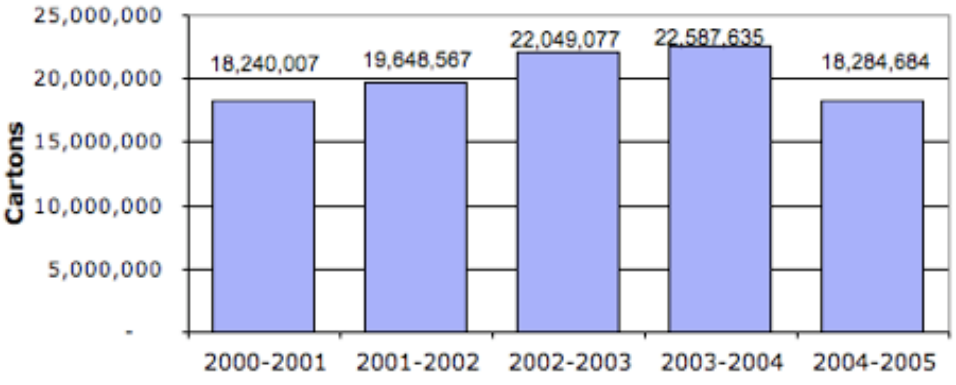


Figure 1.1. Number of cartons of apples exported per season in South Africa. Deciduous Fruit Producers' Trust of South Africa in 2005 (unpublished data)

The number of cartons of apples produced has shown that the total production has fluctuated between seasons (Table 1.1). This could be caused by several factors such as drought, pests and diseases. However, more losses might have been encountered due to currency fluctuation in the year 2004-2005 financial markets than 2003-2004 (unpublished data). A decline in productivity in 2004-2005 might have been affected by low rainfall short in 2005.

Table 1.1. Total apple production from 1991-2005 seasons.

Year	Total production	Local market*	Exports	Processed	Dried
October – September	ton	ton	ton	ton	ton
1991/1992	559,077	187,500	221,250	147,500	2,827
1992/1993	599,316	175,000	253,750	167,500	3,066
1993/1994	632,835	211,250	210,000	210,000	1,585
1994/1995	640,893	226,250	225,000	187,500	2,143
1995/1996	578,711	210,000	213,750	153,750	1,211
1996/1997	704,157	246,250	208,750	247,500	1,657
1997/1998	696,727	251,934	234,573	208,720	1,500
1998/1999	628,619	241,267	185,678	199,826	1,848
1999/2000	692,181	296,193	165,879	229,087	1,022
2000/2001	673,848	248,466	228,199	195,571	1,612
2001/2002	626,107	192,433	245,584	187,290	800
2002/2003	701,663	174,220	273,507	253,046	890
2003/2004*	756,144	227,166	281,998	245,948	1,032
2004/2005*	658,940	237,067	226,614	194,459	800

Deciduous Fruit Producers' Trust of South Africa, 2005 (unpublished data). * = The star superscript means that the results were not completed in the year indicated.

When apples were compared with other deciduous fruit exported in 2004-2005, it was seen that there was higher production of apples than all other deciduous fruit types in South Africa (Table 1.2). Though apple production was lower in comparison to previous seasons, exports were actually increased.

Table 1.2. Export, local market and processed statistics for deciduous fruit types in 2004-2005 seasons.

Item	Local tons	Export tons	Processed tons	Dried tons	Total tons
Apples	240 336	281 998	232 778	1 032	756 144
Pears	45 170	166 648	109 448	3 378	324 644
Grapes	29 927	239 500	-	158 064	427 491
Plums	12 239	47 085	2 545	-	61 869
Peaches & Nectarines	35 576	7 740	172 413	6 387	222 116
Apricots	2 112	5 024	74 742	9 504	91 382

Deciduous Fruit Producers' Trust of South Africa, 2005 (unpublished data).

1.1.2 Pathogens of apples

Apple has been under continuous selection since the start of agriculture (Janick *et al.*, 1996). It has been affected by many environmental stresses such as pests and diseases. There are a variety of pests and diseases associated with apples such as: apple scab, powdery mildew, woolly apple aphid, codling moth, fire blight, bitter rot and crown rot. They are regarded as the main concern in the apple industry in different parts of the world. They reduce the commercial value of apples in the market and contribute to operational costs.

Plants use resistance genes as a defensive mechanism against pathogens. Plants can respond to pathogens by producing proteins that recognise the proteins produced by the pathogen and thus at least in part, reduce pathogenicity (Staskawicz, 2001). This form of gene-for-gene defence implies that plants contain a single dominant resistance gene or multiple genes that produce a specific protein that recognise complementary avirulence genes in the pathogen

(Van der Biezen and Jones, 1998). In some cases many genes can be involved in resistance to same pathogen. If the infected plant lacks resistance genes, the pathogen will cause disease outbreak. This latter event can be more or less controlled by chemicals such as pesticides or fungicides.

1.1.3 Effects of Chemical residues in fruit crops

The use of chemicals against pests and diseases is of major concern in commercial fruit production because chemicals are expensive and environmentally unfriendly. Different methods of detection such as high performance liquid chromatography (HPLC) are being modified due to increased sensitivity required in the chemical analyses of food crops (Mensah *et al*., 1997). Other methods such as gas chromatography and liquid chromatography are also being phased out since they appear error prone and labour intensive. In addition, they do not allow for simultaneous detection of multiple chemical residues at any given time (Ferrer *et al*., 2005; Xiaoliang *et al*., 2006). Due to the seriousness of health care concerns, a multi-residue analysis method to evaluate maximum of 15 chemicals at the same time is used in crop production (Ferrer *et al*., 2005). This method uses liquid chromatography-time-of-flight mass spectrometry (LC-TOF-MS) and it can determine chemical quantity and precise structure of the chemical residue. The consumers prefer apple products with minimal amounts of chemical residues. Maximum residue limit (MRL) recommended in apples and pears by European Union (EU) regulation is 0.01-0.02 mg/kg and 0.02-0.05 mg/kg range respectively (Lacassie *et al*., 1998a; Lacassie *et al*., 1999b). Therefore, adapted and disease resistant cultivars will bring about reduced spraying costs and strike a balance in producing high quality apples for the commercial market's needs.

Breeding apple varieties with multiple, durable resistance will provide an alternative control to the environmentally unfriendly pesticides (Stankiewicz-Kosyl *et al*., 2005).

1.1.4 Apple breeding strategies

Conventional breeding has been used in apple breeding programmes to improve the quality of apple production. The method such as backcrossing has been used to transfer resistance genes or other traits to susceptible cultivar and cultivar with deficiency trait. Due to long juvenile time period (time taken from seedling to fruiting) of apple, the method was labour intensive and time consuming because it could take 10 years before the trait could be observed.

Molecular techniques can be used in apple breeding (e.g. for the identification of woolly apple aphid resistance cultivars) to assist in the identification of individuals with the desired genes. Molecular techniques such as microsatellites or simple sequence repeats (SSR), amplified fragment length polymorphisms (AFLPs), restriction fragment length polymorphic (RFLPs) and random amplified polymorphic DNAs (RAPD) are used to identify DNA markers that can be used in plant breeding programmes through linkage with favourable genes. Plants that have the ability to resist infection by pests using resistance genes to combat the toxicity or morbidity produced by pathogens are referred to as resistant. Molecular technology can provide tools for the selection of these naturally occurring genes that might be systematically pyramided in a cultivar and used as a defensive barrier against pests in apples and other related species.

The construction of linkage maps using DNA molecular techniques will be essential in the future for apple breeding programmes, as marker-assisted selection can be utilized. Individual seedlings possessing a gene of interest can be identified within weeks after germination by using linked molecular markers. DNA marker techniques rely on informative markers to map major or minor genes that confer resistance against pests and diseases.

1.2 Woolly apple aphid (WAA)

Eriosoma lanigerum (Hausm.) (Homoptera: Aphididae) is a pest of apple trees (Crane *et al.*, 1936) that has spread with nursery materials to almost every apple growing country (Georgala, 1953). It is not known when WAA was first introduced into South Africa, but by the year 1894 the pest was already being regarded as destructive in commercial apple production (Georgala, 1953). This insect is not necessarily a leaf-feeder but it attacks one to two year old apple trees at axils, pruning and hail wounds (Asante, 1994). *E. lanigerum* is a bark feeder and it causes injuries by inserting its stylets through the bark and sucking sap from the host plant. The nymphs and adult aphids infest both roots and stems at tender places on the trunk and branches and damaged areas on apples (Asante, 1994). They can be recognised by whitish woolly structures on the axils of the infested apple tree and the colonies themselves are brownish in colour. These woolly structures serve as protection against its natural enemies.

Eriosoma lanigerum's feeding mode leads to gall formation on the roots and lateral shoots. Fungi can invade these galls, when they burst and the wood is exposed, causing perennial canker. The infestation of the roots causes significant reduction in growth and the heavily

infested shoots crack and most of the distorted buds are destroyed. *Eriosoma lanigerum* also infests mature trees causing honeydew exudation from the infected part to drop on the fruit and this allows fungi to colonise. This degrades the quality of the apple and lowers its market value. Generally, infestation by *E. lanigerum* reduces sustainability of the seedlings, weakens mature trees and leads to loss of vitality, and also to poor qualitative and quantitative yields (Asante, 1994).

1.2.1 WAA life cycle

The life cycle of *E. lanigerum* in South Africa it is not well understood but it appears as if it propagates itself entirely by parthenogenesis (Georgala, 1953) which is a form of reproduction in which the females do not have to be fertilised (Figure 1.2). During cooler conditions in South Africa females overwinter on suitably protected spots on the tree, both on the canopy and underground parts. In the environment where it originated (America), aphids lay eggs on the bark of elm trees during autumn and these eggs hatch in spring. The newly hatched aphids migrate to the apple trees. Due to the absence of elm trees in South Africa, the cycle during which eggs are laid does not take place. In early summer *E. lanigerum* gives birth to nymphs, which quickly migrate looking for suitable spots to feed on. During the summer period more generations are produced and the females are produced with wings, allowing them to migrate to another apple tree to start a new infestation (Georgala, 1953). This mode of reproduction might have contributed to the low level of genetic divergence of *E. lanigerum* observed in the apple growing regions in the Western Cape (Timm *et al.*, 2005).

It is proven that tissue infestation by *E. lanigerum* is not equally distributed; shoots are attacked less than root systems. Infestation of the shoots can be consistently estimated from rates of increase in the size of colonies, whereas assessment of the infestation of the roots has to be performed differently because colonies are disturbed during root unearthing. This can be explained by the fact that infestation for roots and shoots is not comparable on a quantitative scale, but the overall observation is that infestation of shoots is lower than that of the roots (Sen Gupta and Miles, 1975).

1.2.2 Biological control of *E. lanigerum*

The introduction of the wasp *Aphelinus mali* in South Africa as a measure to control *E. lanigerum* in 1920 was a success for many years in the apple industry (Lundie, 1939). There are other parasites that are implicated in contributing to biological control of *E. lanigerum* such as lady beetles, syrphid fly larvae and green lacewings (Beers, 1993). Among these different insects *A. mali* has been widely used in orchard practice. This insect has several admirable characteristics such as a short life cycle, unique host selection, the ability to survive for a lengthy period in a cold environment and resistance to orchard sprays. These characteristics make it an important predator. The wasp kills *E. lanigerum* by piercing a hole in the body wall of the live aphid with its ovipositor with which it deposits an egg into the body of the aphid (Figure 1.3). The insect attaches itself to the aphid, which has little chance of avoiding attack as the aphid has its mouthparts attached to the tissue of the apple plant. Three days after injection, a larva emerges from the egg and starts feeding on aphid body fluids until these are exhausted. Ten days after a fully-grown larva purpariates and kills the aphid, but before killing the host it makes a hole in the aphid body cavity. During that time

the aphid becomes restless and moves a distance from its original site and it may end up on a plant other than the apple. In many cases the movement of the aphid before its death creates a confusion of whether *A. mali* was introduced specifically for aphids on apples or it came from other plants that are at close proximity. Wasps suppressed the aphid population to such an extent that spraying against aphids became unnecessary under most South Africa conditions (Lundie, 1939). This method was successfully used until introduction of the pesticide DDT (dichloro-diphenyl-trichloroethane). This insecticide is used to control the Codling Moth, but it also kills *A. mali* and consequently *E. lanigerum* insect numbers increased dramatically, which led to the destruction of apple plants during the growing season. Once the process of Codling Moth control is completed the level of aphids would be extremely high and the damage to the apple tree would already been done (Nel, 1985).

Another factor which makes aphid control by *A. mali* difficult is that only above ground infestation can be efficiently controlled, infestation of underground structures is hard to control and the infested roots will retain a reservoir of *E. lanigerum* (Janick *et al*., 1996). Therefore, this method proved difficult and unreliable to use. Apple breeders chose to use resistance rootstocks for pest or disease as a natural barrier.

1.3 Quest for durable resistance in apples

The fundamental problem with the use of single natural resistance genes in plant breeding is a lack of durability. For example a single resistance gene in the Northern Spy was overcome by *E. lanigerum* after a period of time in the Elgin area (Giliomee *et al*., 1968). The transient nature of resistance is due to development of new strains of pests that overcome the defensive

barrier of the plant. This problem prevents the improvement of yield potential of apples and continuing effort is required to replace old cultivars whose resistance has been overcome with new resistant cultivars (Lamberti *et al*., 1981). Durable disease and pest resistance can be defined as resistance that has remained effective whilst the cultivar possessing this defensive barrier has been widely cultivated in an environment favouring the pest or the disease. This has been widely used in crops by incorporating genetic diversity of the minor or major resistance genes, creating the possibility that different genes can be pyramided (Section 1.1) for resistance against a single pest or disease (Mohan *et al*., 1997). It is important for cultivars in commercial markets to remain resistant so that they retain their commercial value (Hand *et al*., 2003). Unfortunately for apple breeders the pests are adaptive and continually evolve from the parent strain to overcome resistant genes (Cook, 1998).

Pyramiding of different resistance genes into the single cultivar is a reliable way to create cultivars with durable apple disease resistance (Patocchi *et al*., 2005; Gardiner *et al*., 2006). The three resistance genes were discovered from Northern Spy (*Er*1 gene), Robusta 5 (*Er*2 gene) and Aotea (*Er*3 gene). The genes were identified using DNA markers and knowing their position on the genetic linkage map will be important for the apple breeding programme (Gardiner *et al*., 2006). Two genes *Er*1 and *Er*3 were mapped close to *PI-w* on linkage group 8 (LG 8) of genetic linkage map (Gardiner *et al*., 2006; Durel *et al*., 2006). The priority will be to saturate LG 8 with more microsatellite markers in order to identify markers linked to the genes. These resistance genes (*Er*1, *Er*2 and *Er*3) have shown difference levels of resistance with *Er*3 gene showing the lowest resistance amongst them and *Er*1 gene the highest (Sandanayaka *et al*., 2003).

1.4 Resistance to WAA in apple

There are several apple cultivars that have proven to be resistant to *E. lanigerum* under numerous environmental conditions such, as Northern Spy, Winter Majetin and M793. Northern Spy and its derivatives possess a single major *E. lanigerum* resistance gene (*Er*1) (Knight *et al*., 1962). This cultivar is commercially regarded as resistant and, when tested together with other varieties, has shown a higher degree of resistance transmission to the seedlings when used as a parent in breeding lines (Knight *et al*., 1962). *E. lanigerum* does not reproduce but stimulates gall formation on these cultivars (Crane *et al*., 1936).

When Winter Majetin is crossed with susceptible cultivars it gives no clear indication of resistance or susceptibility from the segregation. Northern Spy is regarded as being resistant to *E. lanigerum* although in some instances it showed resistance and became completely susceptible according to Knight *et al*. (1962). It does however have an acceptable degree of resistance under orchard management control. Northern Spy derivatives showed resistance variation depending on the type of strain. The proposed involvement of the resistance *Er*1 gene from the scion cultivar (Northern Spy) was based on the reclassification of symptom classes as used by Knight *et al*. (1962). This variety has been used in breeding programmes for commercial rootstocks, especially in Australia and South Africa, where root damage is prevalent. Northern Spy has been used as a standard parent in *E. lanigerum* resistance breeding because of its genetic makeup and also as one of the parents for the Malling-Merton (MM) series of *E. lanigerum* resistant rootstocks (Cummins *et al*., 1981). Resistance to *E. lanigerum* however does not make it a parent of choice because of its undesirable characteristics such as susceptibility to mildew and lack of appealing characteristics such as size, colour and flavour (Crane *et al*., 1936). These traits made Northern Spy useful as a

standard parent crossed with susceptible cultivars possessing commercially appealing qualities.

Although Northern Spy is commercially regarded as resistant, some *E. lanigerum* strains still infest the cultivar to a certain degree and this implies that the cultivar is not absolutely resistant to the pest (Knight *et al*., 1962). Northern Spy showed varying degrees of damage from strains from different regions, although observations showed the same degree of infestation. Giliomee *et al*. (1968) showed that the propagation of the pest is not necessarily affected in any way in a plant, but the plant showed lower levels of tolerance. Tolerant cultivars are not affected by pest propagation, resulting in little effect on plant survival. Plant breeders are not aiming at using tolerant varieties in breeding programmes, since such varieties may provide a resource of infection for other less tolerant varieties (Russel, 1978).

The first infestation by *E. lanigerum* on Northern Spy, Merton and Malling-Merton rootstock was observed in South Africa 1964/1965 at an experimental farm of the Fruit and Food Technology Research Institute in the Elgin apple district. The year after *E. lanigerum* infestation on Northern Spy and its derivatives, experiments were conducted under more controlled conditions to establish differences in degree of resistance of the various clones. It was discovered that this specific *E. lanigerum* strain had evolved in Elgin to overcome the resistant nature of Northern Spy (Giliomee *et al*., 1968). A recent study has confirmed that the strain from this area has slight genetic divergence compared to strains from the neighbouring regions in the Western Cape (Timm *et al*., 2005).

It has been hypothesized that other susceptible varieties might show different levels of resistance as a result of resistance genes being expressed at lower levels. When these varieties are crossed with Northern Spy, their levels of resistance could be coupled to reach expression levels that will result in strong resistance (Crane *et al*., 1936). The resultant mapping population can be screened for *E. lanigerum* resistance at an early stage in the greenhouse by placing a short piece of infected shoot at the base of the rootstock. When the first round of infestation is done the procedure is repeated after 4-8 weeks to re-infest all the seedlings that have escaped infection. This method has been used to mimic natural infestation in the field but is time consuming and unreliable as some seedlings escape infestation (Bus, 1994; Bus *et al.*, 2000).

1.5 Molecular marker techniques

Molecular markers can be defined as variable DNA sequences that serve as easily locatable points of reference on the genome. These markers are used as tags for specific genes located near them and are easy to identify rather than having to identify the actual resistance genes. The application of DNA molecular marker technology has the potential to make a great improvement in the way apple-breeding programmes are operated by using it in cultivar improvement, pest or disease resistance and cultivar identification. The use of molecular techniques in apple breeding programmes will assist in identifying inferior or deficient and superior characteristics and also facilitate the breeding of new cultivars with commercial market requirements (Kumar, 1999). Molecular marker techniques make it possible to identify markers linked to the desirable genes from related wild varieties and introduce them into the targeted varieties, for example using a backcrossing technique and marker assisted

breeding. It is possible for polygenic characters to be analysed by high-resolution genetic maps using molecular markers and this is a significant advantage over traditional plant breeding methods (Mohan *et al*., 1997).

There are quite a number of molecular marker techniques that can be used for genomic linkage map construction and linkage analysis. These include simple sequence repeat (SSR) or microsatellites, amplified fragment length polymorphisms (AFLPs), random amplified polymorphic DNAs (RAPDs) and restriction fragment length polymorphism (RFLPs) (Table 1.3). All these molecular marker techniques need genomic DNA as a standard requirement for their application. Molecular markers have been used to map genes of minor or major economic importance in a wide range of species and that was the beginning of marker-assisted selection (MAS) (Mohan *et al*., 1997). However, these techniques have shown several advantages over the traditional phenotypic markers that were previously available to plant breeders. Phenotypic markers can only be used at certain periods during the year when the expression levels of certain genes linked to these characteristics are highly expressed. They are influenced by environmental factors and also subjected to post-translational modifications, but DNA markers (or genotypic markers) can be used at any time during the year (Kumar, 1999).

Table 1.3. A comparison of the main features of molecular marker system.

	RAPD	SSR	AFLP	RFLP
Principle	Random PCR amplification of genomic regions	PCR amplification of microsatellites	Restriction digestion, adapter annealing, selective PCR	PCR amplification of restriction digestion
Nature of polymorphism	Base changes, insertions, deletions	Variation in repeat length	Base changes, insertion, deletions	Base changes, insertion, deletions
Level of polymorphism	Medium	Very high	Medium	Medium
Abundance	Very high	Medium	Very high	High
Dominance	Dominant	Co-dominant	Mixed	Co-dominant
Multiplex ratio	5-20	1	50-100	1
Sequence information required	No	Yes	No	Yes
Costs	Low	High	Medium	High

Modified from Breyne *et al*. (1997)

Breeders cross two different varieties with one cultivar carrying the gene of interest and the other lacking it, in order to generate a mapping population. PCR followed by analysis using polyacrylamide gel electrophoresis or capillary electrophoresis can be used to determine the absence or presence of the gene of interest in individual seedlings. Any deviation observed in alleles acquired from parents may be the result of undesirable cross-pollination occurring in outbreeding species (Ortiz *et al*., 2001). This compels breeders to perform the fertilisation process under a more controlled environment to avoid undesirable pollen causing fertilization.

Marker-assisted selection (MAS) is a technique used to infer the possibility of the presence of a gene by identifying markers closely linked to the gene of interest. If there is any correlation between a marker and the gene of interest, then the gene and marker are linked (the "higher" the correlation, the "tighter" the link between the marker and the gene of interest). This increases the prediction power of MAS because the marker and gene are closer (Gardiner *et al*., 1999). It is also useful in quantitative trait loci (QTL) analysis as many loci can be screened at the same time. Most of the molecular markers techniques complement each other depending on their application because there is no ideal technique that has proved useful to a range of investigations. For example a genetic linkage map generated from microsatellite markers need to be saturated by AFLP makers because SSR markers alone leave huge spaces on the chromosomes.

1.5.1 Restriction fragment length polymorphism (RFLP)

RFLP is a hybridization-based molecular technique in which restriction enzymes cleave DNA at precise points producing DNA fragments. The DNA fragments are separated according to molecular size using gel electrophoresis. These fragments can be visualized with labelled probes to detect polymorphisms. If the probe gives a strong hybridisation signal then a number of fragments are revealed and also polymorphism is detected between the different DNAs in question. RFLPs are informative and co-dominant but requires a larger amount of high quality DNA compared to PCR markers. This technique is labour intensive and time consuming because of procedures such as Southern blotting (Kumar, 1999). This technique was developed to generate a complete linkage maps in many organisms (Botstein *et al*., 1980; Lander and Botstein, 1988).

Although the technique has been used in genetic mapping it remain technically complex, labour intensive and difficult to automate and technically demanding when used in routine breeding application (Reiter, 2001; Botha *et al*., 2004). Generally, RFLPs represent a single copy sequence; if there is a small amount of target DNA it will be difficult to detect any signal from bands after hybridisation to the filter. However, to achieve meaningful results there must be complete digestion of the DNA to avoid false results on the gel. Partial digestion gives inconsistent results that cannot be mapped (Young, 2001).

1.5.2 Random amplified polymorphic DNA (RAPD)

RAPD is a PCR-based technique used to amplify a specific region of genomic DNA and the products are resolved on an agarose gel to differentiate banding patterns for individual species. Short oligonucleotides primers of 9 to 10 base pairs are used to amplify specific sites of unknown sequence profiles. Those sequences differ in length; amplification stringencies and differences produced are resolved by their banding pattern on a gel (Kumar, 1999). RAPD markers detect a larger number of genetic polymorphisms on major amplification products. The potential use of RAPD technology as a reliable, rapid, inexpensive screening technique for genotyping is limited by inconsistencies in experimental conditions (Schiliro *et al*., 2001; Doherty *et al*., 2003).

The drawbacks of RAPD are the lack of reliability and reproducibility, this means the DNA fragment from one cultivar can be amplified but not from another cultivar (Breyne *et al*., 1997). The RAPD technique produces small fragments, which bring about a challenge in

determining the presence or absence of bands on an agarose gel due to poor resolution. In this technique minor changes in the reaction conditions lead to altered amplification which in turn might cause misleading interpretation of the data (Tenzer *et al*., 1999).

1.5.3 Amplified fragment length polymorphism (AFLP)

AFLP technology is a technique based on the detection of restriction fragments by PCR amplification that can arguably be perceived as a combination of PCR and RFLP technologies together. Firstly, genomic DNA is completely digested with restriction enzymes and then double stranded adapters are ligated to the ends of the DNA fragments to serve as the template for PCR amplification (Figure 1.4). The adapter sequences and adjacent restriction site serve as primer binding sites for restriction fragments amplification of the two enzymes, one a rare cutter (6 bp) and one a frequent cutter (4 bp). This technique results in predominantly amplified restriction fragments that have rare cutter sequences at one end and frequent cutter sequences at the other end.

- Frequent cutters generate smaller DNA fragments that amplify well and can be electrophoresed on a 6 % polyacrylamide gel.
- Using a rare cutter will reduce fragment numbers and only fragments with cutter sites (rare cutter/frequent cutter) are amplified.
- By using two restriction enzymes it is possible to label one strand of the double strand PCR products. This prevents repetition of bands on the gel of unequal migration of the two strands.
- The use of two restriction enzymes gives flexibility in the number of fragments to be amplified.

- Various combinations can be generated from these large numbers of DNA fingerprints using various selective primers, different in 3′ terminus nucleotides.

There are two frequently used restriction enzymes, *Mse*I as a frequent cutter and *Eco*RI as a rare cutter. The use of *Mse*I is preferred in the AFLP technique because this enzyme cuts frequently in eukaryotic genomes producing fragments of optimal size for PCR amplification and separation on a polyacrylamide gel. The second enzyme, *EcoRI* is preferred because of its reliability as six-cutter enzyme that limits problems related to the incomplete restriction in AFLP fingerprinting. AFLP fingerprinting with incomplete digestion show some unexpected fragments on polyacrylamide gel (Vos *et al*., 1995).

The technology allows a rapid generation of markers that are randomly spread over the genome or confined at specific region with high level of polymorphism. Furthermore, it has been suggested that the use of AFLP is the most efficient method for the generation of larger number of markers linked to a gene of interest (Xu *et al*., 1999).

1.5.4 Microsatellite markers

Microsatellite markers or simple sequence repeats (SSR) are a molecular marker technique based on the length of the motif sequence. They are defined as tandem repeat sequences of DNA bases or nucleotides that contain mono-, di-, tri- and tetra- nucleotide repeats, for example:

Mononucleotide: AAAAAAAAA referred to as a $(A)_9$ motif

Dinucleotide: GAGAGAGAGAGAGA referred to as a $(GA)_7$ motif

Trinucleotide: CTGCTGCTGCTGCTGCTGCTGCTG referred to as a $(CTG)_8$ motif

Tetranucleotide: CTAGCTAGCTAGCTAGCTAG referred to as a $(CTAG)_5$ motif

Microsatellite markers provide a more reliable method for genetic mapping due to their co-dominance, high level of polymorphism, abundance, hypervariability and transferability. They are also multi-allelic due to the variation in the number of repeat units. Polymorphism of trinucleotide repeats was easy to distinguish than the dinucleotide repeats because of the minimum length different between alleles from trinucleotide and dinucleotide. Microsatellite markers can be used in QTLs mapping for apple breeding programmes (Gianfranceschi *et al*., 1998; Yamamoto *et al*., 2002). These markers have been obtained through searches in public databases such as Genbank and EMBL databank or by screening genomic libraries with synthetic oligonucleotides (Ma *et al*., 1996). The reason being that microsatellites are widely distributed on both coding and non-coding regions of the genome (Rakoczy-Trojanowska and Bolibok, 2004).

1.5.4.1 Nature of microsatellites

Microsatellites have interesting characteristics that make them the markers of choice in both plants and animals because of their suitability for automation and high throughput of information (Gianfranceschi *et al*., 1998). They are transferable across closely related species (for example between apples and pears) and also different research laboratories can exchange SSR markers because each locus is defined by its sequence. The transferability of SSR markers depends entirely on the conservation of the flanking region and stability of the repeats during evolution (Holton, 2001). When the SSR marker was assayed through PCR it was possible to obtain different fragment sizes between apple and pear because of differences in repeat numbers. Interestingly, regions of DNA sequence flanking microsatellites in apple

and pear are highly conserved, facilitating the cross species use of SSR markers (Yamamoto *et al*., 2001).

1.5.4.2 Distribution of microsatellites

SSR repeats are found throughout the genomes of most eukaryotic plants and they have high variation in the number of repeat units. The variation is caused by slippage of DNA polymerase during DNA replication or unequal recombination, and this result in differences in the genome sequence (Tenzer *et al*., 1999). This can lead to high levels of polymorphism in the genome, caused by insertions or deletions of one of the repeat units.

SSRs in plant genomes appear to be less abundant than in mammal or insect genomes (Barrier *et al*., 2000). It was shown that microsatellites are distributed at an average of 33 kb in plant nuclear genomes, whereas in humans it is found approximately every 6 kb. Interestingly, more studies were done to compare motif occurrence in some plants. In apple the CT repeat once occur every 120 kb on average, in peach once every 100 kb and in rice once every 225 kb. The CA repeat in apple occurs every 190 kb on average and less frequent in both peach and rice at 420 kb and 480 kb respectively (Canli, 2004).

1.5.4.3 Multiplexing SSR markers

Multiplex PCR is a convenient screening technique that enables many targets of interest to be simultaneously amplified in a single reaction. Development of such high throughput techniques can accelerate the collection and analysis of data for linkage analysis studies. Microsatellite markers can be fluorescently labelled with different dyes, thus facilitating the

multiplexing of the maximum number of markers for analysis on systems such as the ABI 310 PRISM™ Genetic Analyser (Clark *et al*., 2004). Markers can be multiplexed in terms of expected fragment sizes from each locus provided there is no overlap between markers labelled with the same fluorescent dye colour. Multiplexing minimises the expenses, time and genetic material required to collect genotype information to be used in linkage analysis.

A common problem in multiplex PCR is associated with preferential amplification of one target fragment over others. This problem is attributed to the sequences that differentiate between a "good" and "bad" marker. However, as more loci are simultaneously amplified, competition in the pool of enzyme and nucleotides becomes a limiting factor. During optimisation of multiplex PCR, reagents must be adjusted particularly the amount of $MgCl_2$, dNTPs, Taq polymerase and reactions buffer. Other factors like temperature and annealing time also play a crucial role. The effects of the above mentioned requirements play a pivotal role in multiplex PCR because many loci are amplified between 56 ºC–60 ºC (Markoulatos *et al*., 2002). In multiplex PCR the annealing temperature must be dropped so that all target fragments can be amplified. Although, non-specific amplification occurs, it is overcome by an increased number of specific loci in the multiplex reaction. Similarly, when many loci are amplified simultaneously, the loci that are efficiently amplified yield more dominant product than less efficient loci (Henegariu *et al*., 1997).

1.6 Genetic linkage map

A genetic linkage map is defined as the representation of the relative positions of genetic loci on the chromosomes. These positions of the genetic loci are determined by segregation

probability, that is of how often they are inherited together or separated by genetic recombination events. The markers are sequentially aligned to the genetic linkage map and are linearly ordered to reflect the chromosomal structure (Jansen *et al*., 2001). The distances on a genetic linkage map are measured in units called centiMorgans (cM) that define relative distances between markers in which recombination occurs with a frequency of one percent (1 %). A recombination frequency of 1 % means only one in 100 offspring is the combination of two markers different from that in their parents. In contrast, markers that are far apart on the same chromosomes or those that are on different chromosomes are equally to be transmitted together or separately. This would have a recombination frequency of 50 %.

The utilisation of genetic linkage maps in plant breeding facilitates the genomic localisation of genes responsible for monogenic and quantitative trait loci (QTL) by DNA markers (Tanksley *et al*., 1989). It helps in map-based cloning of genes where the products were not known before (Jansen *et al*., 2001; Rouppe van der Voort *et al*., 1999). Linkage maps are also relevant to marker-assisted breeding programmes because regions involved in a specific trait can be linked with known markers.

1.6.1 Genetic linkage map construction

The broader approach of genetic linkage map determination starts with the creation of a F_1 mapping population (seedlings) so that one can study inheritance of genotypes or segregation types by genetic recombination. The mapping population should be created from two different (apple) parents but they must differ in genotype and parents should display high level of heterozygosity (Hemmat *et al*., 1994; Maliepaard *et al*., 1998). A minimum of

hundred seedlings should be enough for data collection, but using a large number mapping population gives accurate and more reliable results.

The software package JoinMap™ 3.0 (Van Ooijen and Voorrips, 2001) was used for the construction of a genetic linkage map. This approach determines the probability of detecting recombination frequency that provides a reliable estimate of tightly linked markers (Cevik and King, 2002). Once the recombination frequencies between loci were determined then the markers were grouped according to their linkage groups. A linkage group is described as a set of markers, where there is strong statistical evidence that markers are linked and possibly residing on the same chromosome. Each linkage group shows different segments of the same group according to different threshold values (LOD score), starting from 2 to 10. LOD stands for logarithm of the odds (to the base 10). LOD score is defined as a statistical estimate of whether two loci are likely to link to each other on a chromosome and are therefore likely to be inherited together as a unit. At LOD score of 2.0 unlinked markers tend to link, but when threshold values are increased weaker linkages separate into subgroups. The strict linkage conditions of a LOD score of at least 3.0 and the recombination distance between linking markers of less than 20 cM, and this would reduce false linkages (Hemmat *et al*., 1994). One segment from each linkage group would be chosen for linkage map construction.

1.7 Apple genetics and genomics

In the recent years apple-breeding projects have been aimed at developing resistant apple cultivars with high fruit quality. Genomic fragments homologous to resistance genes have been sampled in apple and these gene families can be used as sources of markers associated

disease resistance genes (Lee *et al*., 2003; Baldi *et al*., 2004). Research efforts focus on the development of the tools that could enable understanding of functional genomics or proteomics, genetic mapping and micro-arrays. The results of functional genomics will be used to develop novel gene expression markers for molecular assisted apple breeding (Jensen *et al*., 2006).

The most saturated apple linkage maps published so far were from Fiesta × Discovery maps (Figure 1.5) (Liebhard *et al*., 2002; Liebhard *et al*., 2003). The linkage maps were saturated with other DNA markers such as RAPD, RFLPs and AFLPs. Recently efforts have been made to saturate existing apple genetic linkage maps and approximately 300 microsatellite markers have already been mapped on the apple genome. The aim is to identify sufficient microsatellite markers that will cover initial genome genotyping of 100 microsatellite markers with an average distance of 15 cM in between (Figure 1.6). This requires developing apple genetic linkage map of microsatellite markers with less than 20 cM and occasionally allowed to be 25 cM. The preferred map should have marker within every 10 cM on all chromosomes (Silfverberg-Dilworth *et al*., 2006). A minimum of 10 markers per chromosome will be useful based on the framework map of chromosomal average length of 85.6 cM. Microsatellite markers from expressed sequence tag (EST) have been developed in many species such as apples. The use of genomics will improve apple-breeding programmes and also benefit apple linkage maps (Korban *et al*., 2004; Korban *et al*., 2005; Naik *et al*., 2006).

The apple genome project by the HortResearch fruit group in New Zealand, and at the University of Illinois Urbana-Champaign, involves the construction of cDNA libraries from

vegetative, flowering tissues, and tissues responding to pathogen infection (Jensen *et al*., 2006; Korban *et al*., 2005; Newcomb *et al*., 2006). The vegetative tissues were preferred because most phenotypes respond to environment during the growing stages of the plant. The HortResearch fruit database contains over 160719 expressed sequence tags (ESTs) from apples with more than 2000 microsatellite markers (Han *et al*., 2006). The use of microsatellite markers from ESTs can be beneficial as those markers can assist in revealing the location and structure of gene-rich regions in the genome (Naik *et al*., 2006). The database can be used in a wide range of technologies such as micro-array, proteomics and functional analysis. The identification of the location of resistance gene families using molecular techniques in the apple genome will assist in understanding their functions in fruit plants.

`

1.8 Objectives of the study

The objective of this study was to construct an apple (*Malus* × *domestica* Borkh) linkage map from Northern Spy × Cox Orange Pippin (as parents) mapping population (F_1). The Fiesta × Discovery linkage map was as used a reference framework (Liebhard *et al*., 2002; Liebhard *et al*., 2003). The goal was to identify markers linked to the *Er*1 gene responsible for *E. lanigerum* resistance in apple. The specific objectives towards achieving this goal were:

1. Phenotypic data collection and analysis
2. Optimisation of published and predicted microsatellite markers
3. Detection of polymorphisms on optimal microsatellite markers
4. Generation of multiplexes
5. Genotyping

6. Genetic linkage map construction

7. The identification of QTLs for WAA resistance

Figure 1.7 shows a schematic representation of the project strategy for this study.

CHAPTER 2

2.1 Materials and methods

2.1.1 Materials

Apple plant material (leaves) used in this study was collected for the ARC Experimental Farm, Bien Donné in Simondium, Western Cape Province, South Africa.

Apple seedlings from Northern Spy × Cox Orange Pippin mapping population were used in genetic linkage map construction.

2.2 List of chemicals

	Company
500 LIZ™ size standard	Applied Biosystems
Agarose	Promega
Ammonium Persulphate (APS)	Merck
40 % (19:1) Acrylamide: bis-acrylamide	Promega
BioTAQ DNA Polymerase	BioLine
Boric Acid	Saarchem
Bovine Serum Albumin	Roche
Bromophenol Blue	Amersham Life Science
Buffer saturated phenol	Invitrogen
Chloroform	Merck

Dithiothreitol (DTT)	Fermentas
Ethanol 99.7-100 %	Merck
Ethidium Bromide	Sigma
Ethylene Diamine Tetra-acetic Acid (EDTA)	Saarchem
Formaldehyde solution min. 36.5 %	Riedel-de Haen
Formamide	Merck
Iso-amylalcohol	Saarchem
Isopropyl alcohol	BDH
Phenol	Invitrogen
Performance Optimized Polymer 4 (POP-4™)	Applied Biosystems
Propan-2-ol (Iso-propyl alcohol)	Merck
Proteinase K	Roche
RNase A	Roche
Silver Nitrate	Merck
Sodium Borohydride	Saarchem
Sodium Hydroxide	Merck
Spermidine	Sigma
TEMED (N, N, N^1,N^1- tetra methylethelene-diamine)	Promega
Tris (hydroxymethyl) aminomethane	Merck
Urea	Merck
Xylene Cyanol FF	BDH chemicals

2.3 Buffers and solutions

10× TBE	0.9 M Tris, 0.89 M Boric acid, and 0.032 M EDTA, pH 8.3.
1× TBE	90 mM Tris, 89 mM Boric Acid and 3.2 mM EDTA, pH 8.3.
1× TE	10 mM Tris HCl and 1 mM EDTA, pH 7.4.
1 % agarose	1 % agarose (w/v) in 1× TBE.
DNA loading buffer	0.25 % bromophenol blue (w/v), 0.25 % of xylene cyanol (w/v) and 30 % glycerol (v/v) in water.
Chloroform-isoamylalcohol (CIA)	24:1 ratio (v/v).
70 % Ethanol	70 ml absolute ethanol (99.5 %) (v/v) in water.
Proteinase K	20 mg/ml in water.
RNase A buffer	0.1 M sodium acetate, 0.3mM EDTA, pH 4.8.
RNase A (DNase free)	20 mg/ml in RNase A buffer.
2× CTAB buffer	2 % CTAB w/v 1.4 M NaCl, 100 mM Tris-HCl, 20 mM EDTA pH 8, 1 % PVP-40 (w/v) and 2% (v/v) 2-mercaptoethanol in water, pH 8.3.
DTT	325 mM DTT in water.
Formamide buffer	0.5 mM NaOH, 0.029 mM Bromophenol blue, 0.188 mM Xylene, 0.053 mM EDTA and 80 % Formamide (v/v) in water, pH 8.0.
APS solution	10 % ammonium persulphate

Silver stain solutions	Solution B: 5.9 mM $AgNO_3$ in water. Solution C: 375 mM. NaOH, 2.64 mM $NaBH_4$ and 0.4 % Formaldehyde (v/v) in water.
PAGE gel	6 % 19:1 Acrylamide bis-acrylamide, 7 M Urea, 1× TBE, 0.08 % APS and 0.1 % of TEMED.

2.4 Mapping population

The mapping population was produced by hand pollination from Northern Spy × Cox Orange Pippin (104 individuals) at Agricultural Research Council (Bien Donné) plant breeding farm. The cultivar Cox Orange Pippin was used as a pollinator and Northern Spy pollinated in 50 % of the mapping population. The other 50 % Northern Spy was as a pollinator and Cox Orange Pippin pollinated. Ninety six plants were used in this study from initial 104 individual seedlings. Fifty two seedlings of 96 individuals were *in vitro* propagated to produce three blocks of replicate seedling clones. The other 44 seedlings were included in genotypic analysis experiment (Chapter 4). The replicate seedling clones were used for *E. lanigerum* infestation evaluation trials in the greenhouse at Agricultural Research Council (Bien Donné) plant breeding farm towards the end of February 2005.

2.4.1 Phenotypic analysis of infested apple seedling clones with *Eriosoma lanigerum*

Infestation was performed by planting a heavily pre-infested apple seedling (from other breeding crosses) next to each of the replicate seedling clones. Infested seedlings were tied onto these clone seedlings. This allowed *E. lanigerum* to migrate from the infested seedlings

to the seedling clones. Fifty-two Northern Spy clones were planted in one litre plastic planting bags in replicates of three blocks or groups in a greenhouse (20-25 ºC). This means that the each seedling clone was reproduced three times and placed in different groups of three. These seedling replicate clones were available from the initial mapping population of 96 individuals generated for genotypic analysis.

After the first evaluation (after 30 days), all the resistant seedlings were re-infested assuming that they might have escaped infestation. In some instances the pre-infested seedlings died while the infestation process was underway and the infestation compromised. Replanting pre-infested seedlings and tying them to the seedling clones was performed. If the same plant did not show any signs of susceptibility after re-infestation they were classified as resistant.

2.4.2 Data collection and analyses

The response to infestation on seedling shoots was recorded according to a quantitative scoring method and a classification scoring method (23 March 2005). The method of classification and calculation was developed and used at Agricultural Research Council (Bien Donné) plant breeding farm. The quantitative scoring was performed based on the following formula:

$$\frac{\text{Number of leaf axils infested}}{\text{Total number of leaves}} \times 100\ \% = \%\ \text{Infestation}$$

The shoot infestation was classified as follows: class 0 was free from any infestation by *E. lanigerum*, class 1 had visible *E. lanigerum* but no colonisation, class 2 had visible *E.*

lanigerum colonisation with minimal spread on the seedling clones, class 3 seedling clones were heavily infested with *E. lanigerum* and class 4 seedling clones were dead. Root infestation response was also classified and recorded in the similar way as shoot classification method. The only different was that the plants were uprooted before analysis. Data on shoot infestation was collected on two separate occasions (30 days and 60 days), while root analysis was destructive and could only be performed once. After the first round of data collection on shoots (after 30 days) all the uninfected seedling clones were re-infested to minimize the occurrence of mis-classification.

The same number (52) of plants was assessed for root infestation (25 April 2005). When root analysis was performed, the seedlings were uprooted, quantified and classified based on the roots infestation. The classification was based on the presence or absence of *E. lanigerum* on the roots, which was used as a determined factor for susceptible or resistance plants. The presence and the intensity of the whitish woolly material on the roots were classified into classes zero, one, two, three and four. In some instances the roots were too wet and it was difficult to classify the degree of infestation.

2.5 CTAB DNA isolation from apple leaves

Genomic DNA was extracted from apple leaves using the Cetyltrimethylammoniumbromide (CTAB) method (Doyle and Doyle, 1987). Leaves were collected and stored at –20 ºC until analysis. One leaf was put in a clean mortar and liquid nitrogen was added. The leaf was immediately ground using a pestle. The ground material was allowed to thaw at room temperature for about 15 minutes. The ground material was transferred into a 2 ml tube and

1ml of pre-warmed (60 ºC) CTAB was added. The sample was incubated at 62 ºC for 30 minutes to equilibrate. Then 2.75 µl of 20 mg/ml Proteinase K was added to the homogenates and incubated at 37 ºC for 30 minutes. 1 ml of chloroformisoamylalcoholl (CIA) was added, the samples were vortexed briefly and inverted for 10 minutes. The samples were centrifuged at 10 000× g for 10 minutes. 1 ml of the upper aqueous layer was collected and transferred into a new 1.5 ml tubes to which 2.5 µl RNase (10 mg/ml) was added and incubated at 37 ºC for 30 minutes. An equal volume of CIA was added and briefly vortexed, and then the samples were mixed continuously for 5 minutes. The samples were centrifuged for 10 minutes at 10 000× g, and the aqueous layer was transferred into a new 1.5 ml tubes to which 0.6 ml of ice-cold isopropanol was added. The samples were inverted several times and kept at –20 ºC for 20 minutes. The samples were then spun for 10 minutes at 10 000× g and the supernatant was carefully discarded. The pellet was washed twice with 70 % ethanol. The washed pellet was air dried and re-suspended in 100 µl of 1× TE.

In instances where the DNA samples contained proteins and nucleic acids when visualized on the agarose gel, the samples were re-extracted with Phenol:Chloroform (1:1, v:v). In this method an equal volume of Phenol:Chloroform was added to the samples and the tubes were vortexed gently and spun for 10 minutes at 10 000× g. The upper aqueous phase from each tube was transferred into fresh 2 ml tubes and equal volume of Chloroform:Butanol (4:1, v:v), was added. The samples were mixed by inversion and spun for 10 minutes at 10 000× g. The upper phase from each tube was transferred (300 µl) into 2 ml tubes and 200 µl (1/10th of final volume) of 3 M NaOAc (1 mM EDTA) pH 7.4 and 1.5 ml of 100 % EtOH were added and the tubes were incubated for 20 minutes at -20 ºC. After incubation the samples were spun for 10 minutes at 10 000× g at 4 ºC. The supernatant was carefully discarded and

pellet was washed twice with 70 % EtOH, air dried for 15 minutes and dissolved 100 µl 1× TE.

2.5.1 Quantification of DNA

DNA concentrations were estimated from the OD_{260} of the re-suspended DNA (assuming that 1 OD_{260} is given by a DNA solution of 50 ng/µl) using a NanoDrop® ND-1000 spectrophotometer.

2.6 Agarose gel electrophoresis

Samples were analysed on 1 % agarose gel electrophoresis in 1× TBE at 10 V/cm. The DNA was visualized by staining with Ethidium Bromide, illuminating under UV light and photographed on a UVP image system.

2.7 Polymerase Chain Reaction (PCR)

PCR reactions were performed in 1× reaction buffer (0.005 mM of Tris, 0.025 mM of KCl and 0.00075 mM of $MgCl_2$), 1 mg/ml of BSA, 2 mM of $MgCl_2$, 0.1 mM of dNTPs, between 1 and 10 ng of template DNA between 0.016 and 1.4 pmol of primers (depending on the optimal concentration of the primer) and 1 unit of Taq polymerase per reaction in 25 µl with sterile water. Eppendorf Mastercycler® Gradient, Applied Biosystems 2700 and Applied Biosystems 9700 PCR machines were used for PCR reactions. The reaction was performed using the following cycles:

96 ºC for 5 minutes (Initial denaturation)

94 ºC for 40 seconds (Denaturation)
60 ºC for 40 seconds (Annealing) } 35 cycles
72 ºC for 20 seconds (Extension)
72 ºC for 10 minutes (Final extension)

The cycles were followed by incubation at 4 ºC.

2.7.1 Touchdown PCR

Touchdown PCR reactions were undertaken using the standard PCR reaction mix (Section 2.7), with a PCR cycle protocol that reduces the annealing temperature from 60 ºC to 50 ºC at 1 ºC per cycle, followed by 25 cycles with an annealing temperature of 50 ºC. All cycles used a denaturing temperature of 94 ºC and an extension temperature of 72 ºC.

2.7.2 Touchdown gradient PCR

Touchdown gradient were used to optimise primer conditions on an Eppendorf Mastercycler® Gradient. Temperature gradient decreases by 1 ºC in each PCR cycle for 10 cycles. The reaction was performed using the following cycles: (example of 60 ºC to 50 ºC)

96 ºC for 5 minutes (Initial denaturation)
94 ºC for 40 seconds (Denaturation)
60 ºC for 40 seconds (Annealing) } 10 cycles
72 ºC for 20 seconds (Extension)

(After 10 cycles the temperature down by 10 ºC, temperature reduced by 1 ºC with each cycle)

94 ºC for 40 seconds (Denaturation)
50 ºC for 40 seconds (Annealing) } 25 cycles
72 ºC for 20 seconds (Extension)

72 ºC for 10 minutes (Final extension)

The cycles were followed by incubation at 4 ºC.

2.7.3 Multiplex PCR

Fluorescently labelled primers were multiplexed during PCR as a high throughput screening technique. The primers with the same fluorescent dye were multiplexed according to their product sizes. The minimum multiplex made was three or four primer sets per fluorescent dye (fluorescent dyes used were PET, VIC, NED and 6-FAM). Reagents and primers concentration were also optimised. The annealing temperature was reduced to 55 ºC in the multiplex PCR and this would allow all primers to anneal and amplify targeted sites. The extension temperature was also reduced from 72 °C to 65 ºC for 2 minutes to allow long fragments to be fully amplified. The final extension step temperature also was reduced from 72 °C to 65 ºC for 30 minutes to allow all incomplete fragments to be amplified.

2.8 Denaturing PAGE on 6 % polyacrylamide gel

Samples were electrophoresed on a 6 % polyacrylamide gel containing 1× TBE, at 10 V/cm. After electrophoresis the gel was silver stained in solution B for 10 minutes, then washed three or four times with water and the gel was stained in solution C until bands were visible.

2.9 Determination of fragment sizes on ABI 310 PRISM™ Genetic Analyser

PCR products were analysed on ABI 310 PRISM™ Genetic Analyser to determine allele fragment sizes. After completion of the PCR, 3 µl of the PCR product was added to a tube containing pre-mix of 0.5 µl 500 LIZ™ size standard and 25 µl of formamide. These samples were denatured for 5 minutes at 96 ºC and snap cooled in ice water. Samples were analysed using capillary electrophoresis under standard conditions using the POP-4™ polymer. The size standard was used to determine allele fragment sizes based on the peaks obtained from individual samples.

2.9.1 Primer design using Tandem Repeats Finder

Tandem Repeats Finder was used to search for perfect and imperfect repeat sequences (Benson**,** 1999). The necessary filters were used to allow the selection of the repeats, which were important to the primer design. Table 2.1 shows the parameters used for repeat sequence selection. Forward and reverse primers were designed using the regions flanking the repeat, since these regions should be well conserved. Primers were designed such that (i) they end in a G or C, (ii) have a melting temperature (Tm) of about 60 ºC, (iii) they are between 17 and 30 bases long, and (iv) they have a GC content of 50-60 %. The primers were also designed to amplify fragments of between 100 and 500 base pairs. Primers were then synthesised by Applied Biosystems (Foster City CA, USA). The primer sequence located closest to the repeat was labelled with one of four fluorescent dyes viz. 6-FAM, VIC, NED or PET i.e. blue, green, yellow or red respectively. Fluorescently labelled primers make it possible to detect fragment lengths accurately using fluorescence-based DNA detection systems, such as an ABI 310 PRISM™ Genetic Analyser.

Table 2.1. Summary of the filters used for selecting repeat sequences in primer design.

Parameters	Filtering options	Critical limits
Pattern size	=	2, 3 or 4
Score	≥	40
% Matches	≥	90
First Index	≥	20 bases

The pattern size refers to the repeat motif of the sequence. The critical limit of 90 % for percentage matches refers to the number of perfect or imperfect repeats found in a particular sequence i.e. whether or not there are nucleotides present within the repeat which differ from the repeat itself e.g. if a repeat consists of Thymine (T) and Adenine (A) i.e. (TATATATATA), then a limit of 90 % will occur if one of these ten bases are out of sequence i. e. TATATAAATA, where the fourth T is replaced by another nucleotide, in this case an Adenine (A). The sequences with a 90 % percentage match limit have a score of more than 40. Scores of less than 40 tend to generate repeats with more mismatches than what is favoured. The "First Index" in table 2.1 refers to the position in the DNA sequence at which the repeat is found. Usually, the first index is set greater than or equal to 25 bases. This indicates that 24 bases on the left flanking sequence from which to design a forward primer and the 25 positions are where the repeat sequence starts.

2.10 Genotyping analyses

Data from GeneScan® was imported and analysed into GenoTyper® 2.5.2 software, which can handle raw data from capillary electrophoresis. Analysis parameters were set based on the fluorescent dye and fragment sizes obtained from parents used in generating the mapping

population. The fragment sizes from the parents were interpreted as alleles that could be transferred into the segregating population. Each fluorescently labelled SSR marker was scored automatically and unknown genotypes were scored manually. The complete genotyped data was imported from GenoTyper® 2.5.2 software program onto Microsoft Excel. The allele segregation codes used in Genotype® 2.5.2 were changed to JoinMap® codes (Table 2.2). The segregation types used were CP (cross pollinators) type and up to four different alleles may be segregating. The two characters the left of "×" in these codes represent the alleles of the first parent, the two on the right represent those of the second parent; each distinct allele is represented with a different character.

Table 2.2. Segregation types for population type CP, depending on the segregation type of the locus.

Segregation codes	Description	Possible genotypes
<ab×cd>	Locus heterozygous in both parents, four alleles	ac, ad, bc, bd, uu
<ef×eg>	Locus heterozygous in both parents, three alleles	ee, ef, eg, fg, uu
<lm×ll>	Locus heterozygous in one parent	ll, lm, uu
<nn×np>	Heterozygous in the other parent	nn, np, uu

uu = unknown or missing segregation types

2.11 Linkage map construction

Linkage analysis and map construction was performed using JoinMap® 3.0 software package (Van Ooijen and Voorrips, 2001). Figure 2.1 shows JoinMap® 3.0 interface with parameters used for linkage analysis and map construction. The mapping population CP type dataset was

used for linkage analysis and map construction. The similarity thresholds for locus and individual pairs were set at >0.95. The similarity threshold is defined as a lower limit for the similarity of two loci that belong to the same linkage group. LOD groupings threshold values were set at 2 to 10 during analysis. Segregation ratio for markers was set at 2 and the markers with distortion P<2 were excluded from map alignment. The map was calculated using a minimum recombination fraction of 0.30 and LOD value >1.00 with a threshold removal of 5.0 goodness-of-fit. The Kosambi mapping function was used to convert recombination into genetic distances. The markers were added using a minimum of LOD values of 3.0- 6.0 and recombination factor of 0.40.

CHAPTER 3: PHENOTYPIC ANALYSES OF WOOLLY APPLE APHID (WAA) INFESTATION RESPONSE ON SEEDLING CLONES

3.1 Introduction

The objective of this study was to perform phenotypic trials using 52 *in vitro* propagated seedling replicate clones of 96 seedlings mapping population from Northern Spy × Cox Orange Pippin in order to determine resistance response. The seedling replicate clones (52) were infested with *E. lanigerum* and then quantified and classified the resistance response after a period of 30 and 60 days. Data from shoots was collected on at 30 days and 60 days and on the roots at 60 days. The analysis was performed to investigate if any resistance response association pattern observed exists between shoots and roots that data can be used for genetic analysis.

3.2 Shoot infestation by Woolly Apple Aphid (WAA)

Fifty two Northern Spy clones were planted in the plastic planting bags in replicates of three blocks in a greenhouse (20-25 ºC) (Section 2.4.1). The replicate seedling clones were infested with heavily pre-infested susceptible apple seedlings. These pre-infested seedlings were tied onto clone replicate seedlings. This allowed *E. lanigerum* to migrate from the infested seedlings to the replicate seedling clones.

3.2.1 Infestation response data collection

A month after infestation classification and quantification of shoot infection was applied according to four classes as: Clean (class 0 and 1); Mild (class 2); Heavy infestation (class 3) and Dead seedling replicate clones (class 4) (Section 2.4.2). Appendix A and B summarise the phenotypic data collected on three replicate blocks of *in vitro* propagated seedling clones at 30 and 60 days of shoot infestation. Table 3.1 shows the summary of the assessment of infestation on shoots after 30 days and 60 days. Data after 30 days indicated that *E. lanigerum* needed longer time to infest, as there were few replicate seedling clones, which were not infested. The data showed a significant increase of infestation after 60 days. Data on shoot infestation was collected on two separate occasions, while root analysis was destructive and could only be performed once.

Table 3.1. Shoot infestation analysis at 30 and 60 days post infestation.

Assessment	Class					Total
	0	1	2	3	4	
Frequency at 30 days	10	56	37	48	0	151
Frequency at 60 days	27	28	17	48	28	148

3.2.2 Symptoms of WAA shoot infestation

In all susceptible seedlings, newly developed leaf axils were infested, colonised and characterised by a whitish waxy covering of the insects. Figure 3.1-**A**, -**B**, -**C** and -**D** shows the classification and quantification of *E. lanigerum* infestation on *in vitro* propagated seedlings. The resistant seedlings showed no colonisation while the susceptible replicate clones showed a large number of colonies forming specifically in the leaf axils on the seedlings. As the *E. lanigerum* continued to migrate to the top of the susceptible replicate clones, infestation could be observed from the bottom of the stem to the top. In most instances leaves from infested seedlings started to wilt and drop from the plant. Eventually the highly infested seedlings died.

3.3 Analysis of data

SAS Frequency Procedures (calculations not shown) were applied on data recorded on infestation classes (Cary, 1996). A significant level of variance was shown between seedlings concerning the distribution of classes ($P<0.0001$), indicating variation of response to *E. lanigerum* between assessment at 30 days and 60 days (Table 3.1) and also variation between blocks (repeats) (Appendix A and B). More importantly to note is the variation between assessments (30 and 60 days) indicating an increase in *E. lanigerum* colonisation. Clearly, a relatively large number of replicate clones died during the second assessment (60 days), one month after the first assessment. It is also evident that the number of replicate clones in class 0 increased from the first to second assessment and the number of replicate clones in class 1 decreased, which may indicate that these replicate clones were resistant to woolly

aphid *E. lanigerum*. The *E. lanigerum* occurred on the replicate clones during assessment one, but then died. In class 0 replicate seedling clones showed increment from 10-27 between 30 and 60 days and also increased from 10-56 between class 0 and class 1 at 30 days. In class 1 replicate seedling clones showed a decrease from 56-28 between 30 and 60 days and slight different (27-28) between class 0 and class 1 at 60 days. Taking into account the changes in *E. lanigerum* distribution from 30 days to the 60 days assessment, it is advisable to use the 60 days dataset for genetic analyses and interpretation of the phenotypic response. The resistance response within the 60 days dataset indicates a 50:50 segregation pattern, (between the total number of seedlings in classes 0, 1 and 2 and in classes 3 and 4) which fits with findings on the *Er*1 gene described in the "Northern Spy" experiment by Knight *et al*. (1962).

3.4 Root infestation by Woolly Apple Aphid (WAA)

The roots of each replicate seedling clone were observed for visible *E. lanigerum* infestation and classified according to the levels of resistance or susceptibility (Section 3.2) after 60 days. The roots were assessed differently from the shoots as the plants were uprooted (Section 2.4.2). Appendix C summarises the phenotypic data collected on three replicate blocks of *in vitro* propagated seedling clones after 60 days of root infestation. Data from root infestation was scored based on the presence or the absence of *E. lanigerum*. Table 3.2 shows summary of the infestation frequency after 60 days on roots. Figure 3.2 shows a heavily infested and susceptible seedling with gall formation at the roots. This resulted in seedlings wilting and ultimately dying. If the seedlings were resistant to the infestation the root system remained free of *E. lanigerum* infestation.

Table 3.2. Root infestation frequency at 60 days post infestation.

Assessment	Class					Total
	0	1	2	3	4	
Frequency	42	68	26	5	8	148

3.5 Summary

The analysis of resistance and susceptibility from replicate seedling clones was performed successfully by collecting data between March and April 2005. The quantification and classification methods used proved to be effective measures of resistance and susceptibility response in replicate seedling clones. However, in some instances it was noted that infestation occurred on one branch only while other branches remained uninfected, but this phenomenon was restricted to a few replicate clones. The dominant branch or the branch showing the higher rate of infestation was selected for quantification and classification analyses. Most of the susceptible replicate clones showed heavy gall formation on their roots, which might have contributed to the death of seedlings. The highly resistant seedlings did not show any signs of *E. lanigerum* effect on both shoots and roots.

A consistent pattern of association in resistance and susceptibility response from all three replicate blocks and individual seedling replicate clones were observed. It was shown that at 60 days of infestation the data assessment could be more reliable due to the decreased number of resistant and increased number of susceptible seedlings. In the assessment at 30 days susceptible seedlings can be classified as resistant when they escaped infestation or were susceptible to *E. lanigerum* infestation. The difference between the shoot and root

resistance response data might have been caused by expression of different QTLs or expression of different genes. Therefore, these results (at 60 days assessment) could be used for genetic analysis; to calculate the infestation response for *E. lanigerum* and evaluate association patterns between resistance and susceptibility among the replicate seedling clones. It can be concluded that this infestation response data from replicate blocks shoot showed statistical significant and reliability to be used in genetic analysis. However, this data was not enough (52 replicate clones) to be used for QTL analysis and identification of other important trait related to the *E. lanigerum* resistance gene because the trials were only conducted for one season in the greenhouse.

CHAPTER 4: GENERAL OPTIMISATION OF PCR CONDITIONS FOR MICROSATELLITE ANALYSIS

4.1 Introduction

This chapter presents the description of genomic DNA isolation and quantification from apple leaves and optimisation of PCR conditions critical for the application of PCR-based techniques. The quality of genomic DNA largely depends on the DNA extraction method used. The CTAB method that has been applied in this study yields stable and high quality DNA (Section 2.5). The use of high quality genomic DNA in PCR, together with optimal PCR reagents gives consistent results (Stein *et al*., 2001; Khan *et al*., 2004). These conditions enhance optimisation of microsatellite markers thus reducing the chance of unspecific amplification.

The detection of polymorphic information content on SSR markers and multiplex PCR technique were also described in this section. Multiplex PCR products were analysed on an ABI 310 PRISM™ Genetic Analyser (Section 2.9). The analysis of data from GeneScan® Analyser by GenoTyper® 2.5.2 (Section 2.10) was used for allele segregation type's data generation.

4.2 Genomic DNA extraction from apple leaves

Genomic DNA was extracted from 96 seedlings from a Northern Spy × Cox Orange Pippin mapping population, using the CTAB method (Section 2.5). DNA was also extracted from cultivars serving as parents in local breeding programmes. This extraction method yielded genomic DNA of high quality. DNA concentrations were estimated from the OD_{260} of the re-suspended DNA using NanoDrop® ND-1000 spectrophotometer (Section 2.5.1). The quality of genomic DNA was determined by agarose gel electrophoresis (Section 2.6). Figure 4.1 shows the quality of genomic DNA extracted from a representative set of the seedlings from the Northern Spy × Cox Orange Pippin mapping population.

4.3 Primer optimisation

PCR conditions for the microsatellite markers that have been published (66 SSR markers) (Guilford *et al*., 1997; Liebhard *et al*., 2002; Yamamoto *et al*., 2002; Liebhard *et al*., 2003; Silfverberg-Dilworth *et al*., 2006) and predicted (six SSR markers) needed to be optimised in order to achieve specific amplification of target sequences. The predicted microsatellite markers were designed by searching through the databases such as Genbank and EMBL databank at the University of the Western Cape (unpublished data) (Section 2.9.1).

Individual markers were optimised using touchdown gradient PCR (Section 2.7.2) using genomic DNA from a single apple cultivar. The touchdown PCR technique (Section 2.7.1) was used, starting with the high annealing temperature and reduced by 1 ºC per cycle, for example from 60 ºC to 50 ºC and followed by 25 cycles with same annealing temperature for example 50 ºC. Each cycle started with a denaturing temperature of 94 ºC and was followed

by extension at 72 ºC. One apple cultivar (Co-op 22) was selected for primers optimisation to ensure reliability of the results. The PCR products obtained after performing a touchdown gradient (Section 2.7.2**)** was analysed by electrophoresis on a 6 % polyacrylamide gel (Section 2.8). Figure 4.2a shows the effect of the temperature gradient PCR analysis on a predicted primer (A402 or AT000420). Figure 4.2b also shows the effect of temperature gradient on a published primer (A81 or MS06g03). The high intensity of the bands was used as a measure to determine optimal PCR conditions. The desired PCR fragment was selected and the corresponding temperature was used as the optimal working condition. The temperature range of 52 ºC to 67 ºC was used to determine the correct temperature condition. Problems encountered during optimisation included primer dimerisation and DNA denaturation of PCR products. Primer dimers were visible at bottom of the gel just below the bands of interest due to high concentration of primers. Two dark bands or a band represent the 'unwanted' double stranded 'undenatured' product running slightly above the denatured product. Seventy two primer sets were optimised, sixty six were published and six predicted. These primers were further detected for polymorphisms.

4.4 Detection of polymorphism across apple cultivars

When the conditions were optimised polymorphisms were detected in nine apple cultivars (Table 4.1). These nine apple cultivars are frequently used as parents in local breeding programmes and were used in order to determine the polymorphic information content of both published and predicted primers. Polymorphisms were detected based on fragment size variations resulting from differences in the number of tandem repeat sequences. Table 4.1

shows a summary of the nine apple cultivars used in polymorphism detection. Indicated in table 4.1 are the two cultivars used for the mapping population for this project.

Table 4.1. Apple parents cultivars used in primers optimisation and polymorphisms detection.

In bolded type: Cultivars used for generating mapping populations in this study. Other parents are used in mapping population for other projects as indicated.

Cultivar	Used in mapping population for:	Number	Traits
Austin	Anna × Austin	94	Dormancy/Initial time of budbreak
Golden Hornet	Golden Hornet × Golden Delicious	142	Scab, WAA, Mildew and Phytophthora
Co-op 22	Co-op 22 × Autumn Blush	205	Scab resistance
Starking	*	*	*
Northern Spy	**Northern Spy × Cox Orange Pippin**	**104**	Woolly apple aphid, Phytophthora, mildew
Braeburn	Golden Delicious × Braeburn	96	Dormancy/Initial time budbreak
Cox Orange Pippin	**Northern Spy × Cox Orange Pippin**	**104**	Woolly apple aphid, Phytophthora, mildew
Mildew Resistant	Mildew Resistant × Russian Seedling	251	Scab, WAA, Mildew and Phytophthora
Prima	Anna × Prima	99	Fruit quality, resistance for scab, early fruiting

* = Not in mapping population

PCR products were first separated by electrophoresis on a 6 % polyacrylamide gel (Section 2.8) (Figure 4.3a and b) and the fragment sizes of 221 base pairs were observed using primer set

A402. Figure 4.3a shows polymorphisms on nine apple cultivars using primer set A402 on a 6 % polyacrylamide gel with the expected fragment size of 173 base pairs. Figure 4.3b also shows polymorphisms on nine apple cultivars using a published primer (A81 or MS06g03) on a 6 % polyacrylamide gel with the expected fragment sizes of 190 base pairs.

The PCR products that showed polymorphisms were further analysed for exact allele fragment sizes on the ABI 310 PRISM™ Genetic Analyser (Section 2.9). The allele fragment sizes obtained on A81 and A402 primers are summarised on table 4.2. The PCR products for which polymorphism were not easily detected on a 6 % polyacrylamide gel due to the fact that the fragments were larger (e.g. more than 400bp) were resolved by capillary electrophoresis. The products that did not show any variations between different apple parent cultivars by this analysis were not included in further tests (Table 4.1).

Table 4.2. Summary of allele fragment sizes obtained on A81 and A402.

Cultivar	Obtained allele fragment (A81)	Obtained allele fragment (A402)
Austin	156-183	170-172
Golden Hornet	164-178	170-173
Co-op 22	173-182	170-174
Northern Spy	156-164	172
Braeburn	156-179	162-170
Cox Orange Pippin	153-182	162-170
Mildew Resistant	178	170-172
Starking	178	170
Prima	165	170-172

4.5 Multiplex PCR optimisation

Multiplex PCR is defined as the simultaneous amplification of multiple regions of DNA templates by using more than one primer pair in the amplification reaction mixture. Multiplex PCR is a useful technique when working with a large number of microsatellite markers as a high throughput data generating technique (Section 2.7.3). The primers that were used in the multiplex were fluorescently labelled. This enabled analysis of the PCR products of various sizes on capillary electrophoresis.

SSRs producing different sizes of PCR products and labelled with the same fluorescent dye were multiplexed in a single reaction to determine if they can reproduce the products obtained from PCR using the individual primers. PCR products were separated by electrophoresis on a 6 % polyacrylamide gel (Section 2.8) to determine if all markers used were amplified in the multiplex. Figure 4.4 shows the PCR multiplexing product of four SSRs analysed on 9 different apple cultivars. The PCR products were further analysed on a capillary electrophoresis to determine if all of the individual primer PCR products could be observed. Figure 4.5**A** and **B** represent PCR multiplexing product of four markers with the same fluorescent dye on Northern Spy and Cox Orange Pippin cultivars respectively. If all of the multiplexed primers yielded products corresponding to those obtained from single primer PCRs and if there were no 'extra' fragments, then the multiplexing of these primers was successful and that it can be used in mapping population analysis. PCR multiplex products from different fluorescent dyes can be pooled together in capillary electrophoresis. The fifth fluorescent dye was used to label the size standard for all PCR products pooled in the multiplex reaction (Section 2.9). Figure 4.6a shows the representation of 14 fluorescently labelled multiplexed primers products from GeneScan® Analyser from a single seedling plant

for example (13-03). The fluorescent dye colours were pooled in different ratios depending on the intensity of fluorescence of each dye (figure 4.6b). The ratios for fluorescent dye labelled primers were as follows, 2.5:2.5:3.0:4.0 for VIC, 6-FAM, NED and PET respectively.

4.6 Data analysis from GeneScan® Analyser

A total of seventy two microsatellite markers (Table 4.3) were used in this study and analysed by GenoTyper® 2.5.2 software (Section 2.10). Sixty five of the markers were the previously published (Guilford *et al*., 1997; Liebhard *et al*., 2002; Yamamoto *et al*., 2002; Liebhard *et al*., 2003) and seven were predicted from databases such as Genbank and EMBL databank at University of the Western Cape (unpublished) (Section 2.9.1).

GenoTyper® 2.5.2 software analysis parameters were set based on the fragment sizes obtained from parents used in generating the mapping population and the fluorescent dye of the markers. The common problem associated with GenoTyper® 2.5.2 software was the lack of editing tools that can be use to differentiate between free fluorescent dye peak and allele fragment peak (Section 2.10). It was proved that some allele fragment sizes fall within the same size range with free fluorescent dyes but the "true" allele fragment peak was characterised by a sharp peak often with stutter peaks, and the free fluorescent dye peak was characterised by smooth oval shape with no stutter peaks. Primers fluorescently labelled with "PET" (Red) showed free dye peaks were encountered at 137 bp and 142 bp, "NED" (yellow) at 194 bp and "FAM" (blue) at 75 bp and 120 bp allele fragment sizes respectively. There was no fluorescent free dye peaks observed on "VIC" (green). Seedlings where the

genotype was incorrectly assigned during the automated process using GenoTyper® 2.5.2 were scored manually particularly in the mapping population because of the known genotypes from the parents. Table 4.3 shows a summary of the microsatellite markers used in the linkage analysis. Indicated in this Table 4.3 are repeat types, allelic types, fragment sizes range and multiplex numbers. The markers that are in a specific multiplex can be tested in any PCR without giving complicating results. The markers that were not in the multiplexes were used in single PCR reactions and their products were pooled for capillary electrophoresis analysis. The summary of the assigned genotypes for specific SSR marker data used in this study is shown in Appendix D.

4.7 Summary and discussion

The use of good quality genomic DNA (50 ng/µl) for PCR reactions was demonstrated to be an important factor in primer optimisation. DNA concentration was measured using the NanoDrop® ND-1000 spectrophotometer (Section 2.5.1) and the quality of the DNA was determined by agarose gel electrophoresis (Section 2.6) to ensure that it was RNA free and undegraded. The seventy two primers used in this study were optimised at uniform temperature conditions using the touchdown PCR technique. It was illustrated that the use of genomic DNA from single apple cultivar for optimising primers maintains consistency in terms of the results obtained from each experiment. The use of single genomic DNA minimises uncertainties about primers that are difficult to optimise. Polymorphisms were detected using 9 different apple cultivars to determine allelic variation (Figure 4.3a.). It proved difficult to detect polymorphisms with some primers such as A81 (MS06g03) when PCR products were visualised on a 6 % polyacrylamide gel and for this reason they were

resolved by capillary electrophoresis analysis. The majority of the SSRs used for this study were dinucleotide repeats with a few tri- and tetranucleotide SSRs, as summarised in Table 4.3. Detection of polymorphisms from trinucleotide SSRs was easier than dinucleotide SSRs because of the larger size (3 versus 2 repeats) and this resulted in fewer PCR artefacts (Hearne 1992). In some instances polymorphisms were easier to detect by electrophoresis on a 6 % polyacrylamide gel (Figure 4.3a) and then by capillary electrophoresis, than polyacrylamide gel alone. Polymorphic primers working under uniform PCR conditions were multiplexed in a single PCR reaction.

PCR multiplexing has been demonstrated to be a cost effective, high throughput data generation technique when working with a large number of fluorescently labelled SSR markers. Multiplex PCR products of different fluorescent dyes were pooled in a capillary electrophoresis analysis for high throughput data generation as shown in figure 4.6b. The data from GeneScan® Analyser containing different fluorescently labelled primers were analysed by GenoTyper® 2.5.2 software (Section 2.10). Sixty four of the apple SSRs were published (Guilford *et al*., 1997; Liebhard *et al*., 2002; Yamamoto *et al*., 2002; Liebhard *et al*., 2003) and one pear marker (Yamamoto *et al*., 2002) while the other seven were predicted markers at University of the Western Cape (unpublished) (Section 2.9.1). Seventy two markers were polymorphic in one or both parents and were used for linkage map construction (Table 4.3).

CHAPTER 5: GENOTYPING PROCEDURE AND CONSTRUCTION OF LINKAGE MAP

5.1 Introduction

The objective of this chapter was to use genotypic data from the GenoTyper® 2.5.2 software (Section 2.10) to construct a genetic linkage map using JoinMap® 3.0 software package (Van Ooijen and Voorrips, 2001) (Section 2.11). Each marker was screened on the two parents used in mapping population generation to determine which specific alleles were inherited from each parent by each seedling. The segregation types of markers are referred to as unordered alleles present in the parents and inherited by the seedlings (Table 2.2). The genotyped data for all successfully analysed markers was used for linkage map construction using JoinMap® 3.0 software (Section 2.11).

The preliminary genetic linkage map was constructed using 72 microsatellite makers. The preliminary linkage map can use as a framework towards the completion of all 17 chromosomes of apple.

5.2 Construction of genetic linkage map

The development of a genetic linkage map determines whether markers are linked or not after their recombination frequencies have been calculated. The JoinMap® 3.0 software

(Van Ooijen and Voorrips, 2001) uses mathematical and statistical mapping functions to construct the map in a linear orientation and the distances between individual markers are related to the recombination frequencies.

GenoTyper® 2.5.2 was used to score the segregation of alleles from each locus in the progeny (Appendix D). Microsoft Excel was used in order to enable the manipulation of data from all different loci into one single matrix, after which the matrix was converted to a text format in order to be compatible with the JoinMap 3.0 software used for map construction. The software package JoinMap® 3.0 was used for the construction of a genetic linkage map (Section 2.11). The genetic linkage groups of markers were assigned based on the minimum LOD threshold of 2.0. Loci were grouped together to form linkage groups, based on the number of recombinations observed between each pair of loci. The linkage groups were selected based on the number of markers in the linkage group. Figure 5.1 shows three selected linkage groups (A, B and C) used as an example for preliminary linage map construction i.e. (**A**) 6.0/1(5). The first number is LOD value 6.0, linkage group 1 and contains (5) markers in the subgroup. The subgroup with high LOD value was selected because the markers were strongly linked in that subgroup and was used as a representation for linkage group 1, which corresponds to linkage group (LG) 14 of Liebhard *et al*. (2002) and Liebhard *et al*. (2003). The other linkage groups were selected using the same method. A number of loci did not show any linkage with any other marker and were for this reason excluded during map construction. If the data show statistical significance then the linkage groups will stay intact as the LOD threshold increases. If any linkage existed, linked markers were assigned in a linear order with the distances calculated in map units or centiMorgan (cM).

Genetic linkage map distances are listed on the right and markers on the left of the graphical representation of chromosomes. Figure 5.2 shows the preliminary genetic linkage map of Northern Spy × Cox Orange Pippin.

5.2.1 Data output

Seventy two markers were used to construct a linkage map but only 21 markers showed linkage and they produced nine linkage groups with some different segments of the same linkage group (e.g. linkage group 5 and 10). Table 5.1a shows allele fragment sizes, chi squared (χ^2) values and segregation codes used in the preliminary linkage map construction. The allele fragment sizes were converted to JoinMap® codes because JoinMap® 3.0 software is incompatible with allele fragment sizes in base pairs (Table 2.2). Table 5.1b summarises allele ratios found, degrees of freedom and significant differences. Table 5.1a and table 5.1b are two components of the same table in JoinMap® 3.0. For example chi square was calculated for each individual marker based on the possible genotypes (Table 2.2). Data from table 5.1b were used in the calculation of chi square values (χ^2) in table 5.1a. For example χ^2 was calculated as follows,

$$\chi^2 = \frac{(\text{Observed}-\text{Expected})^2}{\text{Expected}}$$

Possible segregation types = [ac:ad:bc:bd]

= **4**

Expected recombination frequency for A42 = $\frac{27+16+21+16}{4}$ = **20**

Then, $\chi^2 = \frac{(27-20)^2}{20} + \frac{(16-20)^2}{20} + \frac{(21-20)^2}{20} + \frac{(16-20)^2}{20}$

$= 2.45 + 0.8 + 0.05 + 0.8$

$= \mathbf{4.1}$

The degree of freedom (Df) for A42 was calculated as follows: where r represents the number of possible segregation types.

$Df = r - 1$

$= 4-1$

$= \mathbf{3}$

Fifty one markers were unlinked due to several reasons (Table 5.2). Firstly, some linkage groups that had few markers did not show any linkage and >25 cM distances existed between linking markers. The markers that were expected to link in certain linkage groups did not link due to the larger number of missing data and incorrectly scored genotypes. The problem might have also been caused by free fluorescent dye scored in some markers.

Twenty markers on the preliminary linkage map were from the published markers and were aligned with the framework map (Liebhard *et al*., 2002; Liebhard *et al*., 2003) together with one predicted marker (A188). The order of the markers on linkage group (LG) 14 was slightly distorted, but the distance between these markers was small (~1.2 cM) and therefore map positions remained unchanged. Figure 5.3 shows the alignment of LG 14 of Discovery (D14) and of Fiesta (F14) with LG 14 of Northern Spy × Cox Orange Pippin. Two other markers (A61 and A64) on the same chromosome (LG 14) were scored in the seedlings and included in this analysis, but did not show linkage with the markers on this linkage group (LG 14). The fact that they did not show significant linkage to any of the markers on this

linkage group might be explained by the fact that they contained a large number of missing genotypes, 19.8% and 13.5% respectively as shown in table 5.2. Most of the segregation types scored were corrected manually, which might have caused the problem.

5.3 Summary and discussion

The preliminary genetic linkage map containing nine linkage groups was constructed in this study. Two linkage groups (LG 5 and LG 10) contained two segments each from the same linkage group were determined and only one linkage group from nine groups consists of five markers and other linkage groups two markers. The linkage group with five linked markers corresponds to LG 14 of the Fiesta × Discovery genetic linkage map.

The other linkage groups could also be aligned to Fiesta × Discovery linkage map and were represented graphically as shown on figure 5.2. The two published markers (A126 and A135) and a predicted marker A188 were the only new markers aligned on the map. The distance between interlinked markers was 14.2, 25.0 and 45.2 cM for A126, A135 and A188 respectively. It can be concluded that the huge distances between linked markers shows weak linkage and short distance indicate stronger linkage. It was also difficult to conclude the marker orders on the map due to the limited number of markers in the linkage groups containing two markers each. If more markers could be incorporated into these groups then the order and distances between the markers could change. In some instances map distances are influenced by the neighbouring markers that can compress or stretch the corresponding map interval (Liebhard *et al*., 2003). The total distance of this preliminary genetic linkage

map for Northern Spy × Cox Orange Pippin is 263.6 cM as compared to framework map with 1145.3 and 1417.1 cM in Fiesta and Discovery respectively.

A total of 21 markers showed linkage with one or more other markers and were used in linkage map construction. Fifty one markers did not show any linkage due to missing data and limited number of markers used. One of five predicted markers (A188) showed linkage with a published marker (A92) on linkage group five but with huge interlinked distance of 45.2 cM between the markers. The size of the mapping population was large enough (96 individuals) to generate a linkage map considering the fact that linkage map from Silfverberg-Dilworth *et al*. (2006) only 44 seedlings were used for map construction. The number of markers that did not show any linkage was high which might be an indication of the high levels of missing genotypes and unknown genotypes, or a combination of the two. Forty three of 51 markers that did not link contained less than 20 % of the missing data, which meant they were expected to link. The remaining six markers contained more than 20% missing data and were not expected to give reliable linkage. Since the mapping population used in this study was generated in two directions (Section 2.4) that might have also contributed to the large number of markers not to link. Each parent was used as a pollinator (male) in 50 % and also as a pollinated (female) in the other 50 % of the mapping population (Section 2.4). It was concluded that large map gaps between linked markers could be reduced with the incorporation of more polymorphic markers. The preliminary linkage map of the Northern Spy × Cox Orange Pippin mapping population can be used as base towards the completion of mapping all 17 chromosomes of apple.

CHAPTER 6: GENERAL DISCUSSION AND CONCLUSION

6.1 Introduction

The objectives of this study were to construct a linkage map of apple from 96 seedlings from a mapping population of Northern Spy × Cox Orange Pippin using microsatellite markers, and also to perform phenotypic trials in the greenhouse using 52 *in vitro* propagated replicate seedling clones from this mapping population for *E. lanigerum* resistance. The approach used both previously published SSR markers (Guilford *et al*., 1997; Liebhard *et al*., 2002; Yamamoto *et al*., 2002; Liebhard *et al*., 2003; Silfverberg-Dilworth *et al*., 2006) and predicted (Section 2.9.1) SSR markers for linkage map construction. Microsatellite markers used in this study were optimised, tested for polymorphisms, multiplexed, tested on the mapping population, genotyped and used for genetic linkage map construction. This chapter discusses the main findings in general, gives a summary of the thesis and highlights the future directions of this study.

6.2 WAA infestation response on seedling replicate clones and analyses

The data for the infestation response for *E. lanigerum* resistance on shoots and roots were collected, classified and quantified according to four classes after 30 and 60 days of infestation (Section 2.4.1). Assessments after 30 and 60 days on shoots have shown the association of resistance response. This has indicated that *E. lanigerum* needs longer to infest, as the number of replicate seedling clones infested at 60 days assessment was increased and it

was in agreement with the findings of Sandanayaka *et al*. (2003). Roots were more difficult to evaluate in comparison to shoots due to difficulty in separating *E. lanigerum* from soil and counting them manually. The resistance response after 30 and 60 days has shown an association pattern from the three replicate blocks. The statistical analysis showed no direct association between root and shoot resistance response (SAS Frequency Procedures calculations data not shown). This might have been caused by expression of QTLs or genes that would be important for *E. lanigerum* resistance in roots being different to those in shoots. It has been shown that resistance to *E. lanigerum* was classified according to different levels of resistance. The most heavily infested and susceptible seedlings showed gall formation at the roots, which affect water and nutrients uptake to the shoots (Brown *et al*., 1991). The distribution of resistance and susceptibility classes within the 60 days dataset indicated a 50:50 segregation pattern (72:76 replicate seedling clones) that fits with the findings on the *Er*1 gene described in the "Northern Spy" experiment by Knight *et al*. (1962). It was also demonstrated that the data assessment at 60 days could be used for genetic analysis to *E. lanigerum* infestation response. The quality of the replicate datasets was sufficient to observe the resistance and susceptible infestation response association pattern amongst the replicate blocks. However, to achieve more reliable data larger numbers of replicates are required to minimise the chances of overlooking important data information. The phenotypic data analysis needs to be performed for three seasons to be used for quantitative trait analysis linking to resistance genes (Evans and James, 2003), to gain greater statistical significance and confidence in the data.

6.3 Optimisation of simplex and multiplex PCR

Microsatellite markers were optimised using simplex PCR on an Eppendorf Mastercycler® Gradient PCR cycler (Section 2.7.2) by the gradient PCR touchdown technique. PCR products were analysed by electrophoresis on a 6 % polyacrylamide gels. The optimised SSR markers were individually tested for polymorphisms on nine apple cultivars and the PCR products were electrophoresed on a 6 % polyacrylamide gels (Section 2.8) and ABI 310 PRISM™ Genetic Analyser (Section 2.9). Fragment allele sizes were determined for polymorphic SSRs. The optimised primers were multiplexed using a touchdown PCR technique optimisation (Section 4.5), which reduced the time and effort required compared with testing primers individually. Multiplexing proved to be a high throughput data generation technique that became important particularly when working with larger number of SSRs (Henegariu *et al*., 1997; Markoulatos *et al*., 2002). Multiplexed PCR products containing different fluorescently labelled primers were pooled in the capillary electrophoresis using different pooling ratios due to some of the fluorescent dyes having higher intensity (Section 4.5). The data from GeneScan® Analyser containing different fluorescently labelled primers were analysed by GenoTyper® 2.5.2 software (Section 2.10). It has been proved difficult to genotype fluorescent dyes with lower intensity such as "PET" (red), in the multiplex due to suppression by other fluorescent dyes such as "VIC" (green), "6-FAM" (blue) and "NED" (yellow).

GenoTyper® 2.5.2 software was important due to its ability to analyse many primers in a single analysis. The parameter specifications were set based on the allele fragment sizes obtained from each parent for a particular marker of specific fluorescent dye. The key components in specification were fluorescent dye and allele fragment sizes. Some markers

showed better peaks (with no stutter peaks) from the parent cultivars and those primers were analysed automatically while the markers with stutter bands were scored manually. The accuracy in which the segregation types were scored was important, as incorrect genotypes might have a huge impact on the linkage map calculation. It also proved difficult in some cases to distinguish between the true allele fragment sizes and the free fluorescent dye peak. If a specific genotype peak fell within the same range with the free fluorescent dye peak of the same fluorescent dye colour then GenoTyper® 2.5.2 interpreted both as true allele fragment peaks, resulting in unknown segregation types. Incorrectly scored genotypes were corrected manually. Seventy two polymorphic microsatellite markers were scored and used in genetic linkage map construction.

6.4 Linkage map construction

Linkage map calculation and map chart construction was performed by JoinMap® 3.0 software (Section 2.11). Preliminary genetic linkage of the Northern Spy × Cox Orange Pippin mapping population was constructed in this study (Section 5.2) from 72 microsatellite markers. Twenty one markers (of 72 markers used) were linked and produced nine linkage groups with some different segments of the same linkage group, and 51 markers did not link. The problem with unlinked markers was caused by the higher percentage (>20 %) of missing genotypes from individual seedlings (Table 5.2). Those individual seedlings that produced half genotypes during analysis (i.e. **au** instead of **ac, u** is unknown) not a full set of genotypes were interpreted as missing data by JoinMap® 3.0 software. This was another factor that influenced the higher rate of the missing genotypes data. If the data contained a large number of incorrect genotypes then the programme showed an error massage when the data file was

uploaded. The software could only specify the location of errors if a few genotypes were incorrect. The difficulty of this software is its lack of editing tools for incorrectly entered genotyped data (Van Ooijen and Voorrips, 2001).

Due to the few linked markers, only one linkage group (LG 14) was used for map alignment with the Fiesta × Discovery linkage map. This linkage group contained five markers, and then three markers were correctly aligned and two markers positions were slightly distorted (~1.2 cM). The changing of the positions might have been caused by transposition from the parents used. The positions can change with the incorporation of new markers in the linkage group. It has been shown (Figure 5.2) that a larger number of markers were unlinked and this resulted with only nine linkage groups for the preliminary linkage map. Therefore, to achieve any linkage there should be more markers on that linkage group. This would result in smaller distances between linked markers on the chromosomes and the map can be useful for analysis of resistance genes. This preliminary genetic linkage map of Northern Spy × Cox Orange Pippin could be use as a framework map with incorporation of more markers.

6.5 Summary and conclusion

This study has demonstrated the important steps followed to construct a genetic linkage map generation from a Northern Spy × Cox Orange Pippin mapping population aiming to map the *Er*1 gene responsible for *E. lanigerum* resistance. CTAB methodology (Section 2.5) has been used for genomic DNA extraction from apple leaves, which was used for PCR. Eppendorf Mastercycler® Gradient was used for optimisation of the predicted and published microsatellite markers using touchdown PCR technique. The optimal markers were analysed

for polymorphisms on nine apple parents. Polymorphic markers were multiplexed for a high throughput data generation. Seventy two polymorphic markers were tested on a Northern Spy × Cox Orange Pippin mapping population and the PCR products data were analysed using an ABI 310 PRISM™ Genetic Analyser. The data from GeneScan® Analyser were analysed by GenoTyper® 2.5.2 software to determine genotypes inherited from the each parent. The data was used for genetic linkage construction using JoinMap® 3.0 software.

This study again had shown the importance of generating larger mapping populations for quantitative studies. Due to the small number of replicate clones (52) used in this study, the data was not sufficient to perform QTL analysis and phenotypic trials should be performed for three seasons (Evans and James, 2003). The phenotypic data at 60 days were enough to be used in genetic analysis and to evaluate the infestation response for *E. lanigerum*. The linkage map was constructed with 72 markers but only 21 markers linked producing nine linkage groups. For apple, this means there were still eight linkage groups required to complete 17 chromosomes of apple (Guilford *et al*., 1997; Liebhard *et al*., 2002; Yamamoto *et al*., 2002; Liebhard *et al*., 2003). The results for the unlinked markers might have been influenced by incorrect genotypes and missing genotypes data from individual seedlings. The attempts to identify markers linked with *Er*1 gene resistance for *E. lanigerum* were not achieved, as there were few markers in that linkage group (LG 8) (Gardiner *et al*., 2006; Durel *et al*., 2006; Silfverberg-Dilworth *et al*., 2006).

6.6 Future work

The generation of more replicate seedling clones is needed for further phenotypic trials. Phenotypic data in the greenhouse and field analysis should be performed more than three seasons at least (Evans and James, 2003) so that can be useful for genetic analysis. Incorporation of more markers on the preliminary genetic linkage map is required and also the saturation of LG 8 with more markers (Durel *et al*., 2006; Silfverberg-Dilworth *et al*., 2006). This could quickly assist in identifying markers tightly linked to *Er*1 and minor QTL genes for *E. lanigerum* resistance. It would also facilitate the pyramiding of *Er*1 with *Er*2, *Er*3, *Er*-m and *Er*-I for *E. lanigerum* durable resistance (Gardiner *et al*., 2006). This could result in marker-assisted selection ultimately being utilised in the local breeding programmes.

REFERENCES

Asante, S. K. (1994). Susceptibility of apple varieties to attack by the woolly aphid, *Eriosoma lanigerum* (Hausmann) (Hemitptera: Pemphegidae). *Plant Protection Quarterly*. 9: 126-130.

Baldi, P., Patocchi, A., Zini, E., Toller, C., Velasco, R. and Komjanc, M. (2004). Cloning and linkage mapping of resistance gene homologues in apple. *Theor Appl Genet*. 109: 231-239.

Barrier, M., Friar, E., Robichaux, R., and Purugganan, M. (2000). Interspecific evolution in plant microsatellite structure. *Genetics*. 241: 101-105.

Beers, E. (1993). Orchard pest management: a resource book for the Pacific Northwest. *Good Fruit Grower, Yakima*. 276: 167-168.

Benson, G. (1999). Tandem repeats finder: a program to analyze DNA sequences. *Nucleic Acids Res.* 27: 573-580.

Botha, A-M., Venter, E., van der Vyver, C., and Kunert, K. J. (2004). Development and application of molecular DNA markers in Africa: a South African review. *S Afri J Bot*. 70: 152-166.

Botstein, D., White, R. L., Skolnick, M. and Davies, R. W. (1980). Construction of a genetic linkage map in man using restriction fragment length polymorphisms. *Amer J Hum Genet*. 32: 314-331.

Breyne, P., Boerjan, W., Gerats, T., Van Montagu, M., and Van Gysel, A. (1997). Applications of AFLP™ in plant breeding, molecular biology and genetics. *Belgian J Bot*. 129: 107-117.

Brown, M. W., Glenn, D. M., and Wisniewski, M. E. (1991). Functional and anatomical disruption of apple roots by the Woolly Apple Aphid (Homoptera: Aphididae). *J Econ Entomol*. 84: 1823-1826.

Bus, V. (1994). Pest and disease resistance in pipfruit rootstocks. *The Orchardist*. 67: 57-60.

Bus, V., Ranatunga, C., Gardiner, S., Bassett, H., and Rikkerink, E. (2000). Marker assisted selection for pest and disease resistance in the New Zealand apple breeding programme. *Acta Hort*. 538: 541-547.

Canli, F. A. (2004). Development of a second generation genetic linkage map for sour cherry using SSR markers. *Pak J Biol Sci*. 7: 1676-1683.

Cary, N. C. (1996). SAS procedures guide. Edition 8. Published by SAS Institute Inc.

Cevik, V., and King, J. (2002). High-resolution genetic analysis of the Sd-1 aphid resistance locus in *Malus* spp. *Theor Appl Genet*. 105: 346-354.

Clark, L. A., Tsai, K. L., Steiner, J. M., Williams, D. A., Guerra, T., Ostrander, E. A., Galibert, F., and Murphy, K. E. (2004). Chromosomome-specific microsatellite multiplex sets for linkage studies in the domestic dog. *Genomics*. 84: 550-554.

Cook, R. J. (1998). The molecular mechanisms responsible for resistance in plant-pathogen interactions of the gene-for-gene type function more broadly than previously imagined. *Proc Natl Acad Sci USA*. 95: 9711-9712.

Crane, M. B., Greenslade, R. M., Massee, A. M., and Tydman, H. M. (1936). Studies on the resistance and immunity of apples to the woolly aphis, *Eriosoma lanigerum* (Hausm.). *J Pomol*. 14: 137-163.

Cummins, J. N., Forsline, P. L., and Mackenzie, J. D. (1981). Woolly apple aphid colonization on *Malus* cultivars. *J Amer Soc Hort Sci*. 106: 26-30.

Doherty, K. R., Zweifel, E. W., Elde, N. C., McKone, M. J., and Zweifel, S. G. (2003). Random amplified polymorphic DNA markers reveal genetic variation in the symbiotic fungus of leaf-cutting ants. *Mycologia*. 95: 19-23.

Doyle, J. J., and Doyle J. L. (1987). A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochemical Bull*. 19: 11-15.

Dreistadt, S. H. J., Clark, J. K., and Flint, M. L. (1994). Pests of landscape trees and shrubs: An integrated pest management guide. Oakland: Univ Calif *Agric Nat Res Publ*. p335-339.

Durel, C. E., Freslon, V., Denance, C., Calenge, F., Laurens, F., Lespinasse, Y., Rat, E., Parisi, L., Bus, V., Dapena de la Fuente, E., Minarro, M., Blaquez, M. D., and Van de Weg, W. E. (2006). Genetic localization of new major and minor pest and disease resistance factors in the apple genome. In 3rd International Rosaceae Genomics Conference. OP5 (abstract), War Memorial Conference Centre Napier, New Zealand. http://www.mainlab.clemson.edu/gdr/community/conferences/RG3_abstracts.pdf

Evans, K. M., and James, C. M. (2003). Identification of SCAR markers linked to Pl-w mildew resistance in apple. *Theor Appl Genet*. 106: 1178-1183.

Ferrer, I., Garcia-Reyes, J. F., Mezcua, M., Thurman, E. M., and Fernandez-Alba, A. R. (2005). Multi-residue pesticide analysis in fruits and vegetables by liquid chromatography-time-of-flight mass spectrometry. *J Chrom.* 1082: 81-90.

Gardiner, S.E., Bus, V., Bassett, H., Goodman, M., Greer, L., Ranatunga, C., Rikkerink, E., and Foster, R. (1999). Identification of molecular markers linked major resistance to apple scab, powdery mildew and woolly apple aphid in apple. In Proceeding of the Plant and Animal Genomes VII Conference, P495 (abstract), San Diego, California. http://www.intl-pag.org/7/abstracts/PAG7356_P185.html

Gardiner, S. E., Bus, V., Changne, D., Ranatunga, C., Legg, W., Bassett, H., Zhou, J., Cook, M., Crowhurst, R., Gleave, A., Rikkerink, E., Patocchi, A., and Durel, C. E. (2006). Mapping of major resistances to woolly apple aphid. In Proceeding of the Plant and Animal Genomes XIV Conference, P495 (abstract), San Diego, California. http://www.intl-pag.org/14/abstracts/PAG14_P495.html

Georgala, M. B. (1953). The woolly aphis of apple and its control. *Farming S Afri*. 21: 21-27.

Gianfranceschi, L., Seglias, N., Tarchini, R., Komjanc, M., and Gessler, C. (1998). Simple sequence repeats for the genetic analysis of apple. *Theor Appl Genet*. 96: 1069-1076.

Giliomee, J. H., Strydom, D. K., and Van Zyl, H. J. (1968). Northern Spy, Merton and Malling-Merton rootstocks susceptible to woolly aphis, *Eriosoma lanigerum*, in the Western Cape. *S Afri J Agric Sci*. 11: 183-186.

Guilford, P., Prakash, S., Zhu, J. M., Rikkerink, E., Gardiner, S., Bassett, H., and Forster, R. (1997). Microsatellites in *Malus* × *domestica* (apple): Abundance, polymorphism and cultivar identification. *Theor Appl Genet*. 94: 249-254.

Han, Y., Gasic, K., Kertbundit, S., Marron, B., Beever, J. E., and Korban, S. S. (2006). Developing genome-wide physical map for the apple genome. In Proceeding of the Plant and

Animal Genomes XIV Conference, P496 (abstract), San Diego, California. http://www.intl-pag.org/14/abstracts/PAG14_P496.html

Hand, P., Kift, N., McClement, S., Lynn, J. R., Grube, R., Schut, J. W., van der Arend, A. J. W., and Pink, D. A. C. (2003). Progress towards mapping QTLs for pest and disease resistance in lettuce. *Eucarpia Leafy Vegetable*. p31-35.

Harris, S A., Robinson, J. P., and Juniper, B. E. (2002). Genetic clues to the origin of the apple. *Trends in Genet*. 18: 426-429.

Hearne, C. M., Ghosh, S., and Todd, J. A. (1992). Microsatellites for linkage analysis of genetic traits. *Trends Genet*. 8: 288-294.

Hemmat, M., Weeden, N. F., Manganaris, A. G., and Lawson, D. M. (1994). Molecular marker linkage map for apple. *J Hered*. 85: 4-11.

Henegariu, O., Heerema, N. A., Dlouhy, S. R., Vance, G. H., and Vogt, P. H. (1997). Multiplex PCR: critical parameters and step-by-step protocol. *Biotechniques*. 23: 504-511.

Holton, T. A. (2001). Plant genotyping by analysis of microsatellites. In "Plant genotyping: the DNA fingerprinting of plants". Edited by Henry, R. J. Published by CAB International. p15-27.

Janick, J., Cummins, J. N., Brown, S. K., and Hemmat, M. (1996). Apples. In "Tree and tropical fruits". Fruit Breed 1. Edited by Janick, J and Moore, J. N. Published by John Wiley and Sons Inc. p1-76.

Jansen, J., de Jong, A. G., and van Ooijen, J. W. (2001). Constructing dense genetic linkage maps. *Theor Appl Genet*. 102: 1113-1122.

Jensen, P. J., Altman, N., Crassweller, R., Makalowska, I., Maximova, S., Praul, C., Travis, J. W., and McNellis, T. W. (2006). Apple tree functional genomics: Getting to the rootstock of it. In Proceeding of the Plant and Animal Genomes XIV Conference, W135 (abstract), San Diego, California. http://www.intl-pag.org/14/abstracts/PAG14_W135.html

Khan, I. A., Awan, F. S., Ahmad, A., and Khan, A. A. (2004). A modified mini-prep method for economical and rapid extraction of genomic DNA in plants. *Plant Mol Biol Reporter*. 22: 89a-89c.

Knight, R. L., Briggs, J. B., Massee, A. M., and Tydman, H. M. (1962). The inheritance of resistance to woolly aphid, *Eriosoma lanigerum* (Hausmann), in the apple. *J Hort Sci*. 37: 207-218.

Korban, S. S., Vodkin, L. O., Liu, L., Aldwinckle, H. S., Carroll, N., Goldsbrough, P., Orvis, K., and Clifton, S. (2004). Towards apple functional genomics: The EST project. In Proceeding of the Plant and Animal Genomes XII Conference, P39 (abstract), San Diego, California. http://www.intl-pag.org/12/abstracts/P01_PAG12_39.html

Korban, S. S., Vodkin, L. O., Liu, L., Aldwinckle, H. S., Ksenija, G., Gpnzales, D. O., Malnoy, M., Thimmapuram, J., Carroll, N. J., Goldsbrough, P., Orvis, K., Clifton, S., Pape, D., Martin, M., and Meyer, R. (2005). Large-scale analysis of EST sequences in apple genome. In Proceeding of the Plant and Animal Genomes XIII Conference, W130 (abstract), San Diego, California. http://www.intl-pag.org/13/abstracts/PAG13_W130.html

Kumar, L.S. (1999). DNA markers in plant improvement: an overview. *Biotechnol Adv*. 17: 143-182.

Lacassie, E., Dreyfuss, M. –F., Daguet, J. L., Vignaud, M., Marguet, P., and Lachâtre, G. (1998a). Multi-residue determination of pesticides in apples and pears by gas chromatography-mass spectrometry. *J Chrom*. 805: 319-326.

Lacassie, E., Dreyfuss, M. –F., Daguet, J. L., Vignaud, M., Marguet, P., and Lachâtre, G. (1999b). Liquid chromatography-electrospray mass spectrometry multi-residue determination of pesticides in apples and pears. *J Chrom*. 830: 135-143.

Lamberti, F., and Taylor, C. E. (1981). Resistance of crops to nematodes. In "Durable resistance in crops". Edited by Lamberti, F., Walter, J. M and Van der Graaff, N. A. Published by NATO ASI series 55. p197-206.

Lander, E. S., and Botstein, D. (1988). Mapping Mendelian factors underlying quantitative traits using RFLP linkage maps. *Genetics*. 121: 185-199.

Lee, S. –Y., Seo, J. S., Rodriguez-Lanetty, M., and Lee, D. –H. (2003). Comparative analysis of superfamilies of NBS-encoding disease resistance gene analogs in cultivated and wild species. *Mol Gen Genomics*. 269: 101-108.

Liebhard, R., Gianfranceschi, L., Koller, B., Ryder, C.D., Tarchini, R., Van de Weg, E., and Gessler, C. (2002). Development and characterisation of 140 new microsatellites in apple (*Malus* × *domestica* Borkh.). *Mol Breed*. 10: 217-241.

Liebhard, R., Koller, B., Gianfranceschi, L., and Gessler, C. (2003). Creating a saturated reference map for the apple (*Malus* × *domestica* Borkh.) genome. *Theor Appl Genet*. 106: 1497-1508.

Lundie, A. E. (1939). The apple growers' insect ally. *Farming S Afri*. 162: 371-374.

Ma, Z. Q., Roder, M., and Sorrells, M. E. (1996). Frequencies and sequence characteristics of di-, tri-, and tetra-nucleotide microsatellites in wheat. *Genome*. 39: 123-130.

Maliepaard, C., Alston, F. H., van Arkel, G., Brown, L. M., Chevreau, E., Dunemann, F., Evans, K. M., Gardiner, S., Guilford, P., van Heusden, A. W., Janse, J., Laurens, F., Lynn, J. R., Manganaris, A. G., den Nijs, A. P. M., Periam, N., Rikkerink, E., Roche, P., Ryder, C., Sansavini, S., Schmidt, H., Tartarini, S., Verhaegh, J. J., Vrielink-van

Ginkel, M., and King, G. J. (1998). Aligning male and female linkage maps of apple (*Malus pumila* Mill.) using multi-allelic markers. *Theor Appl Genet*. 97: 60-73.

Markoulatos, P., Siafakas, N., and Moncany, M. (2002). Multiplex polymerase chain reaction: a practical approach. *J Clin Lab Anal*. 16: 47-51.

Mensah, J. K., Lundanes, E., Greibrokk, T., and Holen, B. (1997). Determination of diflubenzuron in apples by gas chromatography. *J Chrom*. 765: 85-90.

Mohan, M., Nair, S., Bhagwat, A., Krishna, T. G., Yano, M., Bhatia, C. R., and Sasaki, T. (1997). Genome mapping, molecular markers and marker-assisted selection in crop plants. *Mol Breed*. 3: 87-103.

Naik, S., Hampson, C., Gasic, K., Bakkeren, G., and Korban, S. S. (2006). Development and linkage mapping of E-STS and RAG markers for functional gene homologues in apple. *Genome*. 49: 959-968.

Nel, P. L. (1985). Insect pests. In "Deciduous fruits and vines: Pests and diseases and their control". Edited by Nel, P. L. Published by Philip, D: Cape Town, South Africa. p41-45.

Newcomb, R. D., Crowhurst, R. N., Gleave, A. P., Rikkerink, E. H. A., Allan, A. C., Beuning, L. L., Bowen, J. H., Gera, E., Jamieson, K. R., Janssen, B. J., Laing, W. A., McArtney, S., Nain, B., Ross, G. S., Snowden, K. C., Souleyre, E. J. F.,Walton, E. F.,

and Yauk, Y. K. (2006). Analyses of expressed sequence tags from apple. *Plant Physiol*. 141: 147-166.

Ortiz, J. P. A., Pessino, S. C., Bhat, V., Hayward, M. D., and Quarin, C. L. (2001). A genetic linkage map of diploid *Paspalum nottatum*. *Crop Sci*. 41: 823-830.

Patocchi, A., Walser M., Tartarini, S., Broggini, G. A. L., Gennari, F., Sansavini, S., and Gessler, C. (2005). Identification by genome scanning approach (GSA) of a microsatellite tightly associated with the apple scab resistance gene *Vm*. *Genome*. 48: 630-636.

Rakoczy-Trojanowska, M., and Bolibok, H. (2004). Characteristics and a comparison of three classes of microsatellite-based markers and their application in plants. *Cell Mol Biol Lett*. 9: 221-238.

Reiter, R. (2001). PCR-based marker systems. In "DNA-based markers in plants". Edited by Phillip, R. L and Vasil, I. K. N. Published by Kluwer Academic publisher: p9-29.

Rouppe van der Voort, J. N., van Eck, H. J., van Zandvoort, P. M., Overmars, H., Helder, J., and Bakker, J. (1999). Linkage analysis by genotyping of sibling populations: a genetic map for the potato cyst nematode constructed using a "pseudo-F2" mapping strategy. *Mol Gen Genet*. 261: 1021-1031.

Russel, G. E. (1978). Pests-some general considerations. In "Plant breeding for pest and disease resistance". Edited by Russell, G. E. Published by Butterworth and Co LTD: p293-324.

Sandanayaka, W. R. M., Bus, V. G. M., Connolly, P., and Newcomb, R. (2003). Characteristics associated with Woolly Apple Aphid *Eriosoma lanigerum*, resistance of three apple rootstocks. *Entomol Experimentalis et Applicata*. 109: 63-72.

Schiliro, E., Predieri, S., and Bertaccini, A. (2001). Use of random amplified polymorphic DNA analysis to detect genetic variation in *Pyrus* species. *Plant Mol Biol Reporter*. 19: 271a-h.

Sen Gupta, G.C., and Miles, P.W. (1975). Studies on the susceptibility of varieties of apple to the feeding strains of Woolly aphis (Homoptera) in relation to the chemical content of the tissues of the host. *Aus J Agric Res*. 26: 157-168.

Silfverberg-Dilworth, E., Matasci, C. L., Van de Weg, W. E., Van Kaauwen, M. P. W., Walser, M., Kodde, L. P., Soglio, V., Gianfranceschi, L., Durel, C. E., Costa, F., Yamamoto, T., Koller, B., Gessler, C., and Patocchi, A. (2006). Microsatellite markers spanning the apple (*Malus* × *domestica* Borkh.) genome. *Tree Genetics & Genomes*. 2: 202-224.

Stankiewicz-Koyl, M., Pitera, E., and Gawronski, S. W. (2005). Mapping QTL involved in powdery mildew resistance of the apple clone U 211. *Plant Breed*. 124: 63-66.

Staskawicz, B. J. (2001). Genetic of plant-pathogen interactions specifying plant disease resistance. *Amer Soc Plant Physiologists*. 125: 73-76.

Stein, N., Herren, G., and Keller, B. (2001). A new DNA extraction method for high-throughput marker analysis in a large genome species such as *Triticum aestivum*. *Plant Breed*. 120: 354-356.

Tanksley, S. D., Young, N. D., Paterson, A. H., and Bonierbale, M. W. (1989). RFLP mapping in plant breeding: New tools for an old science. *Bio/Technol*. 7: 257-264.

Tenzer, I., degli Ivanissevich, S., Morgante, M., and Gessler, C. (1999). Identification of microsatellite markers and their application to population genetics of *Venturia inaequalis*. *Phytopathology*. 89: 748-753.

Timm, A. E., Pringle, K. L., and Warnich, L. (2005). Genetic diversity of woolly apple aphid *Eriosoma lanigerum* (Hemiptera: Aphididae) populations in the Western Cape, South Africa. *Bull Entomol Res*. 95: 187-191.

Van der Biezen, E. A., and Jones, J. D. (1998). Plant disease-resistance proteins and the gene-for-gene concept. *Trends Biochem Sci*. 23: 454-456.

Van Ooijen, J. W., and Voorrips, R. E. (2001). JoinMap® 3.0 version, software for the calculation of genetic linkage maps. Plant Research International, Wageningen, Nertherlands. http://www.generationcp.org/capcorner/chile_wksp_2005/manuals/manual_03.pdf

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

Xiaoliang, C., Bing, S., Rong, Z., Juan, M., and Xiaoming, T. (2006). Simultaneous Determination of Twelve Glucocorticoid Residues in Milk by Ultra Performance Liquid Chromatography-Electrospray Tandem Mass Spectrometry. *Chinese J Chrom*. 24: 213-218.

Xu, M. L., Melchinger, A. E., Xia, X. C., and Lubberstedt, T. (1999). High-resolution mapping of loci conferring resistance to sugarcane mosaic virus in maize using RFLP, SSR, and AFLP markers. *Mol Gen Genet*. 261: 574-581.

Yamamoto, T., Kimura, T., Sawamura, Y., Kotobuki, K., Ban, Y., Hayashi, T., and Matsuta, N. (2001). SSRs isolated from apple can identify polymorphism and genetic diversity in pear. *Theor Appl Genet*. 102: 865-870.

Yamamoto, T., Kimura, T., Shoda, M., Ban, Y., Hayashi, T., and Matsuta, N. (2002). Development of microsatellite markers in the Japanese pear (*Pyrus pyrifolia* Nakai). *Mol Ecol*. 2: 14-16.

Yamamoto, T., Kimura, T., Shoda, M., Imai, T., Saito, T., Sawamura, Y., Kotobuki, K., Hayashi, T., and Matsuta, N. (2002). Genetic linkage maps constructed by using an interspecific cross between Japanese and European pears. *Theor Appl Genet*. 106: 9-18.

Young, N. D. (2001). Constructing a plant genetic linkage map with DNA markers. In "DNA-based markers in plants". Edited by Phillip, R. L and Vasil, I. K. N. Published by Kluwer Academic publishers: p31-47.

APPENDICES

Appendix A: Phenotypic data analysis of Woolly Apple Aphid (WAA) resistance on three replicate blocks of *in vitro* propagated seedling clones from Northern Spy × Cox Orange Pippin. Data collected 30 days after infestation.

Appendix B: Phenotypic data analysis of Woolly Apple Aphid (WAA) resistance on three replicate blocks of *in vitro* propagated seedling clones from Northern Spy × Cox Orange Pippin. Data collected 60 days after infestation.

Appendix C: Phenotypic analysis on roots after 60 days of WAA infestation.

Appendix D: Microsatellite markers used in the Northern Spy × Pippin Cox Orange map construction.

Figure 1.3. (A-H). Life cycle of *Aphelinus Mali* in aphid (Lundie, 1939).
It shows all the stages involved during aphid destruction.

A. *A. mali* depositing an egg in the body cavity of an aphid.

B. Egg of *A. mali* in the body cavity of an aphid.

C. Egg hatching.

D. Young larva feeding on the body.

E. Full-grown larva in the body of an aphid.

F. Pupa of *A. mali* in a dead aphid.

G. Adult *A. mali* working its way out of a dead aphid.

H. Full-grown adult *A. mali* ready take another round of aphid destruction.

Figure 1.5. Genetic linkage map of the apple progeny ‘Fiesta’ (F) × ‘Discovery’ (D). Linkage groups are numbered from F1 to F17 and D1 to D17 (Liebhard *et al*., 2003).

One hundred and forty eight new SSR markers developed and mapped on this map (data not shown on the linkage map) (Silfverberg-Dilworth *et al*., 2006). The new SSR markers filled the gaps between previously discovered markers to increase large coverage of the apple genome.

Figure 1.6. Set of 102 SSR primer pairs coverage 85% of the apple genome and that with an average distance between markers of 15 cM (Silfverberg-Dilworth et al., 2006).

The underlined markers were developed on this linkage map while other markers were developed from other linkage maps. ? = on loci indicated that no primer pairs were publicly available yet.

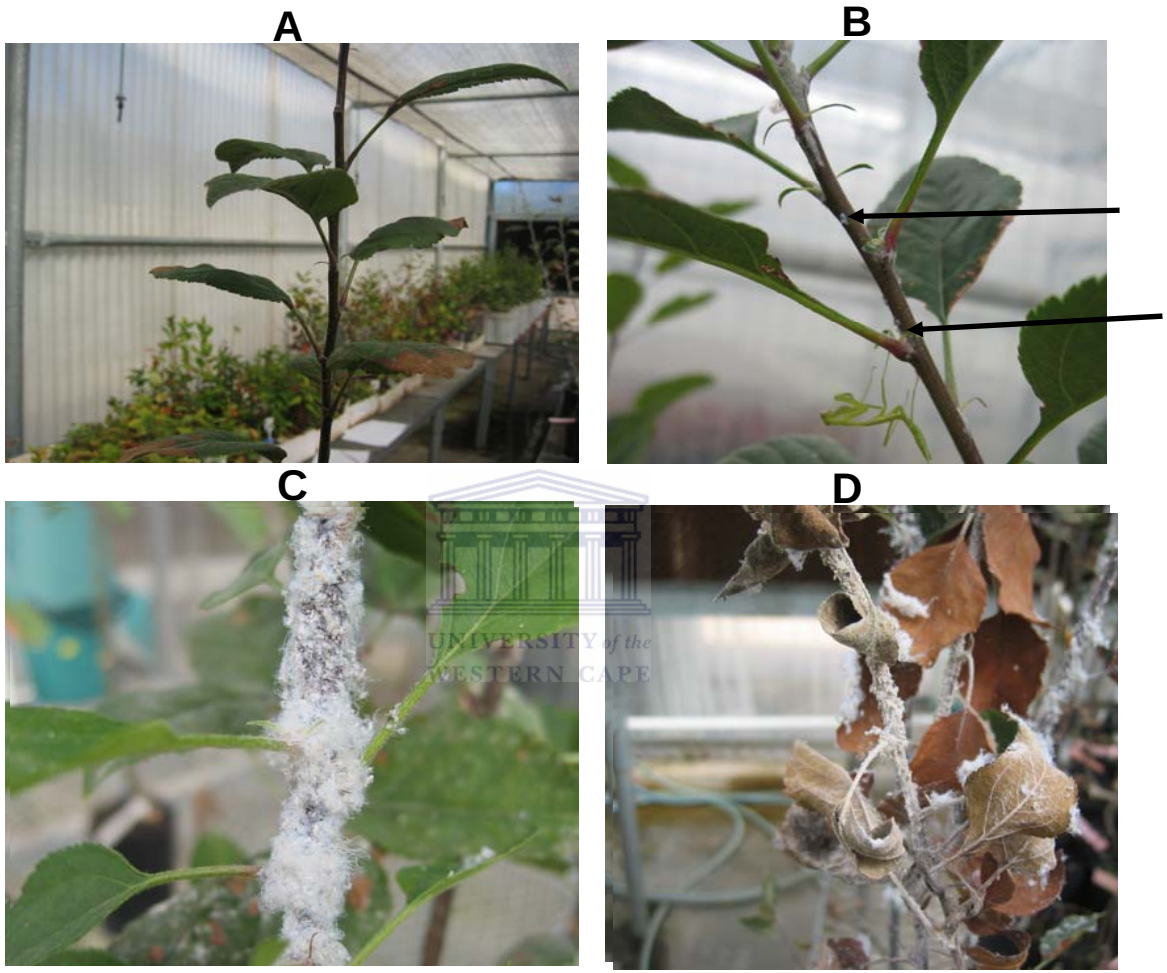


Figure 3.1. Classification and quantification of *E. lanigerum* on shoots. **A** shows class 0 and 1; **B** shows whitish waxy spread infestation on stem and leaf axils, class 2; **C** heavy infestation on the whole plant covered with whitish waxy, class 3 and; **D** shows dead plant due to heavy infestation by *E. lanigerum*, class 4.

Figure 3.2. Heavily infested roots system showing gall formation at the roots.

The galls formed at specific sites where WAA injured the roots.

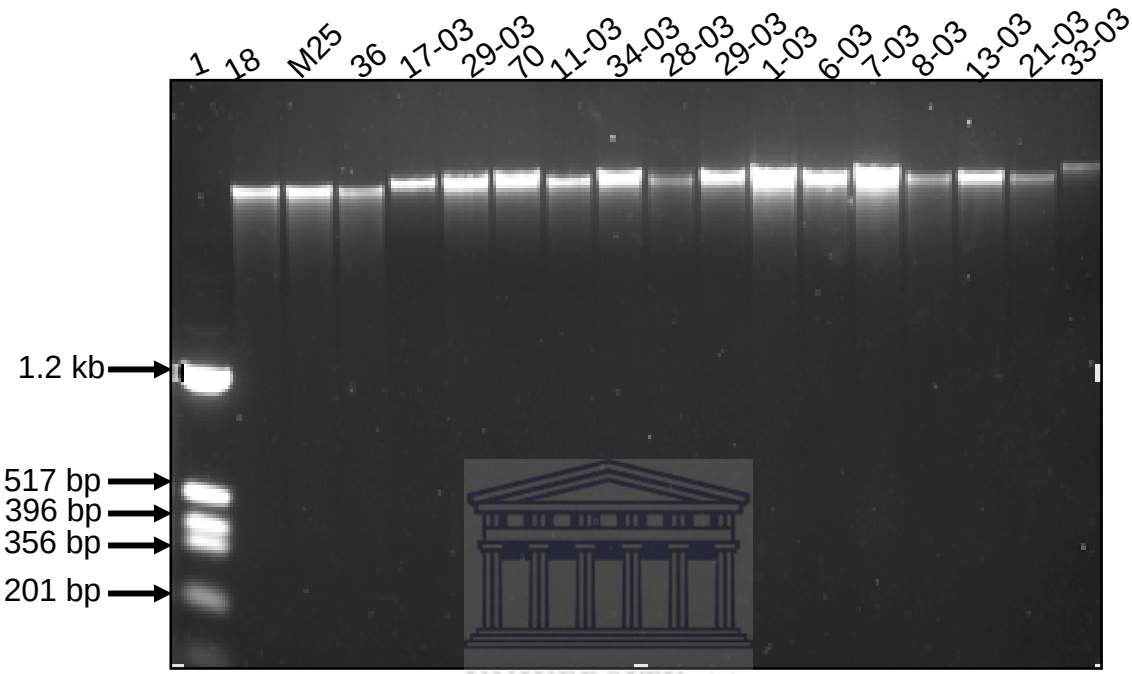


Figure 4.1. Genomic DNA extracted from 96 seedlings clones of Northern Spy × Cox Orange Pippin.

The agarose gel shows DNA from 17 seedling clones part of the 96 clones used in the study. Lane 1: molecular weight marker and lanes 18 to 33-03 represent DNA from the seedling clones.

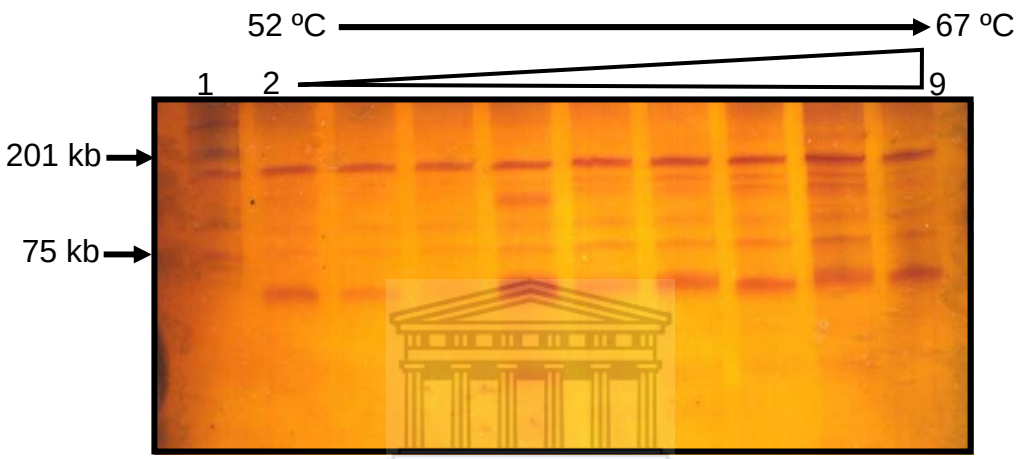


Figure 4.2a. Primer optimisation using touchdown gradient technique.

6 % Polyacryalmide electrophoresis gel shows the effect of temperature gradient when optimising a predicted primer (A81 or MS06g03). Genomic DNA used was from apple cultivar Co-op-22. Lane 1: pTz/ *Hinf*I molecular marker and Lane 2-9: temperature effect on extension.

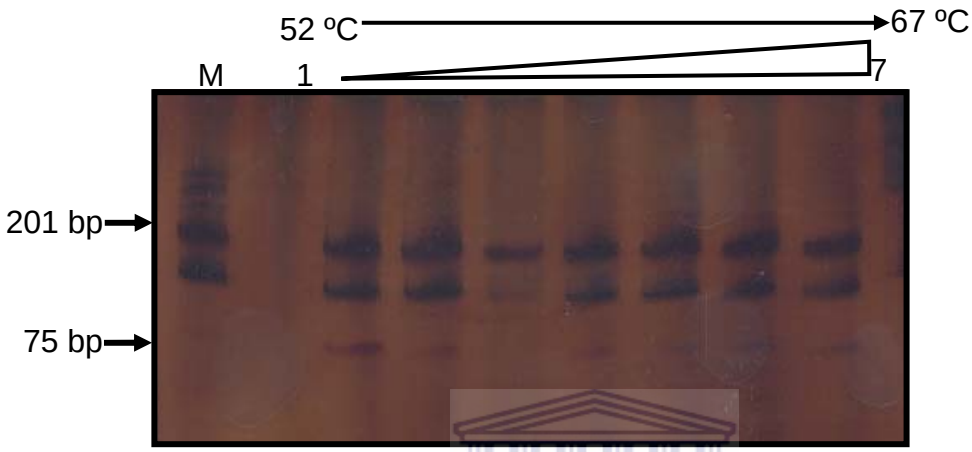


Figure 4.2b. Primer optimisation using touchdown gradient technique.

6 % Polyacryalmide electrophoresis gel shows the effect of temperature gradient when optimizing a published primer (A402 or AT000420). Genomic DNA used was from apple cultivar Co-op-22. In lane M: molecular weight marker and lane 1-7: temperature effect on extension.

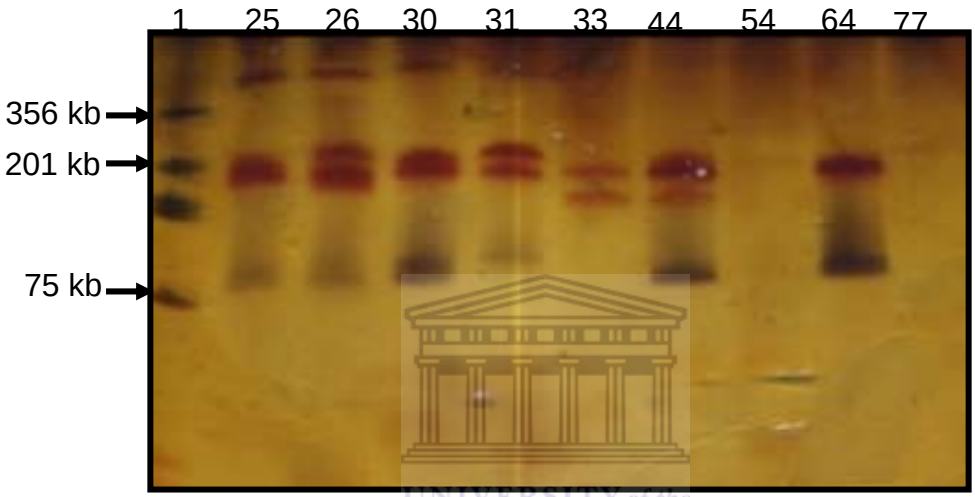


Figure 4.3a. Polymorphisms detection on nine apple cultivars using a predicted primer (A402 or AT000420).

Variations were detected on a 6 % polyacryalmide electrophoresis gel, lane 1: molecular weight marker, 25: Austin, 26: Golden Hornet, 30: Co-op-22, 31: Starking, 33: Northern Spy, 44: Braeburn, 54: Cox Orange Pippin, 64: Mildew Resistant, 77: Prima

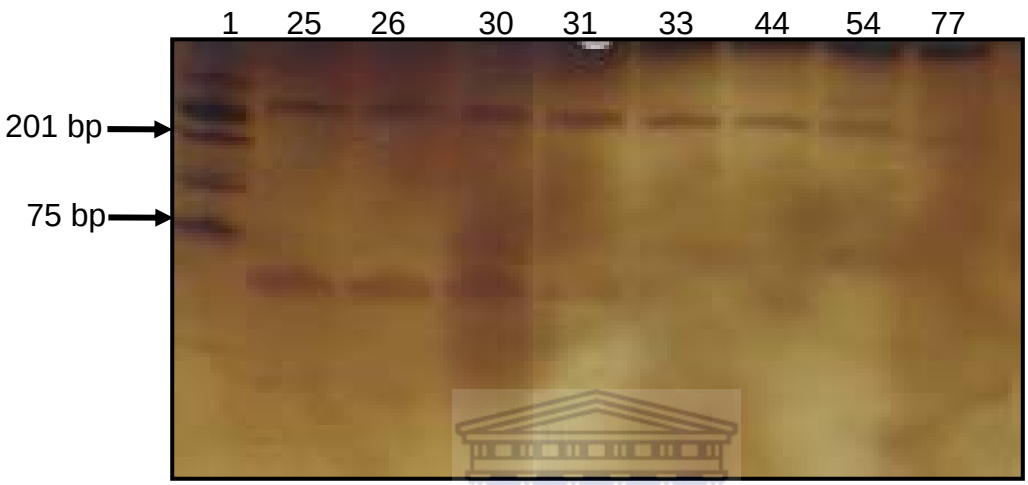


Figure 4.3b. Polymorphisms detection on nine apple cultivars using a published primer (A81 or MS06g03).

Variations were detected on a 6 % polyacryalmide electrophoresis gel, lane 1: molecular weight marker, 25: Austin, 26: Golden Hornet, 30: Co-op-22, 31: Starking, 33: Northern Spy 44: Braeburn, 54: Cox Orange Pippin and 77: Prima.

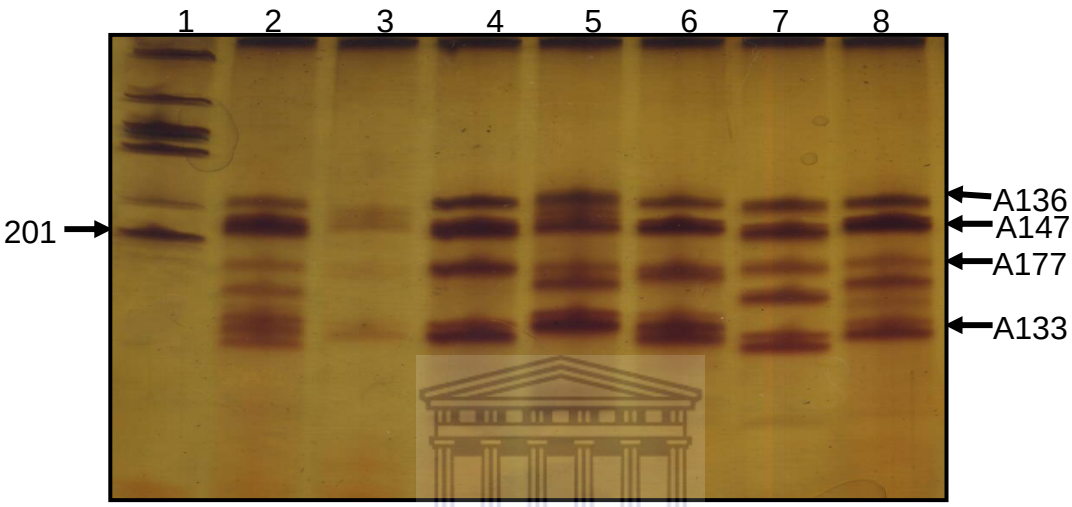


Figure 4.4. PCR multiplex optimisation using four PET labelled primers on seven apple cultivars.

Four primers multiplexed in a single PCR are; A136 (CH01e121), A147 (CH02h11b), A177 (CH05h05), A133 (CH01d03). Apple cultivars used multiplex optimisation in lane 1: molecular weight marker, 2: Golden Hornet, 3: Co-op-22, 4: Starking, 5: Northern Spy, 6: Cox Orange Pippin, 7: Mildew Resistant and 8: Prima.

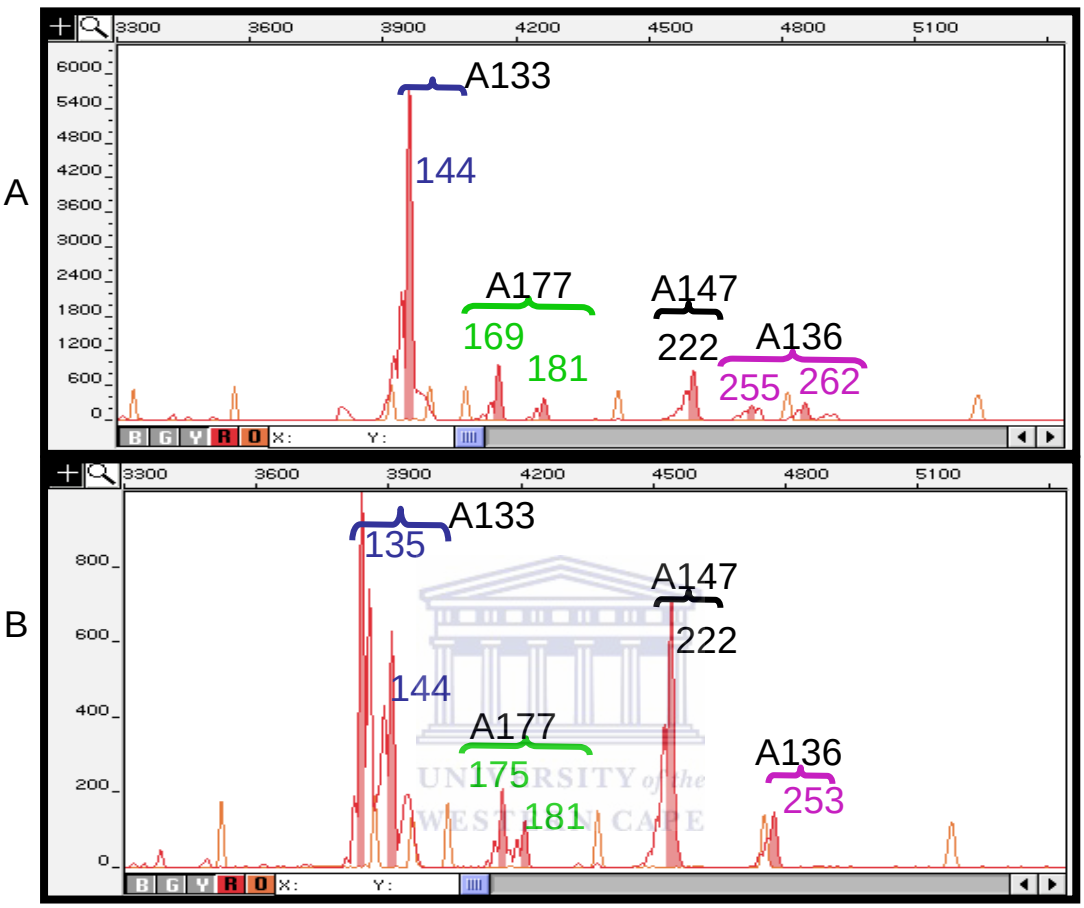


Figure 4.5. Fragment sizes analysis on multiplex PCR product (PET 3).

(A) PET 3 multiplex containing four primers using Northern Spy apple cultivar. (B) PET 3 multiplex containing four primers using Cox Orange Pippin apple cultivar. Primers used (Fig. 4.6) show segregation of alleles expected into the mapping population. (A) Fragment sizes obtained A133:144, A177:169/181, A147: 222, A136: 255/262 and (B) A133:135/144, A177:175/181, A147: 222, A136:253.

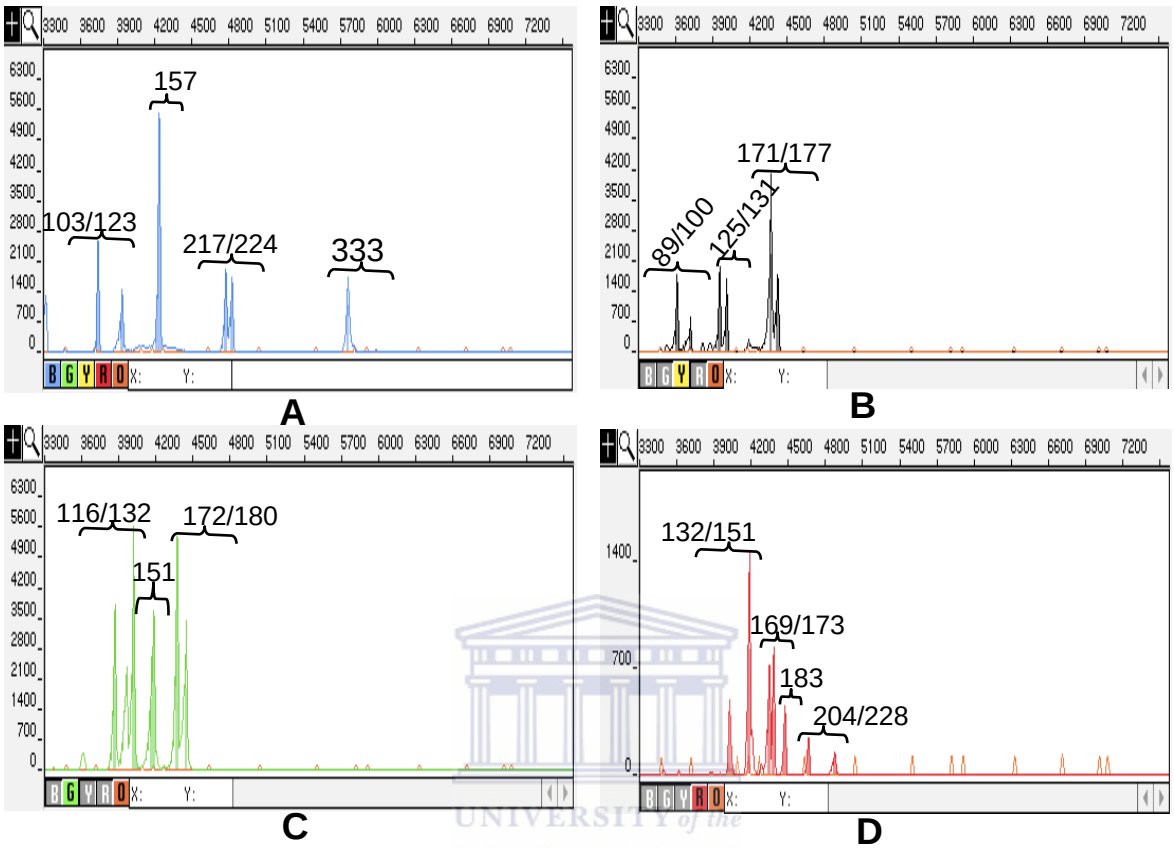


Figure 4.6a. Schematic representation of 14 fluorescently labelled multiplexed primers products from GeneScan® Analyser from a single seedling (13-03).

It shows all the genotypes analysed for each primer on seedling number 13-03. **A**. 6-FAM contains four primer products (A85, A32, A78 and A193). **B**. NED contains three primer products (A115, A102 and A109). **C**. VIC contains three primer products (A91, A84 and A93). **D**. PET contains four primer products (A133, A177, A147 and A136).

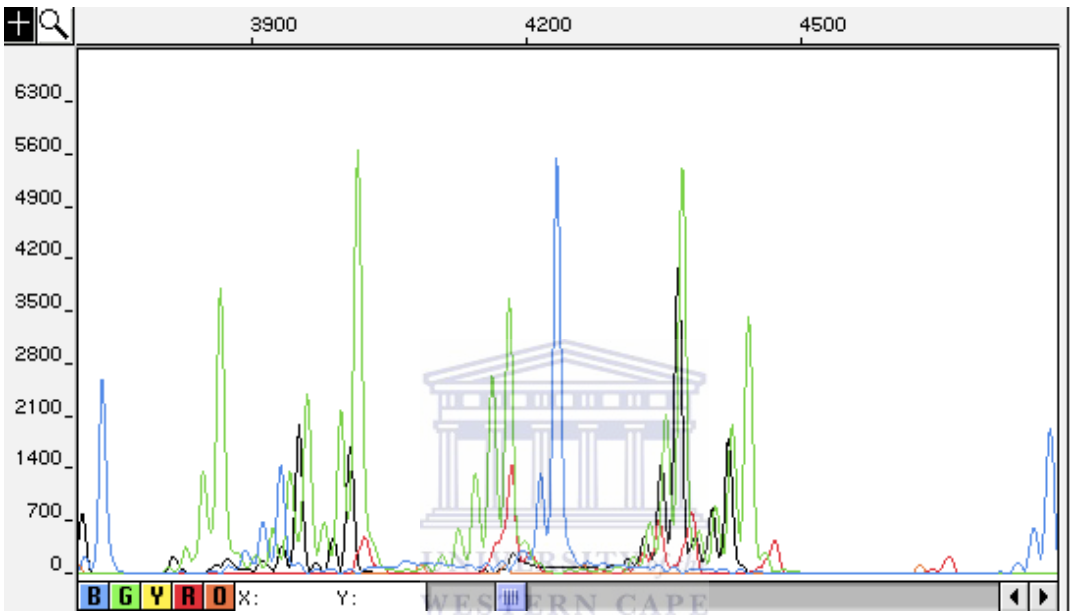


Figure 4.6b. High throughput data generation on different fluorescently labelled markers on seedling 13-03 using GenoTyper® 2.5.2.

Parameters were set based on the fluorescent dye for microsatellite markers in the multiplex and the overall pool contains 14 markers in the analysis. Allele fragment sizes from parents were used to set parameters for expected genotypes on each seedling.

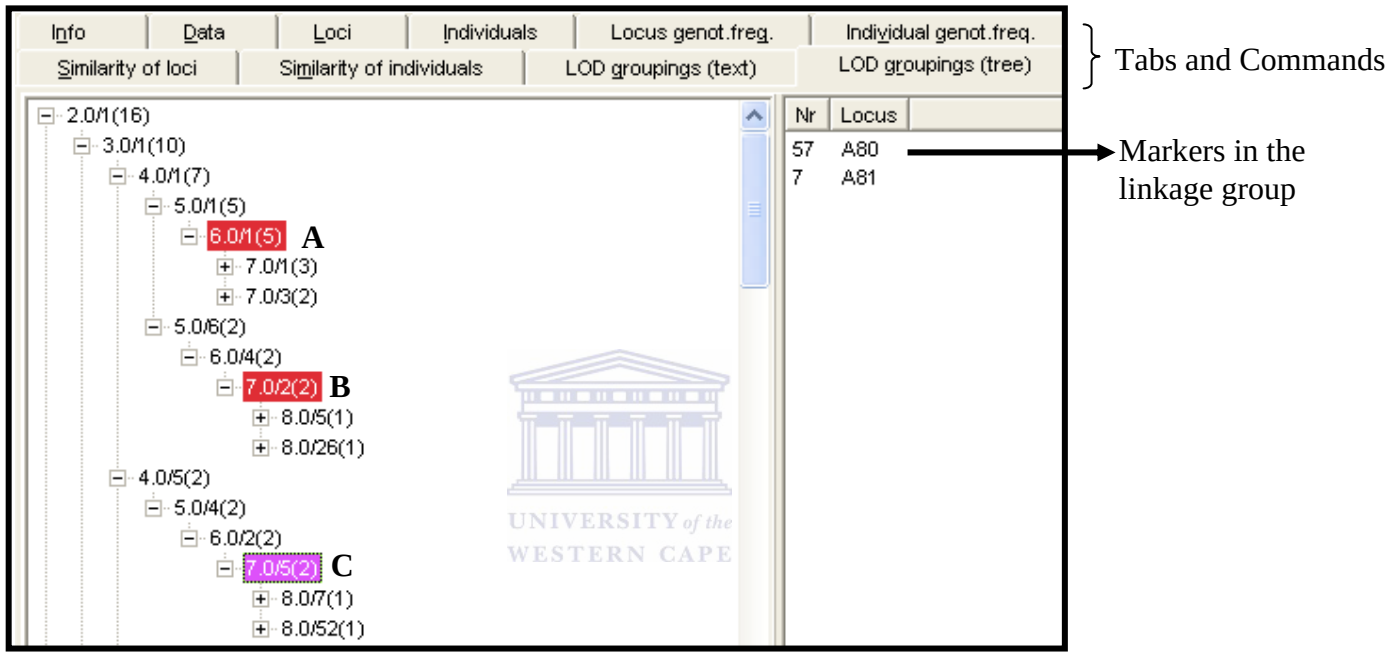


Figure 5.1. Three selected linkage groups used in the analysis of preliminary linkage map

JoinMap® workspace interface with three selected linkage groups (A) LG 14, (B) LG 4 and (C) LG10. The highlighted text box, LOD score (6.0), linkage group number (1) and number of markers in the linkage group.

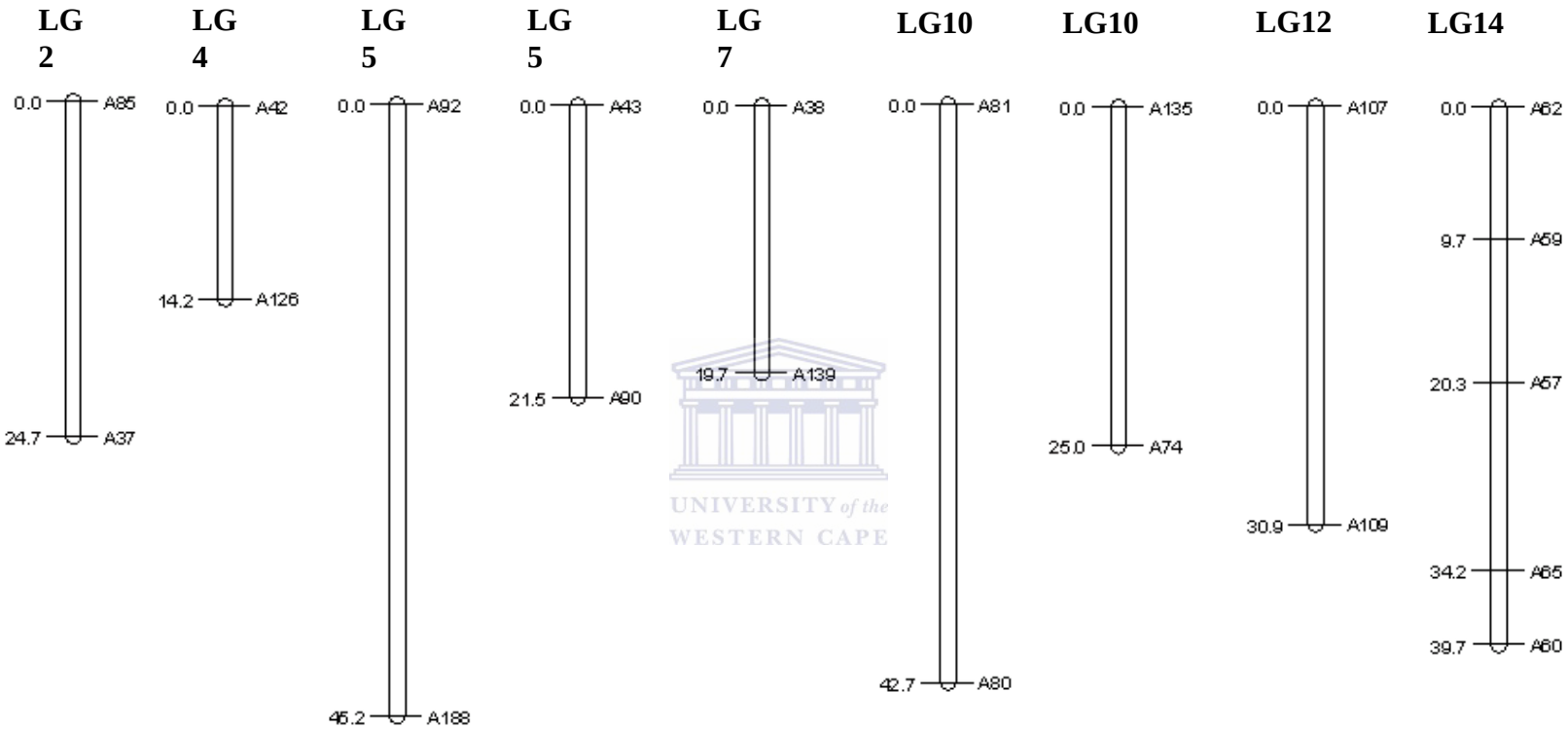


Figure 5.2. Graphical representation of preliminary genetic linkage map of Northern Spy and Cox Orange Pippin.

It shows nine linkage groups (LG) with LG5 and LG10 having two segments of the same linkage groups. Only LG14 contains five marker and other groups contain two each.

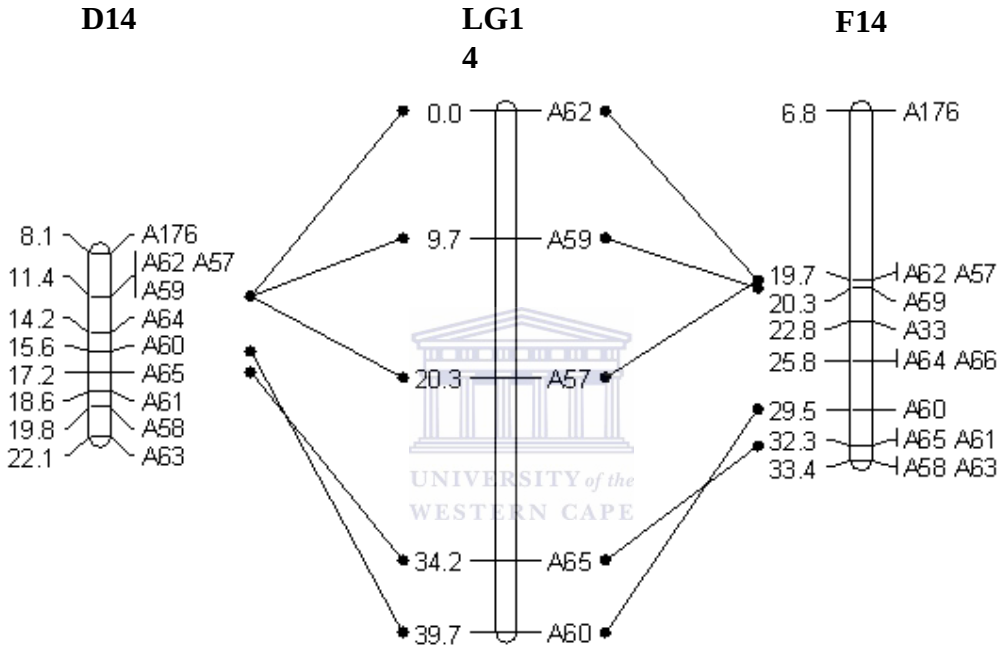


Figure 5.3. Linkage group (LG) 14 of Northern Spy and Cox Orange Pippin alignment with the linkage group of Discovery (D14) and of the Fiesta (F14).

The positions of A60 and A65 were slightly distorted as compared to linkage group D14 and F14 on the framework map.

Appendix A. Continued

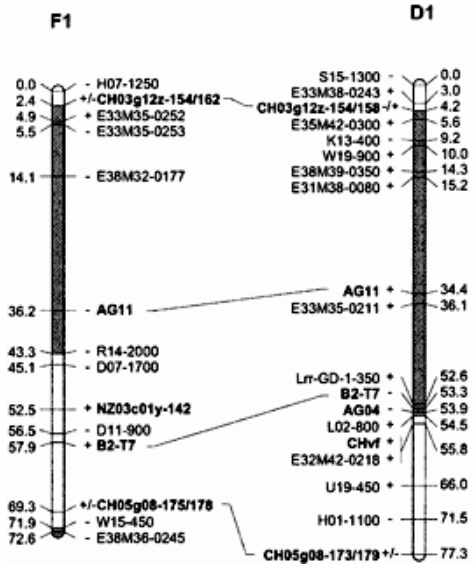
WAA DATA	CLASS			% INFESTATION			FOR CALCULATION OF % INFESTAT				
							BLOCK 1		BLOCK 2		
CLONES NO	BLOCK 1	BLOCK 2	BLOCK 3	BLOCK 1	BLOCK 2	BLOCK 3	# leaves	# total leav	# leaves	total # leav	ŧ
66	3	3	*	40	45	*	12	30	17	38	
43	1	2	1	0	15	0	0	100	4	26	
14	3	3	1	31	24	0	12	39	8	34	
56	0	3	1	0	43	0	0	100	20	46	
30	0	1	3	0	0	45	0	100	0	100	
3	3	3	1	67	28	0	18	27	5	18	
36	1	2	2	0	21	23	0	100	6	29	
24	2	1	2	17	0	4	3	18	0	100	
25	0	2	3	0	5	77	0	100	1	20	
69	1	*	0	0	*	0	0	100	*	*	
80	2	2	2	0	7	13	0	100	1	14	
32	0	0	1	0	0	0	0	100	0	100	
2	*	2	*	*	38	*	0	100	8	21	
10	*	3	*	*	51	*	0	100	18	35	

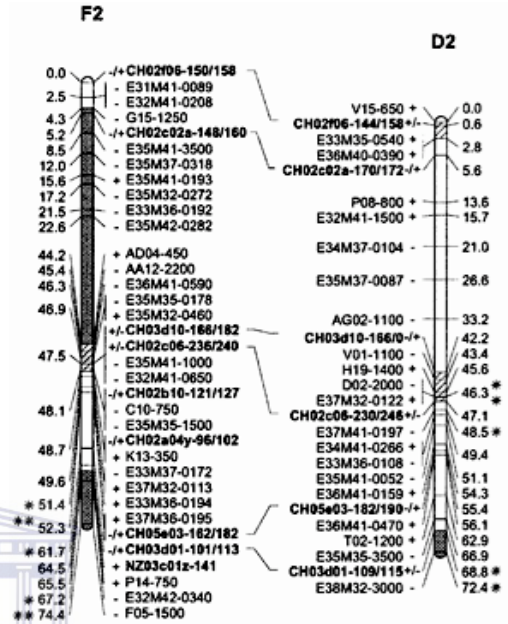
* = dead seedling clones

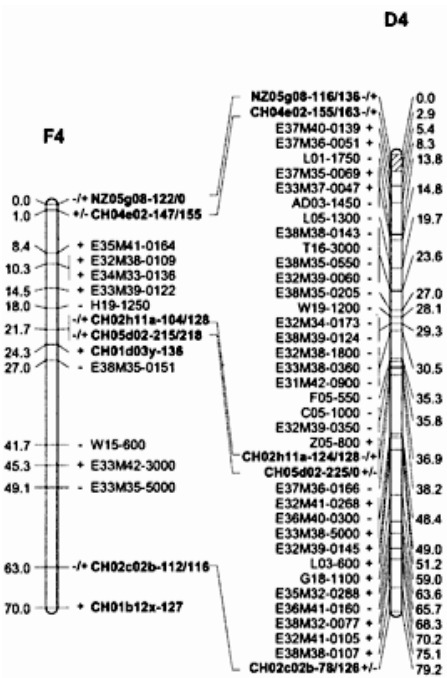
Appendix B. Continued

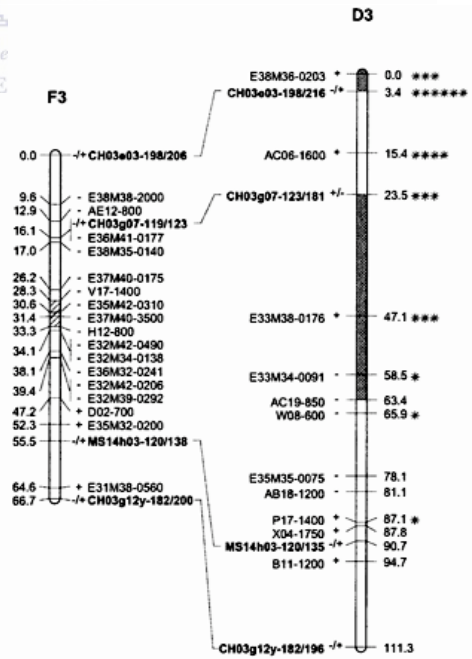
WAA DATA	CLASS			% INFESTATION			FOR CALCULATION OF % INFESTATIO!				
							BLOCK1		BLOCK 2		I
CLONES NO	BLOCK 1	BLOCK 2	BLOCK 3	BLOCK 1	BLOCK 2	BLOCK 3	# leaves in	total # leav	# leaves in	total # leav	# leaves ir
14	3	3	4	61	32	0	20	33	9	28	0
56	0	4	0	0	91	0	0	100	31	34	0
30	0	4	3	0	0	36	0	100	0	100	8
3	4	3	3	0	47	55	0	100	8	17	11
36	0	2	4	0	4	0	0	100	1	25	0
24	2	1	0	0	7	0	0	100	1	15	0
25	0	3	3	0	69	68	0	100	20	29	13
69	1	*	4	4	*	0	4	100			0
80	3	4	3	0	0	38	0	13	0	100	6
32	0	0	2	0	0	17	0	100	0	100	2

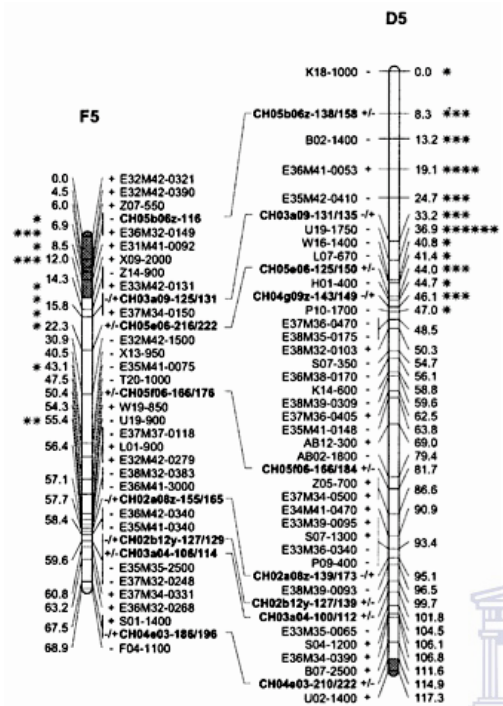
* = dead seedling clones

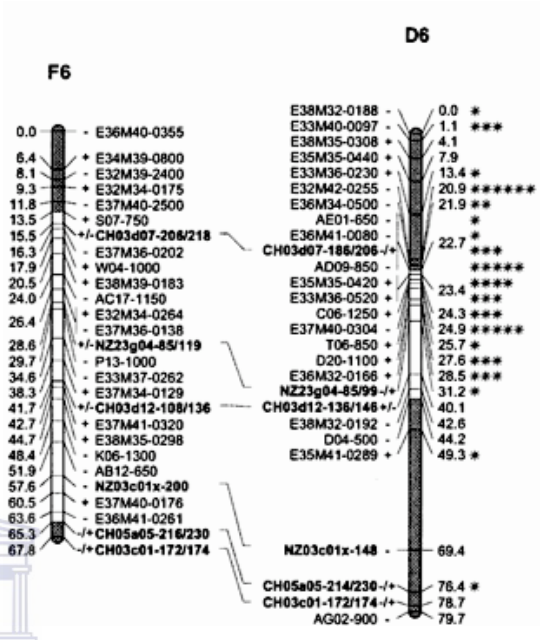


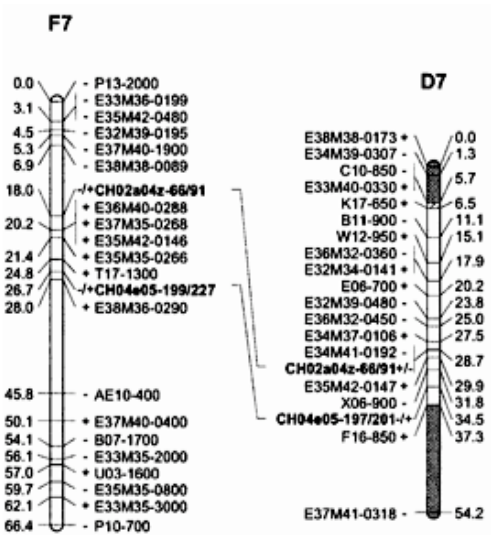


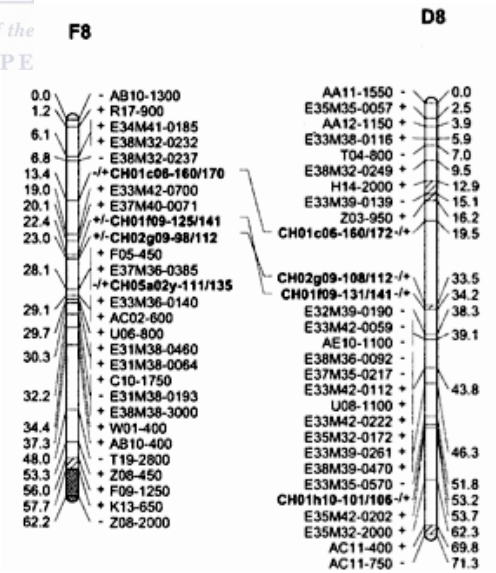


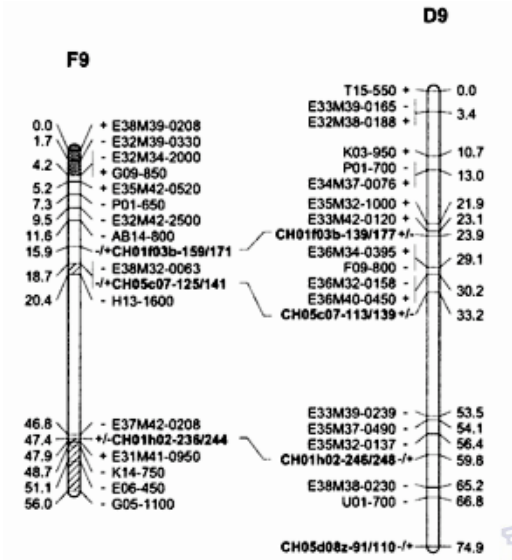


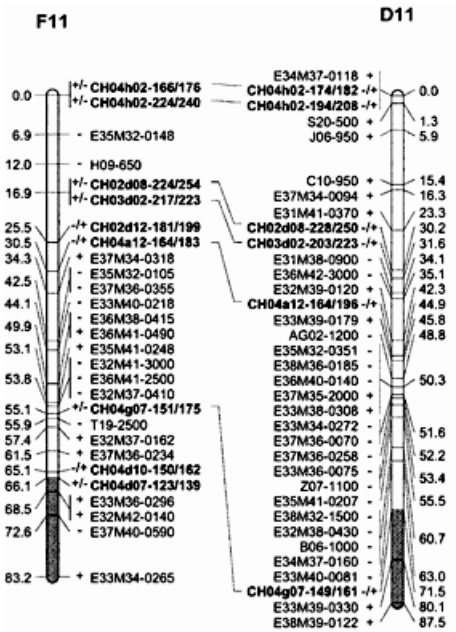


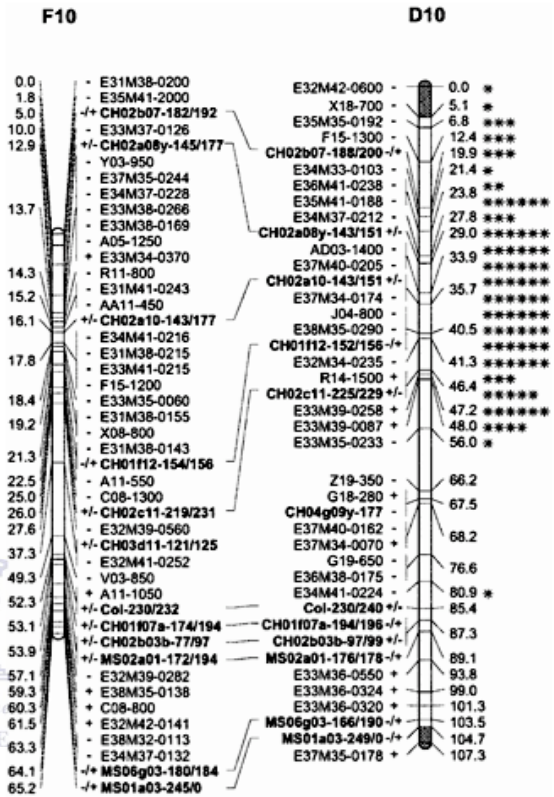


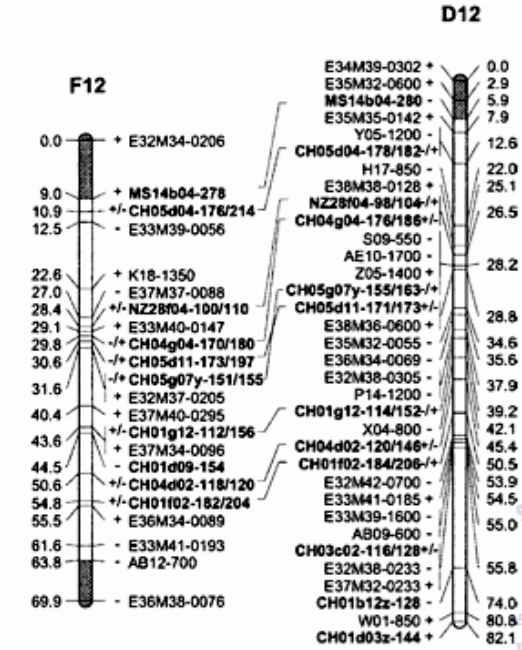


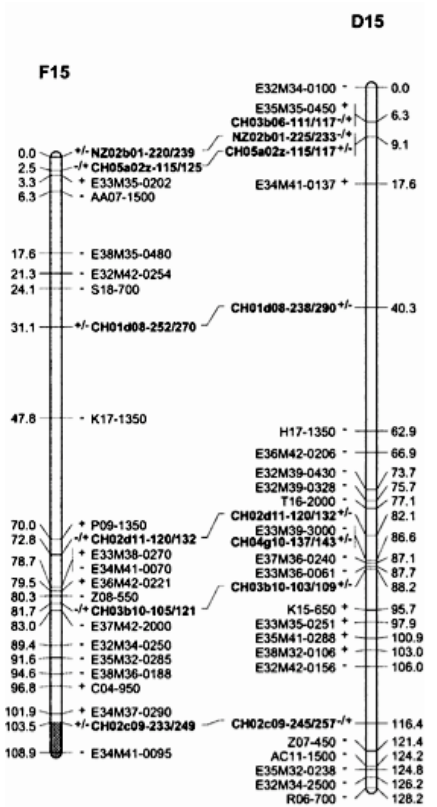


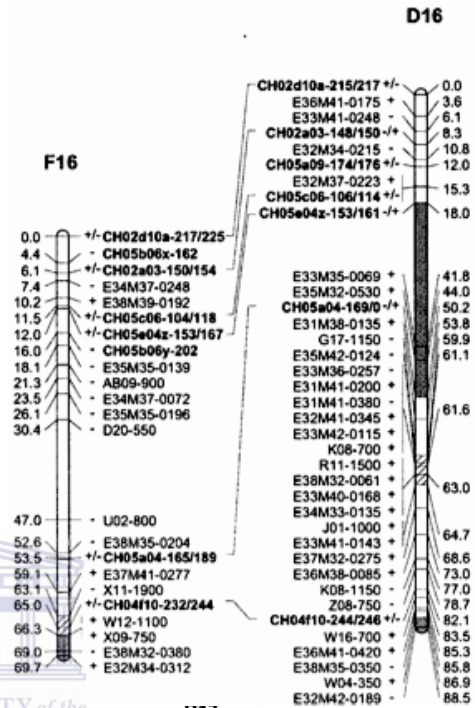


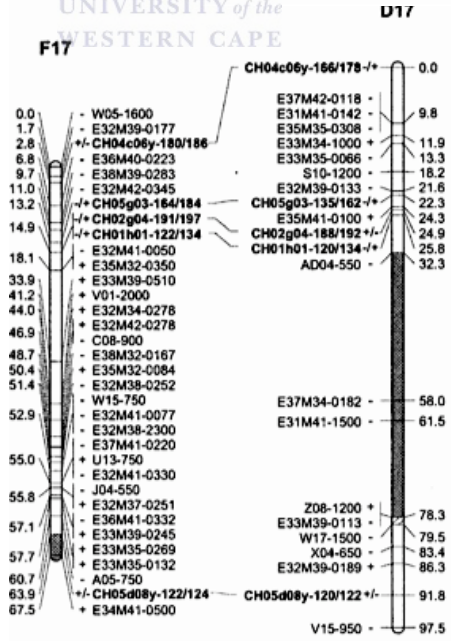


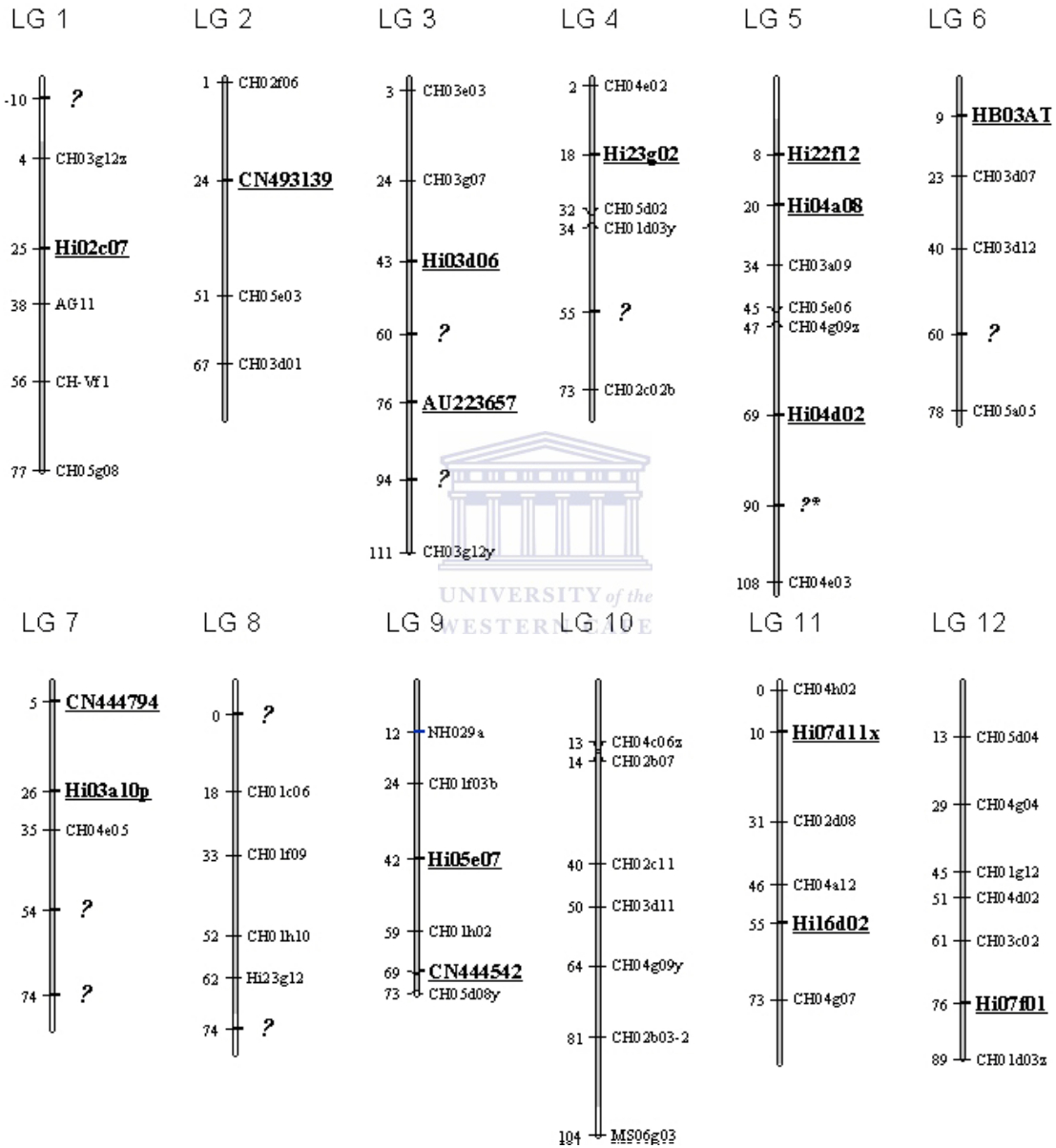


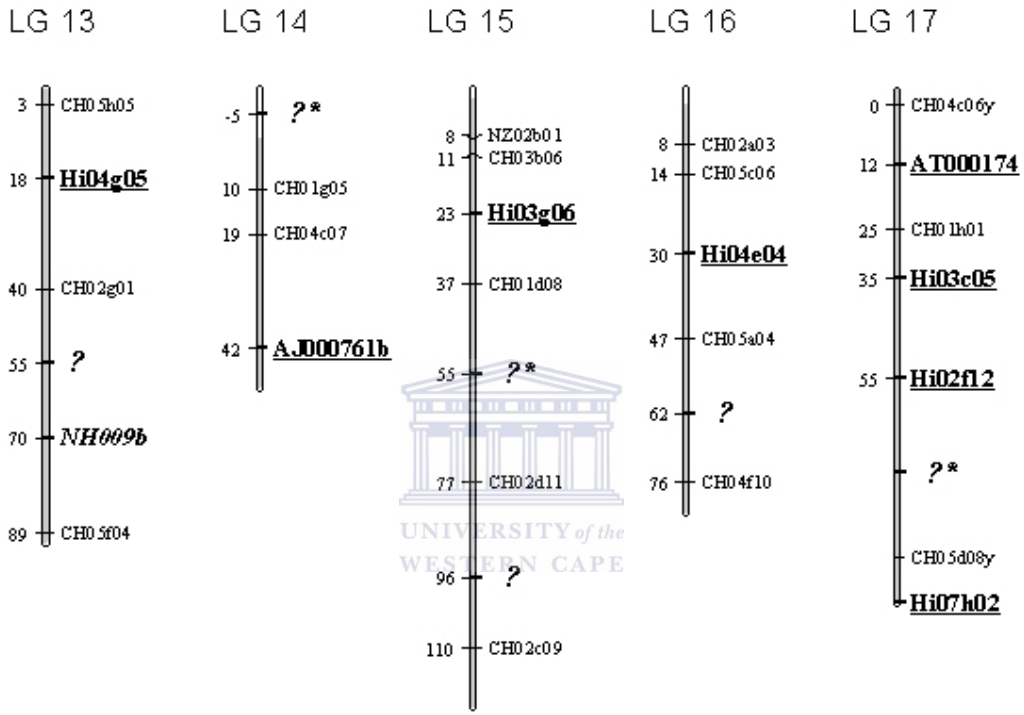












Appendix D: Microsatellite markers used in the Northern Spy × Cox Orange Pippin map construction.

Seedling number	A43	A38	A86	A42	A59	P5	A81	A78	A91	A93	A101
1	pn	uu	np	ac	ad	ml	ad	ll	nn	np	ca
2	nn	nn	np	ac	ad	ll	ad	ll	nn	nn	ad
3	nn	nn	uu	ac	ad	lm	ad	lm	nn	np	ca
7	nn	nn	pn	ac	ad	lm	ad	lm	np	np	ca
8	nn	uu	pn	ac	ad	uu	ad	ll	np	np	ca
10	nn	uu	pn	cb	bd	lm	cb	lm	nn	np	cb
11	pn	np	pn	db	cb	lm	cb	lm	nn	np	ca
13	nn	uu	pn	ac	ad	lm	ad	ll	nn	nn	ad
14	nn	nn	pn	ac	bd	lm	cb	ll	uu	np	ad
15	nn	nn	pn	ac	ad	lm	ad	lm	np	nn	ca
16	nn	nn	np	cb	bd	lm	cb	uu	nn	nn	ca
17	pn	np	np	ac	bd	ll	ad	ll	np	nn	cb
18	nn	nn	pn	ac	ad	lm	ad	lm	np	nn	ca
22	pn	np	np	uu	bc	lm	ad	lm	nn	nn	ca
23	pn	np	np	ad	cb	lm	ad	ll	nn	np	ad
24	pn	nn	np	cb	ad	ll	cb	lm	nn	nn	uu
25	nn	nn	pn	ac	ad	lm	ad	lm	np	nn	ad
26	nn	uu	pn	ac	ad	lm	ad	uu	np	nn	ca
27	nn	nn	pn	ac	ad	uu	ad	lm	np	np	ad
28	nn	np	np	ac	ca	ll	bd	ll	np	nn	ca
30	pn	np	np	cb	ac	lm	ad	lm	nn	uu	ca
31	pn	np	np	uu	ca	ll	ad	lm	nn	nn	ca

Appendix D: Continued

Seedling number	A43	A38	A86	A42	A59	P5	A81	A78	A91	A93	A101
32	nn	np	np	ad	bc	ll	cb	lm	nn	nn	ad
35	nn	np	np	ac	ac	ll	ca	lm	np	nn	cb
36	nn	np	pn	db	bc	lm	bd	lm	nn	np	ca
37	pn	uu	np	cb	bd	ll	cb	uu	np	np	ad
38	pn	np	np	ac	ad	ll	ad	lm	uu	nn	ad
39	uu	uu	uu	cb	ad	lm	bd	ll	pn	uu	bd
40	pn	np	np	uu	ad	ll	ad	ll	np	nn	ad
42	nn	nn	np	ad	ca	lm	cb	lm	nn	nn	ca
43	pn	np	pn	ac	ad	ll	bd	uu	nn	nn	ca
44	nn	np	pn	uu	ad	lm	ca	ll	np	nn	ad
46	pn	nn	pn	ad	cb	ll	ad	ll	np	nn	ca
56	nn	np	pn	ac	cb	lm	cb	lm	nn	nn	cb
58	nn	nn	pn	ac	ad	lm	ad	lm	np	nn	cb
59	pn	nn	pn	ad	ad	lm	ad	lm	nn	nn	uu
60	nn	np	pn	cb	bd	ll	cb	ll	np	np	cb
63	nn	np	np	cb	ad	lm	ad	lm	nn	np	cb
64	nn	nn	np	cb	ad	ll	ad	ll	np	nn	ad
65	nn	nn	np	cb	ad	lm	ad	ml	nn	np	uu
66	nn	np	np	db	cb	ll	cb	ll	nn	nn	uu
67	pn	np	pn	ac	ad	ll	cb	ll	np	np	cb
68	nn	np	np	ca	ad	lm	uu	ll	np	nn	cb
69	pn	np	np	ac	cb	uu	cb	lm	np	np	cb

Appendix D: Continued

Seedling number	A43	A38	A86	A42	A59	P5	A81	A78	A91	A93	A101
70	nn	np	np	ac	ad	lm	ad	lm	np	nn	ca
76	nn	np	np	ac	ad	uu	ad	ll	np	np	cb
77	nn	np	np	ac	ad	lm	ad	lm	np	nn	cb
80	pn	np	np	ac	cb	uu	cb	ll	nn	nn	bd
111	pn	nn	pn	ad	ad	ll	cb	ll	nn	uu	cb
1-03	pn	uu	pn	cb	ad	ll	ca	lm	nn	np	ad
2-03	nn	np	pn	ad	bd	lm	uu	lm	np	nn	ad
4-03	nn	nn	np	ac	ad	ll	ca	uu	np	nn	ca
5-03	pn	np	np	cb	ad	ll	ad	lm	np	np	bd
6-03	pn	np	pn	cb	cb	ll	cb	ll	np	np	bd
7-03	nn	np	np	db	bd	ll	ad	ll	np	nn	ad
8-03	pn	np	np	uu	uu	lm	ad	lm	np	np	bd
9-03	pn	np	pn	db	ad	ll	cb	ll	uu	nn	ad
10-03	pn	nn	np	ad	uu	ll	ad	ll	np	np	ad
11-03	pn	nn	np	db	ad	lm	ad	lm	np	nn	uu
13-03	pn	nn	np	uu	uu	ll	ca	lm	np	np	bd
14-03	nn	np	np	cb	bd	lm	ad	lm	nn	nn	cb
15-03	nn	np	np	cb	bd	ll	ca	ll	nn	nn	ca
16-03	pn	np	np	cb	ad	ll	ca	ll	nn	np	cb
17-03	pn	nn	pn	cb	ca	lm	bd	ll	nn	np	ca
18-03	nn	np	np	ad	bd	lm	bd	ll	np	uu	ca
19-03	nn	np	pn	db	bd	lm	cb	ll	nn	np	cb

Appendix D: Continued

Seedling number	A43	A38	A86	A42	A59	P5	A81	A78	A91	A93	A101
20-03	pn	nn	pn	cb	ac	ll	cb	uu	nn	uu	cb
21-03	nn	np	pn	db	bc	ll	ad	lm	np	nn	cb
22-03	pn	np	np	db	ca	ll	bd	lm	pn	nn	cb
23-03	pn	np	np	ad	cb	ll	ad	ll	nn	nn	ad
24-03	pn	nn	pn	db	bc	ll	cb	lm	np	nn	bd
27-03	pn	np	np	ad	ad	uu	bd	ll	nn	np	ad
28-03	nn	np	np	uu	bd	lm	uu	lm	pn	nn	ca
29-03	pn	np	pn	ad	bd	lm	ca	ll	np	nn	ad
30-03	pn	nn	np	uu	uu	ll	ca	ll	nn	nn	bd
32-03	pn	uu	np	ad	cb	ll	ad	ll	np	nn	ad
33-03	pn	nn	np	cb	bc	ml	bd	ll	nn	np	ad
34-03	nn	nn	np	uu	ac	uu	bd	lm	np	np	ca
35-03	nn	np	np	db	bd	lm	ca	ll	np	nn	ad
36-03	pn	uu	np	uu	uu	uu	cb	lm	np	np	uu
37-03	pn	uu	np	db	cb	ll	cb	lm	nn	nn	bd
38-03	pn	np	np	uu	ad	ml	cb	ll	nn	np	cb
39-03	nn	nn	uu	db	ac	lm	cb	ll	np	np	bd
40-03	pn	np	np	uu	uu	uu	uu	ll	nn	nn	cb
41-03	nn	np	np	db	bc	ll	bd	ll	nn	np	ad
42-03	pn	np	np	db	bd	lm	bd	lm	np	np	ad
43-03	pn	np	np	ad	ca	ll	bd	lm	nn	nn	bd
44-03	pn	uu	np	uu	cb	ll	uu	lm	np	nn	ad

Appendix D: Continued

Seedling number	A43	A38	A86	A42	A59	P5	A81	A78	A91	A93	A101
45-03	pn	uu	uu	uu	bd	ll	cb	uu	np	np	uu
46-03	pn	nn	np	uu	ad	ll	uu	lm	np	uu	cb
47-03	pn	nn	np	ad	bd	lm	cb	lm	np	nn	ca
48-03	pn	nn	np	db	cb	lm	ad	lm	nn	nn	cb
CP	pn	uu	np	ad	uu	ll	ca	lm	uu	nn	cb
M25	pn	nn	np	cb	ad	lm	uu	lm	np	np	bd
MM109	pn	np	nn	cb	cb	uu	cb	ll	np	nn	cb
RG	pn	np	nn	uu	bd	lm	uu	ll	nn	np	bd

Appendix D: Continued

Seedling number	A115	A65	A92	A121	A32	A107	A109	A10	A60	A120	A85
1	ac	nn	eg	ll	nn	ml	lm	ll	nn	bd	lm
2	bd	nn	ee	ll	nn	ll	uu	uu	np	ad	ll
3	uu	nn	ee	lm	nn	ll	uu	lm	nn	ad	ll
7	bd	nn	ee	ll	pn	ll	ll	lm	nn	ad	ll
8	ac	nn	ee	lm	nn	ll	ll	lm	nn	ad	ll
10	ac	nn	eg	lm	nn	ll	ll	lm	nn	ac	lm
11	uu	nn	ef	lm	nn	ml	lm	lm	nn	bd	ll
13	bc	nn	ee	lm	pn	ll	ll	lm	nn	ad	ll
14	bc	nn	eg	ll	nn	ll	ll	lm	nn	ac	lm
15	bc	nn	ee	lm	pn	ml	ll	lm	nn	ad	ll
16	ac	nn	ee	lm	pn	ll	ll	lm	nn	ac	lm
17	bd	nn	eg	lm	nn	ml	lm	lm	nn	ad	lm
18	ac	nn	ee	ml	nn	ll	ll	lm	nn	ad	ll
22	bc	np	eg	lm	nn	ml	lm	ml	np	ac	lm
23	uu	np	eg	ll	nn	ll	ll	uu	np	ac	lm
24	ac	nn	ee	uu	nn	ll	uu	ll	nn	bd	ll
25	bc	nn	ee	lm	pn	ll	ll	lm	nn	ad	ll
26	ac	nn	ee	ll	nn	ll	ll	lm	nn	ad	ll
27	bd	nn	ee	lm	nn	ml	ll	uu	nn	ad	ll
28	ac	np	ee	lm	pn	ml	ll	ll	np	ac	lm
30	ac	np	ee	ll	nn	ll	ll	ll	np	ad	lm
31	ac	np	ee	lm	nn	ll	ll	lm	np	ad	lm

Appendix D: Continued

Seedling number	A115	A65	A92	A121	A32	A107	A109	A10	A60	A120	A85
32	bc	np	ee	lm	pn	ll	ll	lm	np	bd	ll
35	bd	nn	ef	ll	pn	ll	lm	ll	nn	ad	ll
36	ac	np	gf	lm	nn	ml	lm	lm	np	ad	ll
37	ad	nn	ee	uu	pn	ml	ll	lm	nn	ad	lm
38	bc	nn	eg	lm	pn	ml	lm	lm	nn	ad	lm
39	bc	nn	eg	ll	nn	ll	ll	uu	nn	cb	lm
40	ac	nn	eg	lm	nn	ll	ll	uu	nn	ad	lm
42	ac	nn	ee	ll	nn	ll	ll	ll	nn	ac	ll
43	uu	nn	gf	ll	pn	ml	ll	lm	nn	ac	ll
44	bc	np	ef	ml	nn	ll	ll	lm	np	bd	ll
46	bc	np	ef	lm	pn	ml	uu	uu	np	bd	ll
56	bc	nn	eg	lm	pn	ll	ll	ll	nn	ad	ll
58	uu	nn	ee	ll	uu	ll	ll	ml	nn	uu	uu
59	uu	np	ef	lm	nn	ml	lm	ml	np	bd	ll
60	ac	nn	eg	lm	nn	ll	uu	uu	nn	ac	ll
63	bd	uu	ef	lm	pn	ml	lm	lm	nn	cb	lm
64	ad	nn	ef	uu	nn	ml	lm	lm	nn	uu	uu
65	bc	nn	ef	ll	uu	ml	lm	lm	nn	cb	lm
66	ac	pn	ef	ll	pn	ll	ll	ll	np	cb	lm
67	ac	nn	gf	lm	pn	ml	ll	ll	nn	ac	ll
68	bd	nn	ef	lm	nn	ll	ll	uu	np	uu	ll
69	bd	uu	ef	ll	nn	ml	lm	ml	nn	bd	lm

Appendix D: Continued

Seedling number	A115	A65	A92	A121	A32	A107	A109	A10	A60	A120	A85
70	ac	nn	ee	ll	nn	ml	lm	ll	nn	uu	uu
76	bd	nn	ee	lm	nn	ml	lm	ll	nn	cb	lm
77	bd	nn	ee	lm	nn	ml	ll	ll	nn	cb	lm
80	bd	nn	ef	ll	uu	ml	lm	lm	nn	bd	lm
111	bd	uu	eg	ll	pn	ml	ll	ll	nn	uu	ml
1-03	bd	nn	eg	ll	nn	ll	lm	ml	nn	bd	ll
2-03	ac	np	ef	lm	nn	ml	ll	ml	np	bd	ll
4-03	bd	uu	ee	uu	nn	ml	lm	lm	nn	bd	lm
5-03	ad	nn	gf	ll	nn	ll	ll	ll	nn	ac	lm
6-03	uu	np	ee	ll	nn	ll	ll	ll	np	ad	ll
7-03	bc	nn	eg	lm	nn	ml	lm	l m	nn	ad	ll
8-03	bd	nn	gf	lm	nn	ml	ll	uu	nn	uu	uu
9-03	bd	nn	gf	uu	nn	ml	ll	l m	nn	bd	lm
10-03	ac	uu	eg	lm	nn	ll	ll	uu	nn	cb	lm
11-03	bd	np	gf	lm	pn	ll	lm	lm	np	bd	lm
13-03	bc	uu	ee	ll	pn	uu	ll	ll	uu	uu	lm
14-03	bd	nn	ee	ll	nn	ml	lm	lm	nn	cb	lm
15-03	ac	nn	gf	lm	nn	ml	lm	lm	nn	cb	lm
16-03	bd	nn	ef	lm	nn	ll	ll	lm	nn	ad	lm
17-03	uu	np	ee	ll	pn	ml	lm	ll	np	ad	ll
18-03	bc	np	gf	lm	pn	ml	ll	lm	np	bd	lm
19-03	bc	np	ef	ll	nn	ml	lm	uu	uu	uu	uu

Appendix D: Continued

Seedling number	A115	A65	A92	A121	A32	A107	A109	A10	A60	A120	A85
20-03	bd	nn	ef	ll	nn	ll	lm	lm	np	ad	lm
21-03	ac	np	eg	ll	nn	ll	ll	ll	np	bd	lm
22-03	bc	uu	uu	lm	nn	ll	ll	lm	np	ad	lm
23-03	uu	np	ee	ll	nn	ml	lm	ll	np	cb	ll
24-03	bc	np	ee	ll	uu	ll	ll	uu	np	ac	lm
27-03	ac	nn	ef	ll	pn	ll	lm	lm	nn	uu	lm
28-03	bc	pn	eg	lm	nn	ml	uu	uu	np	uu	lm
29-03	bc	nn	ef	ll	nn	ll	lm	uu	nn	cb	lm
30-03	bc	uu	ee	lm	pn	ml	uu	ll	np	uu	uu
32-03	bd	np	gf	ll	pn	ll	uu	ml	np	uu	uu
33-03	uu	pn	ef	ll	pn	ll	ll	lm	np	ad	lm
34-03	bd	np	ef	lm	nn	ll	ll	ll	nn	bd	lm
35-03	bd	nn	eg	lm	pn	uu	uu	uu	nn	ad	lm
36-03	bc	np	ee	lm	pn	ml	ll	ll	uu	bd	lm
37-03	bd	np	eg	ll	nn	ml	lm	ll	nn	ad	lm
38-03	ac	nn	ef	uu	nn	ll	uu	uu	nn	ad	lm
39-03	ac	np	ee	ll	nn	ml	ll	uu	np	cb	lm
40-03	bd	np	eg	lm	uu	ml	lm	ll	uu	uu	ll
41-03	ac	np	eg	lm	pn	ml	ll	ll	np	bd	lm
42-03	bc	nn	eg	ll	nn	ll	ll	lm	nn	ad	ll
43-03	ac	nn	gf	ll	nn	ll	ll	lm	uu	ad	lm
44-03	uu	nn	eg	ll	pn	ml	ll	uu	nn	cb	lm

Appendix D: Continued

Seedling number	A115	A65	A92	A121	A32	A107	A109	A10	A60	A120	A85
45-03	bc	uu	eg	lm	nn	ll	uu	uu	nn	ad	lm
46-03	bc	nn	eg	ll	pn	ml	lm	lm	nn	bd	lm
47-03	ac	np	ef	uu	nn	ll	ll	lm	np	ad	lm
48-03	uu	np	eg	ll	nn	ll	ll	ll	np	cb	lm
CP	bc	np	gf	uu	nn	ml	ll	ll	np	bd	uu
M25	ac	uu	ee	lm	nn	ll	ll	ll	nn	bd	lm
MM109	ac	np	ee	uu	nn	ll	lm	ll	uu	ad	ll
RG	uu	np	eg	lm	pn	ll	ll	uu	np	uu	uu

Appendix D: Continued

Seedling number	A62	A75	A49	A162	A135	A103	A126	A111	A104	A57	A74
1	ad	bd	ll	lm	lm	eg	ml	ef	nn	ad	lm
2	uu	bd	uu	uu	uu	fe	lm	fg	uu	uu	uu
3	ad	ad	lm	ll	ll	fe	lm	fg	nn	ad	ll
7	ad	bd	lm	ll	ll	fe	lm	fg	nn	uu	lm
8	ad	cb	lm	ll	ll	fe	lm	eg	nn	ad	ll
10	db	bd	lm	ll	uu	fg	ll	fg	np	bd	ll
11	cb	cb	ll	uu	ll	ee	ll	eg	nn	cb	ll
13	ad	bd	lm	lm	ll	fe	uu	ee	nn	ad	ll
14	db	bd	lm	ll	ll	fg	ll	fg	np	bd	ll
15	ad	bd	lm	ll	ll	fe	lm	fg	nn	ad	ll
16	db	bd	lm	ll	lm	ee	ll	fg	np	bd	lm
17	ad	ad	ll	lm	lm	ee	lm	ee	nn	cb	lm
18	ad	bd	lm	ll	ll	fe	lm	fg	nn	ad	ll
22	cb	cb	ll	lm	lm	eg	lm	fg	np	cb	lm
23	ac	bd	uu	uu	lm	eg	ml	fg	pn	cb	uu
24	ad	ad	ll	ll	ml	eg	ll	ee	uu	ad	ll
25	ad	bd	lm	ll	ll	fe	uu	fg	nn	ad	ll
26	ad	bd	lm	ll	ll	ee	lm	fg	nn	ca	uu
27	ad	bd	uu	uu	ll	fe	lm	ef	uu	ad	ll
28	ac	ca	ll	ll	lm	fg	ml	ef	nn	ca	lm
30	ad	bd	lm	lm	ml	fg	ll	eg	nn	ca	ll
31	ac	ca	ll	lm	ll	fg	uu	eg	nn	ca	uu

Appendix D: Continued

Seedling number	A62	A75	A49	A162	A135	A103	A126	A111	A104	A57	A74
32	cb	bd	lm	ll	ll	eg	ml	ee	nn	ca	uu
35	ac	bd	ll	lm	ll	fg	uu	ef	nn	ca	ll
36	cb	cb	ll	lm	lm	eg	ll	fg	nn	cb	lm
37	db	ca	lm	uu	uu	eg	ll	ee	np	bd	ll
38	ad	cb	ll	lm	lm	ee	lm	eg	nn	ca	lm
39	db	bd	uu	lm	lm	ee	ll	ee	np	ad	lm
40	ad	cb	uu	uu	ml	ee	uu	fg	nn	ad	lm
42	ac	bd	ll	lm	ll	eg	ml	fe	np	ca	ll
43	ac	ad	lm	lm	lm	fg	ml	eg	np	ad	lm
44	ac	cb	lm	uu	ll	fe	ll	eg	nn	cb	uu
46	ad	ca	uu	uu	uu	fe	ll	eg	np	ca	ll
56	cb	bd	lm	lm	lm	fe	ll	ef	nn	cb	ll
58	ad	bd	lm	ll	ll	ee	uu	fe	nn	uu	lm
59	ac	ad	ll	lm	ll	fe	lm	ee	nn	ca	ll
60	ad	ad	lm	uu	uu	fe	ll	eg	nn	bd	lm
63	ad	ca	lm	lm	lm	eg	ll	ee	nn	ad	lm
64	ad	cb	lm	lm	lm	eg	ll	eg	nn	ad	lm
65	ad	bd	lm	uu	lm	eg	ll	ef	nn	ad	lm
66	cb	bd	ll	lm	ll	ee	ml	ef	nn	cb	ll
67	ad	cb	lm	ml	ll	fe	uu	eg	nn	ad	ll
68	ad	cb	lm	uu	lm	fe	uu	eg	nn	uu	uu
69	cb	cb	ll	lm	lm	fe	lm	ef	np	cb	lm

Appendix D: Continued

Seedling number	A62	A75	A49	A162	A135	A103	A126	A111	A104	A57	A74
70	ad	cb	ll	lm	lm	ee	ml	eg	nn	ad	lm
76	ad	uu	ll	lm	uu	ee	uu	ee	nn	uu	lm
77	ad	cb	ll	lm	lm	ee	uu	eg	nn	ad	uu
80	cb	cb	ll	lm	uu	fe	lm	ef	np	cb	lm
111	ac	bd	uu	ll	lm	uu	lm	fg	nn	ca	ll
1-03	ad	bd	ll	ll	lm	ee	ml	uu	nn	ad	lm
2-03	db	cb	ll	lm	lm	ee	uu	eg	np	bd	lm
4-03	ad	cb	lm	lm	ll	fg	ll	eg	np	ad	ll
5-03	ad	bd	ll	lm	ll	ee	ll	eg	np	ad	ll
6-03	ad	bd	lm	uu	lm	fg	ll	eg	nn	cb	ll
7-03	db	ca	lm	lm	uu	eg	ll	ee	nn	bd	lm
8-03	ad	ca	lm	lm	ll	fg	ll	ee	np	ca	lm
9-03	ad	bd	lm	ll	uu	fe	ll	ef	np	ad	ll
10-03	uu	cb	uu	uu	lm	ee	uu	eg	nn	bd	lm
11-03	ac	uu	ll	ll	lm	eg	ll	ee	nn	ca	lm
13-03	uu	ca	lm	lm	lm	uu	ll	uu	nn	cb	uu
14-03	db	bd	lm	ll	lm	eg	ll	ee	nn	bd	lm
15-03	db	bd	ll	lm	ll	fg	ll	ee	np	bd	ll
16-03	ad	uu	lm	ll	ll	ee	ll	eg	nn	ca	ll
17-03	ac	bd	lm	lm	ll	ee	ll	eg	nn	ca	ll
18-03	db	cb	lm	uu	ll	fg	lm	fg	nn	uu	uu
19-03	uu	bd	uu	lm	lm	uu	ll	ef	nn	ac	uu

Appendix D: Continued

Seedling number	A62	A75	A49	A162	A135	A103	A126	A111	A104	A57	A74
20-03	ac	bd	ll	ll	ll	fg	ll	eg	nn	ca	ll
21-03	cb	bd	lm	lm	lm	fe	ll	eg	nn	cb	ll
22-03	ac	ca	uu	uu	ml	fe	uu	eg	uu	uu	ll
23-03	cb	cb	lm	lm	ll	fg	ll	eg	nn	cb	ll
24-03	uu	cb	lm	ll	ll	ee	ll	eg	nn	cb	ll
27-03	ad	ad	lm	lm	ml	ee	ll	ee	np	ad	ll
28-03	db	ad	uu	lm	ll	fg	ml	ee	np	bd	ll
29-03	db	cb	lm	ll	lm	fe	ml	ee	np	bd	lm
30-03	cb	ad	lm	lm	ml	eg	uu	fg	np	cb	uu
32-03	ac	cb	uu	ll	uu	ee	ll	eg	np	cb	uu
33-03	cb	cb	ll	lm	lm	fg	ll	eg	nn	cb	uu
34-03	ad	ca	lm	lm	lm	ee	ll	ef	nn	cb	lm
35-03	uu	bd	lm	lm	uu	eg	ll	eg	np	bd	lm
36-03	uu	ca	ml	ml	ml	ee	ll	fg	uu	uu	lm
37-03	cb	ad	ll	ll	uu	fg	ll	eg	np	cb	ll
38-03	ad	bd	uu	uu	uu	fe	ll	eg	np	uu	uu
39-03	ad	uu	uu	lm	ll	fg	ll	fg	uu	ca	lm
40-03	db	ca	ll	ll	ll	fg	uu	eg	nn	ca	uu
41-03	cb	uu	ll	ll	ll	eg	ll	ee	np	cb	lm
42-03	db	bd	lm	lm	lm	fe	ll	eg	nn	ca	uu
43-03	ac	bd	ll	lm	ll	uu	ml	ee	uu	cb	lm
44-03	cb	ad	ll	lm	ll	fg	ml	eg	nn	ca	ll

Appendix D: Continued

Seedling number	A62	A75	A49	A162	A135	A103	A126	A111	A104	A57	A74
45-03	da	ca	uu	uu	ll	fe	uu	ee	nn	cb	ll
45-03	da	ca	uu	ll	lm	ee	uu	uu	nn	ad	ll
47-03	cb	ad	lm	lm	ll	ee	ml	eg	np	cb	ll
48-03	cb	bd	ll	lm	ll	fg	ll	ee	np	uu	ll
CP	ad	bd	lm	lm	ml	eg	ll	uu	uu	uu	uu
M25	ac	bd	ll	lm	lm	fe	ll	fg	nn	ad	ml
MM109	uu	bd	uu	uu	uu	fe	ll	eg	np	cb	lm
RG	uu	ca	lm	lm	uu	fe	ll	uu	nn	bc	ll

Appendix D: Continued

Seedling number	A113	A188	A64	A73	A130	A37	A90	A139	A165	A227	A98
1	nn	db	bd	nn	ll	lm	nn	nn	ml	uu	ef
2	uu	uu	bd	np	ll	ll	pn	nn	ml	uu	eg
3	uu	cb	bd	nn	ll	ll	pn	nn	ml	gf	eg
7	uu	da	bd	np	ll	ll	pn	nn	ml	gf	eg
8	pn	uu	ac	uu	ll	ll	pn	nn	ml	gf	ge
10	np	ac	bc	np	ll	uu	nn	nn	ml	uu	eg
11	nn	ad	ca	nn	ll	uu	uu	uu	uu	uu	eg
13	np	cb	ac	uu	ll	uu	nn	nn	ml	uu	eg
14	np	ca	bc	np	ll	lm	pn	nn	ml	gf	eg
15	pn	cb	bc	np	ll	ll	pn	nn	ml	ge	ee
16	uu	cb	ac	np	ll	lm	pn	nn	ml	gf	ee
17	nn	ca	bc	np	ml	lm	nn	pn	ml	uu	eg
18	pn	cb	bd	nn	uu	ll	pn	nn	ml	gf	eg
22	pn	da	ca	np	ll	lm	nn	pn	uu	uu	eg
23	np	uu	bd	np	ml	lm	nn	pn	ml	gf	eg
24	pn	cb	bd	nn	ll	lm	nn	nn	ll	ge	eg
25	pn	cb	bc	np	uu	lm	nn	nn	ml	gf	ee
26	np	uu	bd	nn	uu	ll	pn	nn	ml	uu	eg
27	pn	cb	bd	nn	ll	ll	pn	nn	ml	uu	eg
28	pn	cb	bd	nn	ll	lm	nn	pn	ml	ee	ee
30	np	ca	uu	np	ml	ml	nn	nn	ml	ef	eg
31	np	uu	ad	uu	ml	lm	nn	pn	uu	uu	ef

Appendix D: Continued

Seedling number	A113	A188	A64	A73	A130	A37	A90	A139	A165	A227	A98
32	uu	uu	bd	np	ml	lm	pn	pn	ml	ee	eg
35	nn	cb	ad	np	ml	lm	pn	pn	ll	ee	ee
36	np	bd	bd	np	ml	ll	pn	pn	ll	uu	ee
37	np	cb	ac	np	ml	lm	nn	nn	ml	gf	ef
38	nn	ca	ac	nn	ml	lm	nn	pn	ml	gf	eg
39	np	cb	bd	nn	ll	lm	nn	nn	ml	gf	ef
40	np	ca	uu	uu	uu	lm	pn	nn	uu	uu	ef
42	np	cb	bd	np	ml	lm	nn	pn	ll	uu	ef
43	np	db	bd	nn	ll	uu	uu	pn	ll	ge	eg
44	np	uu	ac	uu	uu	lm	nn	uu	uu	uu	eg
46	nn	ca	bc	nn	ml	ll	nn	nn	ll	uu	ef
56	pn	db	ac	np	ml	ll	pn	pn	ml	ee	ef
58	nn	da	bd	nn	ll	lm	uu	nn	ml	uu	ef
59	nn	ca	ca	nn	ml	ml	nn	nn	uu	gf	eg
60	pn	db	ca	np	ml	uu	uu	uu	uu	ef	eg
63	np	db	bd	np	ll	lm	pn	nn	ll	ge	eg
64	np	db	bd	nn	ll	uu	uu	nn	uu	uu	eg
65	np	db	bd	np	ll	lm	pn	nn	ll	uu	eg
66	np	cb	ac	np	ml	lm	pn	pn	ll	uu	ef
67	np	ad	uu	uu	uu	ll	pn	pn	ll	gf	eg
68	np	uu	ad	uu	uu	ll	nn	nn	uu	uu	ef
69	pn	cb	ca	nn	ll	lm	nn	pn	ll	ee	eg

Appendix D: Continued

Seedling number	A113	A188	A64	A73	A130	A37	A90	A139	A165	A227	A98
70	nn	uu	ca	np	ml	uu	uu	pn	ml	gf	eg
76	nn	cb	ac	nn	uu	ll	pn	nn	uu	uu	uu
77	np	cb	bc	uu	ml	lm	pn	pn	ml	uu	eg
80	np	ca	ad	np	ml	lm	nn	pn	ll	ee	eg
111	np	da	ad	nn	ml	lm	nn	uu	ml	uu	eg
1-03	np	ca	bd	np	ll	lm	nn	nn	ml	uu	eg
2-03	nn	cb	ac	uu	ml	lm	uu	uu	ll	uu	eg
4-03	nn	cb	ac	uu	ml	lm	pn	nn	ml	uu	eg
5-03	pn	da	uu	uu	ll	uu	uu	pn	ml	ee	ef
6-03	np	cb	uu	np	ml	uu	nn	nn	ml	uu	eg
7-03	nn	db	bd	np	ml	ll	pn	pn	ml	ee	eg
8-03	nn	uu	ad	np	ml	lm	nn	uu	ml	ee	ef
9-03	nn	db	bd	nn	ml	uu	uu	nn	ll	ee	ef
10-03	nn	ca	ac	uu	ll	lm	nn	nn	ll	uu	eg
11-03	nn	cb	uu	nn	ml	lm	pn	nn	ll	uu	eg
13-03	np	uu	da	np	ll	lm	nn	nn	ml	ge	eg
14-03	pn	cb	bd	nn	ll	ml	nn	nn	ll	uu	ef
15-03	nn	db	ac	np	ll	lm	pn	pn	ll	uu	eg
16-03	nn	cb	uu	np	ml	lm	nn	nn	ml	gf	eg
17-03	np	cb	bc	np	ml	lm	pn	uu	ml	gf	ef
18-03	uu	ad	bd	np	ll	lm	pn	pn	ll	ge	eg
19-03	np	uu	bd	nn	ml	uu	uu	uu	ll	uu	ef

Appendix D: Continued

Seedling number	A113	A188	A64	A73	A130	A37	A90	A139	A165	A227	A98
20-03	pn	ca	bc	nn	ml	ll	nn	nn	ll	ee	eg
21-03	np	db	bd	nn	ml	uu	uu	uu	ml	ef	ee
22-03	nn	uu	uu	np	ll	uu	uu	uu	ml	uu	ef
23-03	pn	cb	ca	np	ll	ll	pn	pn	ll	ee	eg
24-03	np	ca	bc	np	uu	lm	nn	uu	ml	uu	eg
27-03	nn	ca	bd	np	ll	lm	nn	nn	ml	ge	ef
28-03	nn	uu	uu	np	ml	ll	pn	pn	ll	ee	ef
29-03	nn	db	ca	np	ml	ll	nn	pn	ll	ef	ef
30-03	np	uu	bc	np	uu	ll	nn	nn	ll	ge	eg
32-03	np	ad	uu	uu	uu	lm	nn	nn	ll	uu	eg
33-03	uu	uu	ca	nn	ll	lm	uu	nn	ml	ef	eg
34-03	nn	cb	ad	nn	ll	lm	nn	nn	ll	uu	ee
35-03	uu	uu	ac	uu	ml	lm	pn	pn	ll	uu	eg
36-03	np	ca	ad	nn	uu	lm	nn	uu	ll	ge	eg
37-03	pn	db	db	np	ml	lm	nn	nn	ml	ge	eg
38-03	uu	uu	cb	np	ml	uu	uu	nn	ml	ef	eg
39-03	nn	cb	ac	uu	ll	lm	nn	nn	uu	ge	ef
40-03	np	uu	ca	uu	uu	uu	nn	uu	ml	uu	eg
41-03	pn	db	bd	np	ml	lm	pn	pn	ll	ee	eg
42-03	nn	uu	ac	np	ll	uu	uu	uu	ml	uu	ef
43-03	nn	uu	uu	nn	ml	uu	uu	uu	ml	uu	eg
44-03	nn	ca	cb	np	ml	lm	nn	nn	uu	ee	ee

Appendix D: Continued

	A113	A188	A64	A73	A130	A37	A90	A139	A165	A227	A98
45-03	nn	db	uu	np	ml	ll	nn	nn	ml	uu	ee
46-03	nn	cb	uu	np	ll	ll	nn	nn	ll	uu	ef
47-03	nn	cb	ac	np	ll	lm	nn	uu	ml	gf	eg
48-03	np	ca	bc	np	ml	uu	uu	nn	ml	ee	ef
CP	uu	ca	bc	uu	uu	ll	pn	nn	ml	uu	ef
M25	nn	cb	bc	nn	ml	lm	pn	pn	ml	ef	ee
MM109	nn	cb	bd	np	ll	uu	uu	uu	ml	uu	eg
RG	nn	ca	ad	np	ml	ll	nn	uu	ll	ee	ef

Appendix D: Continued

Seedling number	A96	A106	A77	A94	A102	A52	A133	A41	A29	A67	A61
1	ml	ml	cb	ll	lm	np	nn	nn	nn	ad	lm
2	ll	ll	bd	ll	ll	np	nn	pn	pn	ad	lm
3	ll	ll	cb	ll	ml	pn	nn	pn	pn	ad	lm
7	ml	ll	cb	ll	ll	np	nn	pn	pn	ad	lm
8	ll	ll	cb	ll	ll	pn	nn	pn	nn	ad	lm
10	ml	ll	ad	lm	ml	pn	pn	nn	nn	cb	ll
11	ml	ll	db	ll	ml	pn	pn	nn	nn	uu	uu
13	ll	ll	ac	ll	lm	uu	pn	nn	nn	uu	uu
14	ml	ll	cb	ll	ll	np	nn	nn	pn	bc	ll
15	ll	ll	cb	ll	ll	np	nn	uu	uu	ad	lm
16	ll	ll	ad	ll	ll	np	nn	nn	pn	cb	ll
17	ml	ll	ac	ll	lm	pn	uu	pn	nn	bc	ll
18	ml	ll	cb	ll	ll	uu	nn	pn	pn	ad	lm
22	ll	ll	ac	ll	lm	np	pn	nn	nn	ad	ll
23	ml	uu	cb	ll	ll	uu	nn	uu	uu	ac	lm
24	ml	uu	ad	ll	ml	np	uu	uu	pn	ac	uu
25	ml	ll	ad	ll	ll	np	uu	pn	uu	uu	ll
26	ml	uu	cb	ll	lm	np	uu	pn	nn	ad	ll
27	ml	ml	ad	ll	ll	uu	uu	pn	nn	ad	lm
28	ml	ll	db	lm	lm	pn	pn	nn	nn	cb	lm
30	ml	ll	ca	ll	lm	nn	nn	nn	nn	uu	lm
31	ml	ll	ca	ll	ml	uu	uu	pn	nn	bd	uu

Appendix D: Continued

Seedling number	A96	A106	A77	A94	A102	A52	A133	A41	A29	A67	A61
32	ml	ll	ad	lm	ll	pn	nn	nn	nn	bd	ll
35	ll	ll	ad	ll	ll	uu	nn	pn	pn	bd	ll
36	ml	ll	db	lm	lm	np	np	nn	nn	ad	ll
37	ll	ml	ad	ll	ll	uu	nn	pn	nn	cb	ll
38	ml	ll	ad	ll	ll	np	nn	uu	nn	ac	ll
39	ml	ll	ad	ll	ll	nn	pn	nn	nn	ad	lm
40	ll	uu	ac	ll	lm	np	nn	nn	nn	cb	ll
42	ml	ll	cb	ll	lm	nn	np	nn	nn	cb	ll
43	ml	ll	db	lm	ll	np	nn	nn	nn	ac	lm
44	ll	ll	ac	ll	ll	np	pn	pn	pn	ad	uu
46	ll	uu	ad	lm	ml	uu	uu	nn	nn	ad	lm
56	ml	ll	cb	ll	ll	np	nn	pn	nn	cb	lm
58	ll	ll	ad	ll	uu	uu	pn	pn	nn	ad	lm
59	ll	ll	ad	lm	ml	pn	nn	uu	uu	uu	uu
60	ll	ll	bd	lm	ll	np	nn	pn	nn	ad	lm
63	ll	ll	ac	ll	ml	nn	nn	nn	nn	ad	lm
64	uu	ll	ad	ll	ml	nn	pn	uu	uu	ac	uu
65	ml	ll	ad	ll	uu	nn	nn	nn	pn	ad	ll
66	ml	ml	cb	lm	ll	nn	uu	pn	pn	uu	ll
67	ml	ll	ad	lm	lm	uu	nn	nn	nn	bd	lm
68	ll	ll	ac	ll	ll	np	uu	nn	uu	ac	uu
69	ll	ll	ac	ll	ll	np	nn	pn	nn	ad	ll

Appendix D: Continued

Seedling number	A96	A106	A77	A94	A102	A52	A133	A41	A29	A67	A61
70	ll	ll	ad	lm	ml	uu	uu	pn	nn	ad	ll
76	ll	ll	ac	lm	ll	np	pn	pn	nn	ad	ll
77	ll	ll	ca	lm	ll	np	pn	pn	nn	ad	lm
80	ll	ll	ad	ll	lm	nn	pn	pn	nn	ad	ll
111	ll	ll	bd	ll	lm	uu	pn	nn	nn	uu	ml
1-03	ll	ll	db	ll	lm	nn	pn	pn	nn	ad	lm
2-03	ll	ll	db	ll	lm	np	pn	nn	nn	ad	lm
4-03	ml	ll	db	lm	ll	uu	uu	pn	nn	db	ll
5-03	ll	ml	db	lm	lm	nn	nn	nn	nn	cb	lm
6-03	ll	ll	bd	ll	lm	uu	pn	pn	nn	uu	uu
7-03	uu	ll	cb	ll	lm	nn	pn	nn	pn	uu	ll
8-03	ml	ll	db	ll	lm	np	pn	nn	pn	ac	ll
9-03	ml	uu	da	lm	ml	nn	pn	nn	pn	uu	ll
10-03	ll	ml	db	ll	lm	nn	pn	pn	nn	ac	lm
11-03	ll	ml	db	lm	ml	uu	nn	pn	nn	bd	lm
13-03	ml	ll	db	lm	ll	np	pn	uu	uu	uu	ll
14-03	ml	ll	db	lm	lm	nn	pn	nn	uu	bd	uu
15-03	ml	uu	db	ll	ll	np	nn	pn	nn	bd	ll
16-03	ll	ml	db	lm	lm	np	nn	pn	nn	ad	lm
17-03	ll	ml	db	ll	ml	nn	pn	pn	nn	uu	ll
18-03	ll	ll	db	ll	lm	np	pn	nn	nn	bd	ll
19-03	ml	ll	db	uu	ml	uu	pn	pn	nn	ad	uu

Appendix D: Continued

Seedling number	A96	A106	A77	A94	A102	A52	A133	A41	A29	A67	A61
20-03	ll	ml	uu	ll	ll	pn	nn	pn	nn	ac	lm
21-03	ll	ll	db	ll	lm	uu	pn	nn	nn	uu	uu
22-03	ll	ll	db	ll	ll	nn	pn	nn	nn	uu	uu
23-03	ll	ll	bd	lm	ll	nn	pn	pn	nn	ad	ll
24-03	uu	ml	db	ll	ml	nn	pn	nn	nn	ac	lm
27-03	ml	ll	db	lm	ll	np	nn	pn	nn	ca	ll
28-03	ll	ml	db	lm	ll	np	nn	nn	pn	cb	ll
29-03	ll	ml	db	lm	ml	uu	nn	pn	nn	bd	ll
30-03	lm	ml	db	ll	lm	np	pn	uu	uu	bd	lm
32-03	ml	uu	ad	ll	ll	np	pn	nn	nn	ad	ll
33-03	ll	ll	db	lm	lm	np	pn	nn	nn	ac	ll
34-03	ll	ml	uu	ll	ml	np	pn	nn	nn	bd	lm
35-03	uu	ll	db	ll	lm	np	pn	nn	nn	bd	ll
36-03	ll	uu	db	lm	lm	np	pn	nn	nn	bd	ll
37-03	ll	uu	db	ll	ml	np	np	nn	nn	bd	ll
38-03	ll	uu	ad	ll	ml	np	nn	nn	nn	cb	lm
39-03	ll	uu	db	ll	lm	pn	uu	pn	uu	cb	uu
40-03	ll	ll	db	ll	lm	nn	nn	nn	pn	bd	uu
41-03	ll	ml	db	lm	ml	nn	np	uu	uu	ad	ll
42-03	ll	uu	bd	ll	lm	np	pn	uu	uu	ac	uu
43-03	uu	ll	db	uu	ll	np	nn	nn	uu	uu	uu
44-03	ll	ll	ad	ll	lm	nn	uu	nn	uu	uu	ll

Appendix D: Continued

Seedling number	A96	A106	A77	A94	A102	A52	A133	A41	A29	A67	A61
45-03	ll	ll	ac	ll	ll	uu	uu	uu	uu	uu	uu
46-03	uu	uu	ad	lm	ll	uu	uu	nn	nn	bc	uu
47-03	ll	ll	ad	ll	ll	pn	pn	pn	pn	uu	ll
48-03	ll	ll	bd	lm	ml	pn	nn	nn	nn	ad	ll
CP	ll	ml	ad	lm	lm	np	nn	nn	nn	ad	ll
M25	ml	ll	cb	ll	lm	nn	uu	nn	pn	bc	ll
MM109	ll	ll	ad	lm	lm	nn	uu	nn	nn	ad	ll
RG	ll	ll	ad	ll	ll	pn	uu	pn	nn	cb	lm

Appendix D: Continued

Seedling number	A71	A80	A79	A88	A89	A145	A84	A15	A253	A53	A184
1	eg	nn	ml	fg	cb	pn	np	np	nn	ll	nn
2	eg	nn	ll	ee	db	uu	np	nn	nn	ml	np
3	eg	nn	ll	fe	da	pn	nn	np	nn	ml	pn
7	eg	nn	ml	eg	db	nn	uu	np	nn	ml	pn
8	eg	nn	ll	eg	db	pn	np	np	nn	lm	nn
10	ee	np	ml	gf	db	nn	np	nn	np	ml	uu
11	ee	nn	ll	gf	db	nn	np	nn	np	uu	uu
13	ee	nn	ml	eg	db	nn	np	nn	np	ml	pn
14	ee	np	ml	ge	db	nn	np	np	np	ml	nn
15	eg	nn	ml	gf	db	nn	np	np	nn	ml	pn
16	ee	np	ll	gf	db	uu	nn	nn	np	ml	nn
17	ee	nn	ll	eg	db	pn	uu	np	nn	ml	nn
18	fg	nn	ml	gf	db	nn	uu	np	nn	uu	uu
22	ee	np	ml	gf	db	pn	np	np	np	ll	uu
23	eg	nn	ml	fe	da	uu	uu	np	np	ml	pn
24	ee	np	ml	eg	ad	np	uu	np	uu	ll	pn
25	eg	nn	ml	gf	db	pn	pn	np	np	ml	nn
26	eg	nn	ml	gf	uu	nn	nn	nn	nn	ml	pn
27	fe	nn	ll	ee	uu	pn	nn	np	nn	ll	pn
28	uu	np	ml	gf	db	pn	nn	pn	nn	ml	pn
30	ee	nn	ml	ee	db	nn	nn	np	nn	ml	uu
31	ee	nn	ml	gf	db	nn	uu	np	np	ll	nn

Appendix D: Continued

Seedling number	A71	A80	A79	A88	A89	A145	A84	A15	A253	A53	A184
32	uu	np	ml	gf	db	nn	nn	np	nn	ll	pn
35	ee	nn	uu	gf	db	pn	nn	np	pn	ml	nn
36	uu	np	ll	gf	db	nn	pn	np	np	ml	pn
37	ee	np	uu	gf	db	pn	pn	np	nn	ml	nn
38	ee	nn	ml	gf	db	pn	np	nn	uu	ll	nn
39	ee	np	ml	gf	db	np	np	nn	np	ml	nn
40	ee	uu	ll	eg	ad	nn	np	uu	np	uu	nn
42	ee	np	ll	eg	bd	pn	nn	np	np	ml	pn
43	uu	nn	ml	gf	db	pn	uu	np	nn	ll	nn
44	ee	uu	ml	ee	db	uu	np	np	nn	ml	nn
46	ef	nn	ml	eg	db	pn	nn	nn	nn	uu	nn
56	ee	uu	ml	eg	db	pn	nn	pn	nn	ll	pn
58	fg	nn	ll	gf	db	nn	nn	np	nn	ml	pn
59	eg	nn	ml	eg	db	pn	nn	nn	uu	ml	nn
60	uu	np	ml	ee	bd	uu	nn	nn	uu	ml	nn
63	uu	nn	uu	gf	db	pn	uu	np	np	ll	nn
64	uu	nn	ml	eg	da	nn	nn	nn	np	ll	pn
65	uu	nn	ll	ee	bd	uu	nn	np	np	ml	nn
66	ee	np	ml	gf	db	nn	nn	np	uu	ml	uu
67	ee	nn	ml	gf	db	nn	pn	np	nn	ml	pn
68	ee	nn	ll	gf	da	uu	nn	np	uu	ll	pn
69	uu	nn	ll	gf	db	pn	uu	np	np	ll	nn

Appendix D: Continued

Seedling number	A71	A80	A79	A88	A89	A145	A84	A15	A253	A53	A184
70	ee	nn	ll	eg	db	pn	uu	np	nn	ll	nn
76	ee	nn	lm	gf	db	pn	np	nn	np	ml	nn
77	ee	nn	ml	gf	db	pn	nn	np	nn	ll	pn
80	uu	nn	ml	eg	db	pn	nn	np	np	ml	pn
111	ee	np	ll	gf	uu	np	np	np	np	uu	nn
1-03	ee	nn	ml	fg	db	nn	np	np	np	ml	nn
2-03	ee	uu	ml	eg	db	uu	np	nn	np	ll	np
4-03	uu	uu	ml	eg	db	nn	uu	np	np	ll	pn
5-03	ef	nn	ml	eg	db	nn	nn	np	nn	ll	pn
6-03	uu	np	ml	ee	ca	uu	np	np	np	ll	uu
7-03	ee	np	ml	ee	db	pn	nn	np	np	ll	uu
8-03	ee	nn	ml	ee	uu	nn	np	np	np	uu	nn
9-03	uu	nn	ll	gf	db	pn	nn	nn	np	ll	pn
10-03	ee	uu	ml	gf	db	uu	nn	nn	np	ll	nn
11-03	ee	nn	ll	gf	db	nn	np	np	nn	ml	nn
13-03	ee	nn	ll	fe	uu	pn	np	pn	np	ml	nn
14-03	eg	nn	ml	eg	db	nn	np	np	nn	ll	nn
15-03	eg	uu	ml	eg	db	nn	nn	np	nn	ll	nn
16-03	ee	np	ml	gf	da	nn	np	np	nn	ml	nn
17-03	ee	np	ml	gf	db	nn	uu	np	nn	uu	pn
18-03	ee	np	uu	eg	cb	uu	nn	np	nn	ml	nn
19-03	eg	np	ml	fe	db	pn	np	pn	uu	ll	pn

Appendix D: Continued

Seedling number	A71	A80	A79	A88	A89	A145	A84	A15	A253	A53	A184
20-03	uu	np	ll	eg	db	nn	np	nn	uu	ml	nn
21-03	ee	nn	ml	fg	db	nn	np	nn	np	ll	nn
22-03	ee	np	uu	uu	uu	uu	nn	np	uu	ll	uu
23-03	uu	nn	ll	gf	db	nn	nn	nn	np	ml	pn
24-03	ee	nn	ml	eg	da	nn	np	np	nn	uu	uu
27-03	ee	np	ll	gf	db	pn	nn	np	np	ll	nn
28-03	ee	nn	ml	gf	db	uu	nn	np	nn	lm	pn
29-03	eg	np	ml	gf	db	nn	nn	np	np	uu	nn
30-03	ee	nn	ml	fe	uu	nn	np	np	nn	uu	uu
32-03	uu	uu	ll	gf	uu	uu	nn	np	nn	ml	pn
33-03	eg	np	ml	gf	db	nn	nn	np	np	ll	uu
34-03	ee	np	ll	fe	db	np	nn	np	nn	ml	pn
35-03	ee	uu	ml	ee	db	nn	np	nn	uu	ml	uu
36-03	ee	uu	ll	gf	uu	pn	np	nn	nn	uu	nn
37-03	ee	nn	ml	gf	db	uu	np	nn	nn	ml	nn
38-03	uu	nn	ml	ee	uu	uu	np	nn	nn	ml	nn
39-03	ee	np	ml	ee	bc	nn	np	uu	np	ml	nn
40-03	ee	uu	ml	gf	db	pn	pn	uu	np	ll	uu
41-03	uu	uu	ll	gf	db	nn	nn	nn	nn	ml	uu
42-03	eg	np	ml	gf	bd	nn	nn	nn	np	ml	nn
43-03	ee	np	ml	ee	bc	nn	nn	np	uu	uu	nn
44-03	ee	np	ll	eg	db	nn	nn	np	uu	ll	nn

Appendix D: Continued

Seedling number	A71	A80	A79	A88	A89	A145	A84	A15	A253	A53	A184
45-03	ee	uu	uu	fe	da	nn	uu	uu	np	uu	pn
46-03	ee	nn	uu	ef	uu	nn	uu	nn	np	ml	pn
47-03	ee	nn	ml	gf	db	nn	uu	nn	np	ml	pn
48-03	ee	nn	ll	eg	db	pn	uu	np	np	ll	pn
CP	ee	nn	ml	fg	uu	pn	np	np	nn	ll	nn
M25	eg	nn	ml	eg	uu	nn	np	np	nn	ml	nn
MM109	uu	nn	ll	eg	db	pn	np	np	nn	ml	nn
RG	ee	nn	ml	fg	db	pn	np	nn	nn	ml	np

Appendix D: Continued

Seedling number	A402	A428	A14	A140	A136	A44
1	eg	ll	ad	eg	lm	gf
2	uu	uu	ac	ef	ll	uu
3	gf	ml	ad	ee	ll	uu
7	uu	uu	bc	ee	ll	gf
8	gf	ml	uu	uu	ll	uu
10	uu	uu	uu	uu	ll	eg
11	uu	uu	uu	uu	lm	uu
13	uu	ml	ad	uu	lm	uu
14	ee	ml	db	ee	ll	eg
15	uu	ml	ac	fe	ll	uu
16	gf	ml	ac	fe	lm	gf
17	uu	uu	ac	eg	ll	eg
18	uu	ml	bd	ef	ll	eg
22	gf	ml	uu	uu	ll	uu
23	ee	lm	ca	eg	lm	eg
24	eg	ml	bd	fg	uu	ee
25	uu	uu	bd	ef	lm	gf
26	gf	ll	ca	fe	ll	gf
27	uu	uu	ad	ge	lm	uu
28	ee	ml	ac	uu	lm	gf
30	uu	uu	uu	fg	lm	gf
31	eg	uu	ac	uu	lm	gf

Appendix D: Continued

Seedling number	A402	A428	A14	A140	A136	A44
32	uu	uu	uu	ee	ll	uu
35	gf	ml	ac	uu	ll	gf
36	ee	ml	cb	fg	lm	ee
37	gf	ml	ad	fg	lm	gf
38	uu	uu	ad	uu	uu	eg
39	gf	ll	ac	ef	lm	eg
40	fg	ll	cb	eg	ll	eg
42	eg	ml	db	uu	ll	gf
43	ee	ml	ad	uu	uu	uu
44	ee	ml	uu	eg	lm	eg
46	uu	uu	cb	eg	uu	gf
56	ee	ml	bd	eg	uu	ee
58	gf	ml	ac	ee	lm	gf
59	fg	ml	db	gf	ll	eg
60	fg	ml	bd	uu	ll	eg
63	ee	ll	ad	ge	lm	ef
64	ge	uu	uu	eg	lm	ee
65	ee	ml	ac	ef	lm	gf
66	uu	ml	db	fg	uu	ef
67	fg	ml	uu	eg	lm	eg
68	eg	ll	bd	eg	lm	ee
69	ee	ll	ac	uu	ll	ee

Appendix D: Continued

Seedling number	A402	A428	A14	A140	A136	A44
70	ee	ml	db	ee	lm	gf
76	ge	ml	uu	uu	lm	gf
77	fg	ll	uu	uu	lm	uu
80	ee	ml	bd	eg	lm	ee
111	eg	ml	ac	ge	lm	uu
1-03	eg	ml	db	ee	lm	eg
2-03	fg	lm	uu	eg	lm	ef
4-03	uu	uu	bc	ee	uu	gf
5-03	eg	ml	bd	fg	lm	gf
6-03	ee	ml	uu	fg	lm	gf
7-03	uu	uu	uu	fg	ll	eg
8-03	uu	uu	ad	uu	lm	eg
9-03	gf	ml	uu	ef	lm	uu
10-03	eg	ml	bc	ee	ll	ee
11-03	ee	ml	ad	eg	lm	ee
13-03	gf	ml	bd	uu	lm	uu
14-03	fg	ll	db	uu	lm	ef
15-03	uu	ml	uu	uu	lm	uu
16-03	fg	ml	ac	eg	ll	eg
17-03	ee	ml	ac	fg	lm	gf
18-03	eg	ll	ac	ee	lm	ee
19-03	eg	ml	bd	uu	lm	eg

Appendix D: Continued

Seedling number	A402	A428	A14	A140	A136	A44
20-03	uu	uu	uu	fg	ll	eg
21-03	uu	uu	bd	eg	lm	eg
22-03	uu	uu	ac	fg	ll	uu
23-03	fg	ml	ca	ee	lm	gf
24-03	uu	uu	uu	uu	lm	ee
27-03	uu	uu	ca	fe	uu	gf
28-03	ee	ll	ad	ef	ll	fe
29-03	ge	ml	ca	fg	ll	ef
30-03	uu	uu	ad	fe	lm	uu
32-03	ee	ll	ad	eg	ll	ee
33-03	uu	uu	da	ef	lm	uu
34-03	fg	uu	bc	ee	lm	ee
35-03	uu	uu	ca	gf	lm	eg
36-03	eg	ml	uu	ee	lm	uu
37-03	eg	ll	uu	ge	ll	eg
38-03	uu	uu	bd	gf	ll	gf
39-03	eg	ll	db	uu	uu	gf
40-03	uu	uu	uu	uu	ll	eg
41-03	uu	uu	bd	uu	ll	ee
42-02	uu	uu	uu	ee	lm	gf
43-03	fg	ml	ad	ee	uu	ee
44-03	eg	ll	bd	ee	uu	fe

Appendix D: Continued

Seedling number	A402	A428	A14	A140	A136	A44
45-03	gf	ml	bc	uu	uu	gf
46-03	gf	ml	ac	uu	uu	eg
47-03	eg	ml	da	fg	ll	gf
48-03	gf	ml	cb	eg	ll	eg
CP	fe	ll	cb	uu	ll	uu
M25	uu	uu	cb	uu	uu	uu
MM109	ge	ml	cb	ef	uu	gf
RG	eg	ll	bd	ge	uu	gf

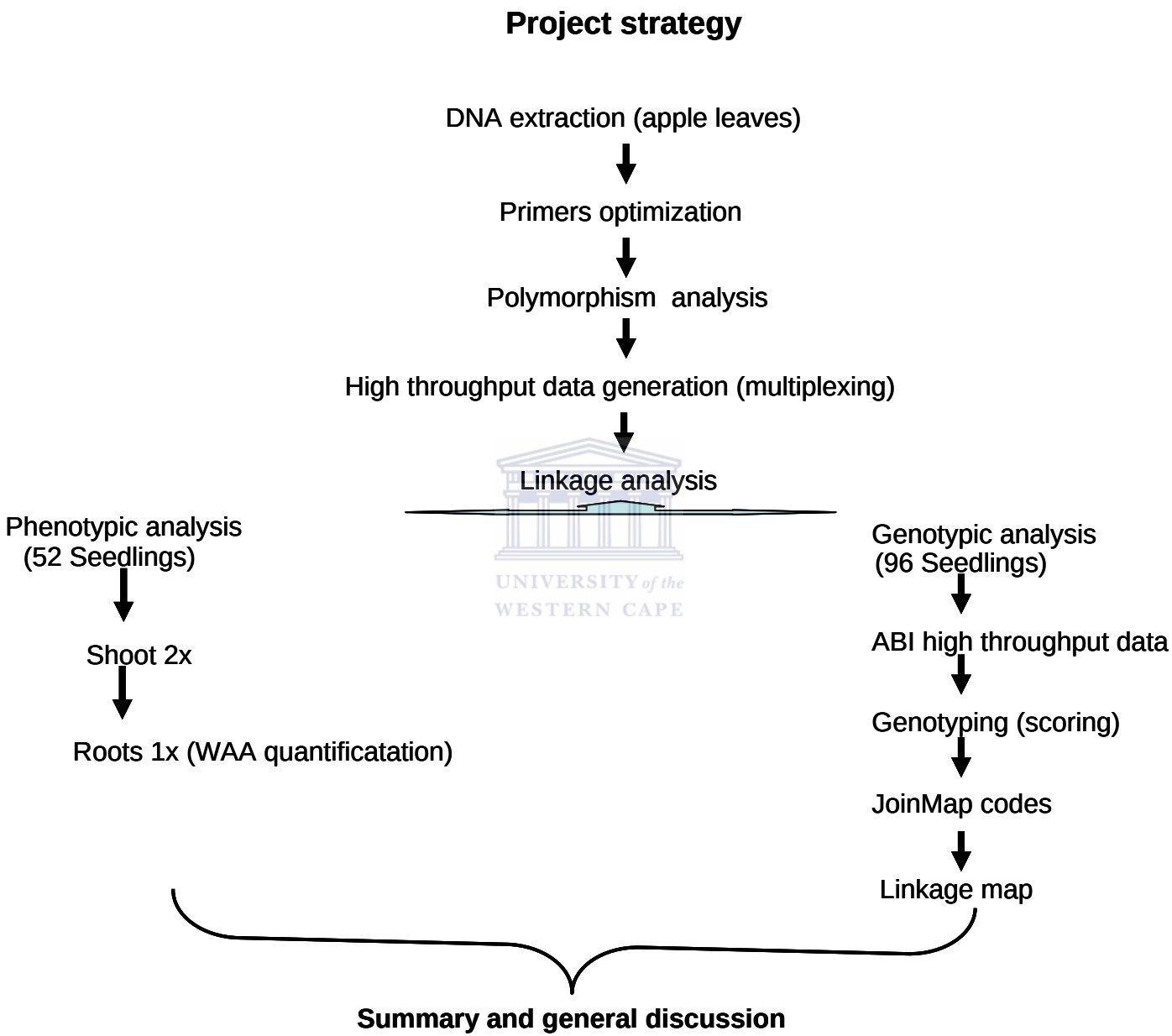


Figure 1.7. Schematic representation of the project strategy.

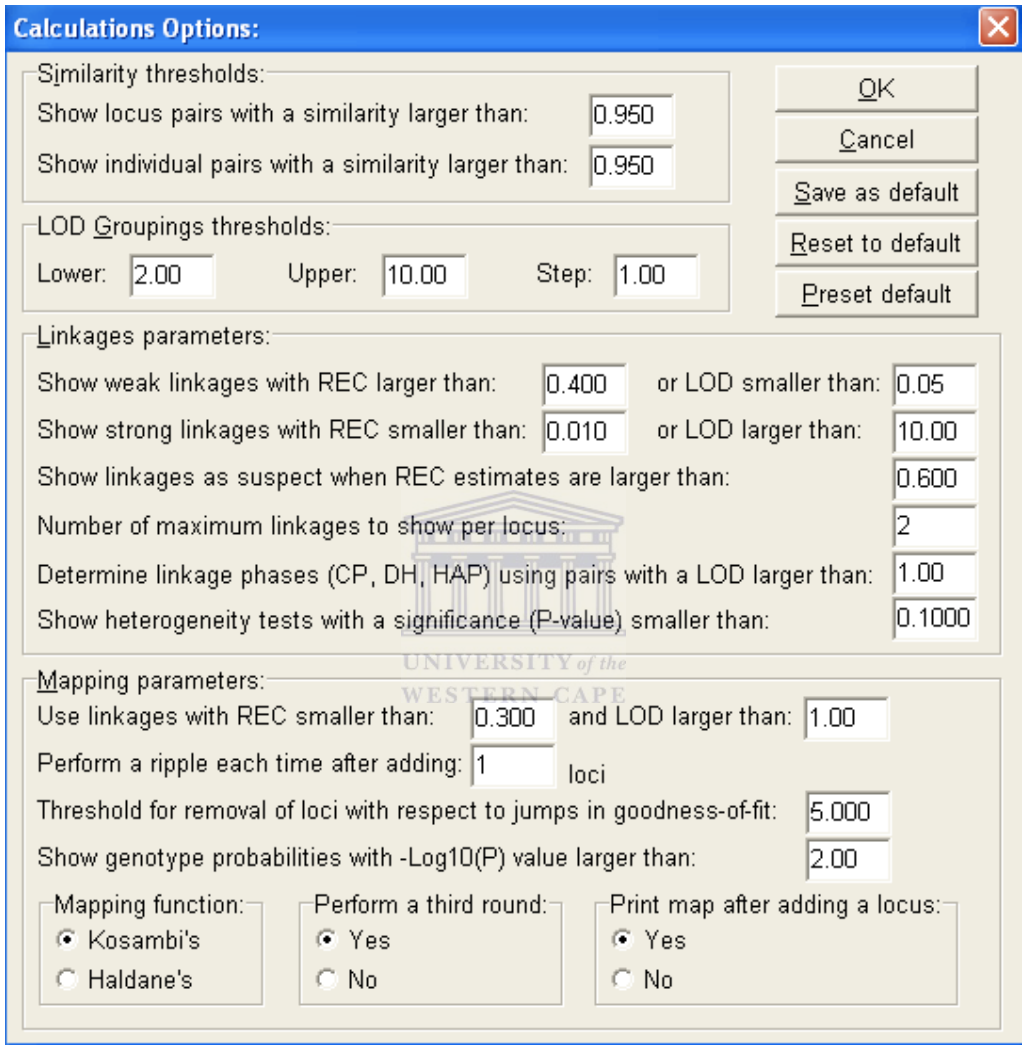
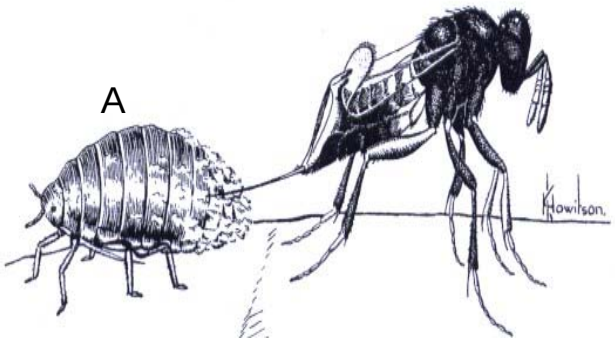
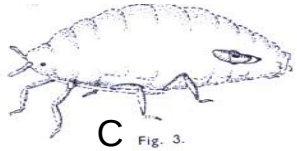


Figure 2.1. JoinMap® 3.0 interface with calculation parameters used in linkage analysis and map construction.


A


C
Fig. 3.

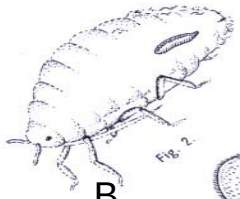
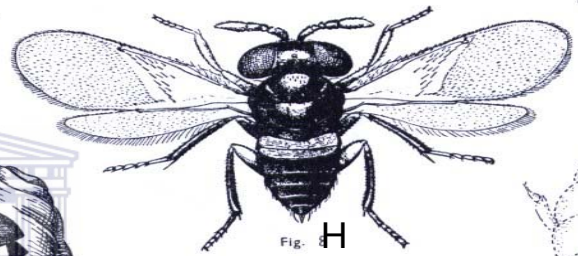
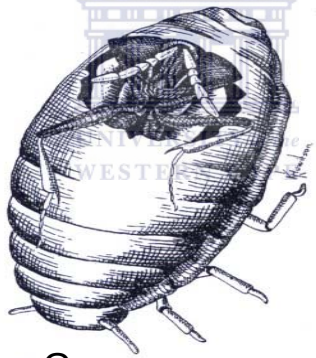
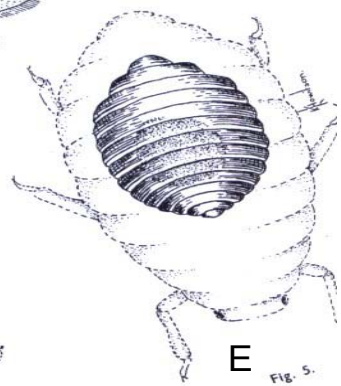


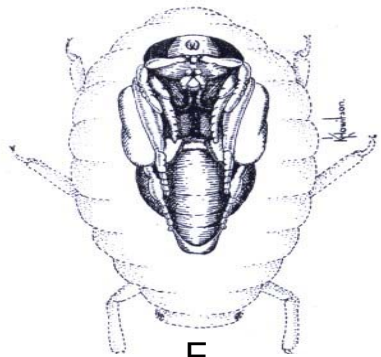
Fig. 2.
B


Fig. 4.
D


H


G


E
Fig. 5.


F

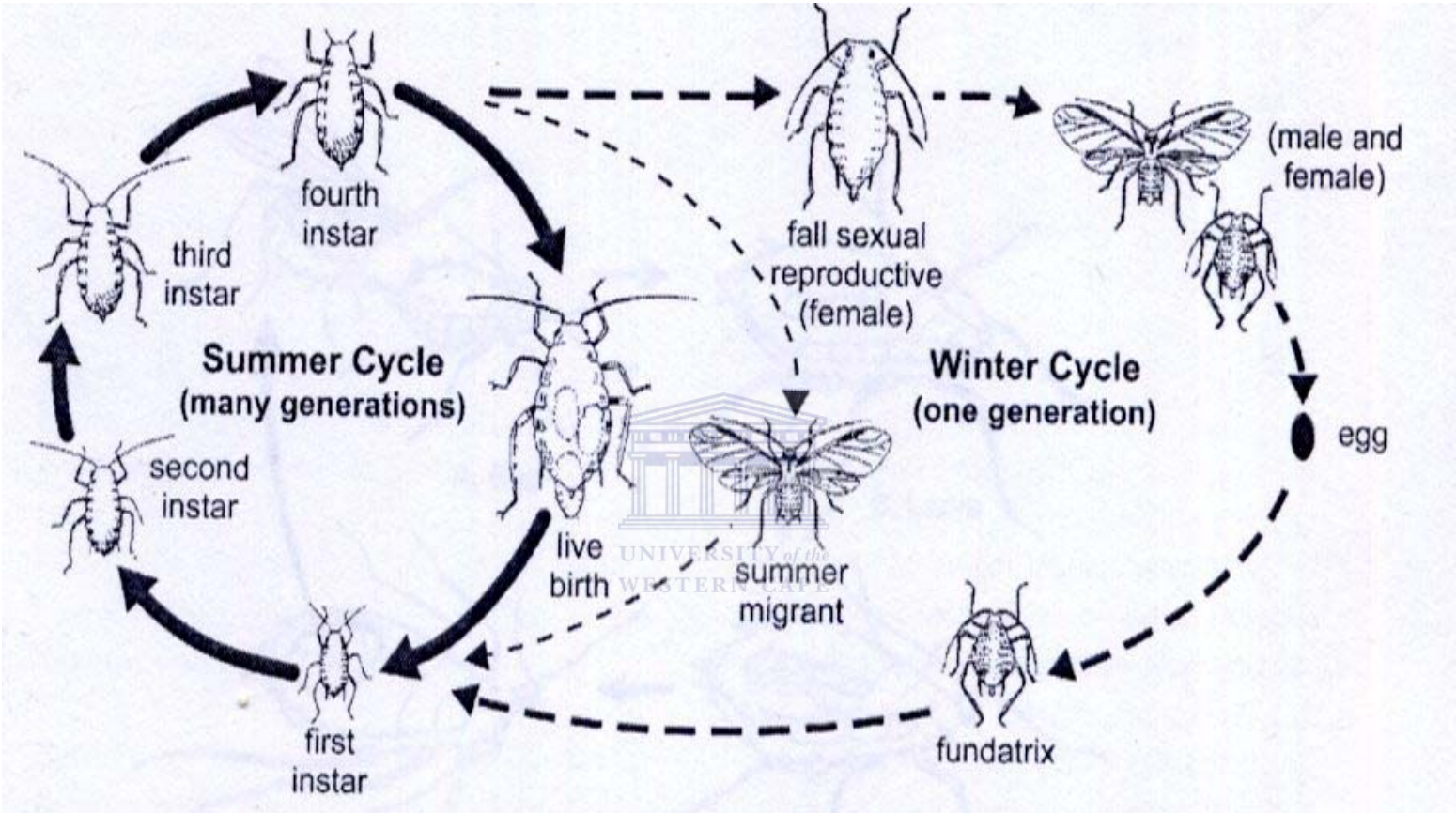


Figure 1.2. General life cycle of WAA. Asexual reproduction occurs during most of the summer cycle (From Dreistadat *et al*., 1994). Some aphid species produce a generation of sexual individuals that overwinter eggs as shown in the winter cycle.

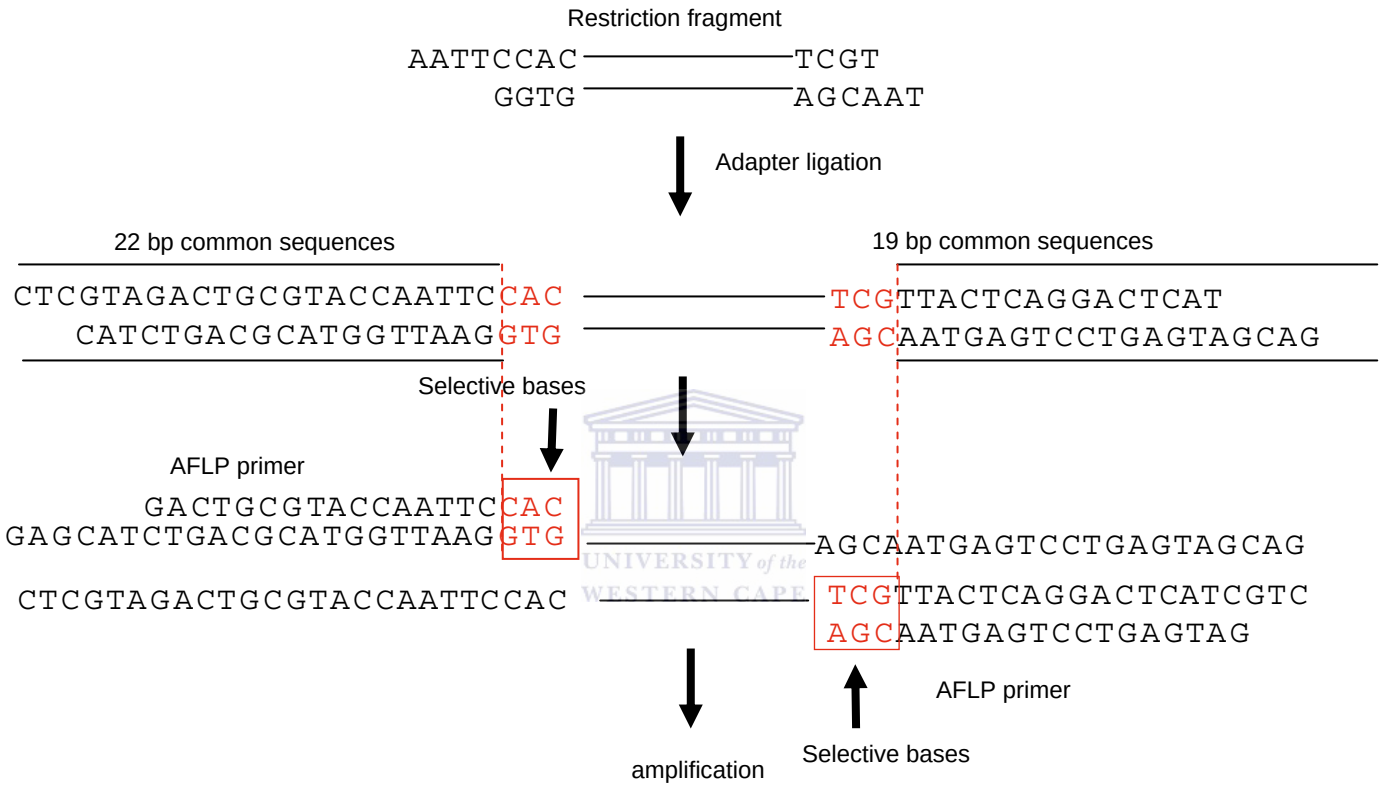


Figure 1.4. Schematic representation of the AFLP technique (from Vos *et al*., 1995).

Top: *EcoR1-Mse1* restriction fragment with its 5´ protruding ends. Center: the fragment after ligation of the EcoR1 and Mse1 adapters. Bottom: both strands of the fragment with their corresponding AFLP primers. The 3´ ends of the primers and their recognition sequence in the *EcoR1- Mse1* fragment are highlighted.

Appendix C. Continued

WAA DATA	OBSERVATION OF WAA						CLASSIFICAT	
	Block 1		Block 2		Block 3			
Clones No	Blk1 Present	Blk 1 absent	Blk2 Present	Blk2 Absent	Blk3 Present	Blk3 Absent	BLOCK 1	BLOCK 2
8	x		x		x		1	1
76		x	*	*	x		0	*
39		x	x		x		0	2
13	x		*	*	*	*	4	*
40		x	x		*		0	1
59	x		x			x	1	1
23	x		x		*	*	1	1
17	x			x	*		1	0
27		x	x		x		0	1
66	x			x	*	*	1	0
43	x		x		x		1	1
14		x	x		*		0	3
56		x		x	x		0	0
30	x		x		x		1	2
3	x		x			x	1	1
36	x		x			x	1	1
24		x	x		x		0	2
25	x		x		x		2	1
69	x		*	*		x	1	*
80		x	x			x	0	2
32			x			x	1	1
35	*	*	x		*	*	*	1
10	*	*		x	*	*	*	4

X = Represent the presence or absence of the WAA infestation during data collection

* = Represent the dead seedlings

Each block was divided by two parts, for example present and absent.

Table 4.3. Microsatellite primer sequences, type of repeat, multiplex number, reference number and linkage group on which they map

Primer	Acc No	Forward primer sequences (5'-3')	Reverse primer sequence (5'-3')	Repeat type	Linkage Group (LG)	Multiplex No	Reference No
A10	02b1	ccg tga tga caa agt gca tga	atg agt ttg atg ccc ttg ga	Di	15	N5	2
A14	23g4	ttt ctc tct ctt tcc caa ctc	agc cgc ctt gca tta aat ac	Di	6	F5	2
A15	28f4	tgc ctc cct tat ata gct ac	tga gga cgg tga gat ttg	Di	12	N8	2
A29	AT000141	gaa ata aac acc gag taa aca g	tgc tat ctg gtt ttc ttt tag c	Tetra	5	V6	2
A32	CH05g08	cca aga cca agg caa cat tt	ccc ttc acc tca ttc tca cc	Di	1	F10	1
A37	CH02c061	tga cga aat cca cta cta atg ca	gat tgc gcg ctt ttt aac at	Di	2	V1	1
A38	CH05e03	cga ata ttt tca ctct gac tgg g	caa gtt gtt gta ctg ctc cga c	Di	2	*	1
A41	CH02c02b	tgc atg cat gga aac gac	tgg aaa aag tca cac tgc tcc	Di	4	V1	1
A42	CH05d02	aaa ctc cct cac ctc aca tca c	aat agt cca atg gtg tgg atg g	Di	4	N2	1
A43	CH04e03	ttg aag atg ttt ggc tgt gc	tgc atg tct gtc tcc tcc at	Di	5	F8	1
A44	CH05e06	aca cgc aca gag aca gag aca t	gtt gaa tag cat ccc aaa tgg t	Di	5	F4	1
A49	CH05c07	tga tgc att agg gct tgt act t	ggg atg cat tgc taa ata gga t	Di	9	N9	1
A52	CH02d08	tcc aaa atg gcgt acc tct c	gca gac act cac tca cta tct ctc	Di	11	F1	1
A53	CH04g07	ccc taa cct caa tcc cca at	atg agg cag gtg aag aag ga	Di	11	V10	1
A57	CH01g05	cat cag tct ctt gca ctg gaa a	gac aga gta agc tag ggc tag gg	Di	14	V4	1
A59	CH03d08	cat cag tct ctt gca ctg gaa a	tag ggc tag gga gag atg atg a	Di	14	F8	1
A60	CH03g04	atg tcc aat gta gac acg caa c	ttg aag atg gcc taa cct tgt t	Di	14	V8	1
A61	CH04c07	ggc ctt cca tgt ctc aga ag	cct cat gcc ctc cac taa ca	Di	14	N2	1
A62	CH04f06	ggc tca gag tac ttg cag agg	atc ctt aag cgc tct cca ca	Di	14	N5	1
A64	CH05e05	tcc tag cga tag ctt gtg aga g	gaa acca cca aac cgt tac aat	Di	14	N6	1
A65	CH05g11	gca aac caa cct ctg gtg at	aaa ctg ttc caa cga cgc ta	Di	14	F2	1
A67	CH02c09	tta tgt acc aac ttt gct aac ctc	aga agc agc aga gga gga tg	Di	15	N6	1
A71	CH01h11	gaa aga ctt gca gtg gga gc	gga gtg ggt ttg aga agg tt	Di	17	N7	1
A73	CH01f12	ctc ctc caa gct tca acc ac	gca aaa acc aca ggc ata ac	Di	10	F5	1
A74	CH02a10	atg cca atg cat gag aca aa	aca cgc agc tga aac act tg	Di	10	N7	1

Table 4.3. Continued

Primer	Acc No	Forward primer sequences (5'-3')	Reverse primer sequence (5'-3')	Repeat type	Linkage Group (LG)	Multiplex No	Reference No
A75	CH02b03b1	ata agg ata caa aaa ccc tac aca g	gac atg ttt ggt tga aaa ctt g	Di	10	F2	1
A77	CH03d11	acc cca cag aaa cct tct cc	caa ctg caa gaa tcg cag ag	Di	10	*	1
A78	COLa	agg aga aag gcg ttt acc tg	gac tca ttc ttc gtc gtc act g	Di	10	*	1
A79	MS01a03	agc agt ata ggt ctt cag	tgc gta gat aac act cga t	Di	10	*	1
A80	MS02a01	ctc cta cat tga cat tgc at	tag aca ttt gat gag act g	Di	10	N8	1
A81	MS06g03	cgg agg gtg tgc tgc cga ag (20)	gcc cag ccc ata tct gct (18)	Di	10	*	1
A84	CH02f061	ccc tct tca gac ctg cat atg	act gtt tcc aag cga tca gg	Di	2	V2	1
A85	CH03d01	cgc acc aca aat cca act c	aga gtc aga agc aca gcc tc	Di	2	F4	1
A86	CH03d10	ctc cct tac caa aaa cac caa a	gtg att aag aga gtg atc ggg g	Di	2	*	1
A88	CH02h11a	cgt ggc atg cct atc att tg	ctg ttt gaa ccg ctt cct tc	Di	4	V10	1
A89	CH04e02	ggc gat gac tac cag gaa aa	atg tag cca agc cag cgt at	Di	4	*	1
A90	CH02b121	ggc agg ctt tac gat tat gc	ccc act aaa agt tca cag gc	Di	5	*	1
A91	CH03a04	gac gca taa ctt ctc ttc cac c	tca agg tgt gct aga caa gga g	Di	5	V2	1
A92	CH03a09	gcc agg tgt gac tcc ttc tc	ctg cag ctg ctg aaa ctg g	Di	5	V6	1
A93	CH05f06	tta gat ccg gtc act ctc cac t	tgg agg aag acg aag aag aaa g	Di	5	*	1
A94	CH03d12	gcc cag aag caa taa gta aac c	att gct cca tgc ata aag gg	Di	6	V5	1
A96	CH01h101	tgc aaa gat agg tag ata tat gcca	agg agg gat tgt ttg tgc ac	Di	8	N1	1
A98	CH02d121	aac cag att tgc ttg cca tc	gct ggt ggt aaa cgt ggt g	Di	11	*	1
A101	CH04d07	tgt cct cca atc tta acc cg	cac aca gac gac aca ttc acc	Di	11	F10	1
A102	CH04d10	gag gga tct gta gct ccg ac	tgg tga gta tct gct cgc tg	Di	11	*	1
A103	CH04h02	gga agc tgc atg atg aga cc	ctc aag gat ttc atg ccc ac	Di	11	*	1
A104	CH01d09	gcc atc tga aca gaa tgt gc	ccc ttc att cac att tcc ag	Di	12	*	1
A106	CH03c02	tca cta ttt acg gga tca agc a	gtg cag agt ctt tga caa ggc	Di	12	*	1
A107	CH04d02	cgt acg ctg ctt ctt ttg ct	cta tcc acc acc cgt caa ct	Di	12	*	1
A109	CH05d11	cac aac ctg ata tcc ggg ac	gag aag gtc gta cat tcc tca a	Di	12	N6	1

Table 4.3. Continued

Primer	Acc No	Forward primer sequences(5'-3')	Reverse primer sequence (5'-3')	Repeat type	Linkage Group (LG)	Multiplex No	Reference No
A111	CH03h03	aag aaa tcg gat cca aaa caa c	tcc ctc aaa gat tgc tcc tg	Di	13	F1	1
A113	CH01d08	ctc cgc cgc tat aac act tc	tac tct gga ggg tat gtc aaa g	Di	15	N7	1
A115	CH03b10	ccc tcc aaa ata tct cct cct c	cgt tgt cct gct cat cat act c	Di	15	N4	1
A120	CH05e04	aag gag aag acc gtg tga aat c	cat gga taa ggc ata gtc agg a	Di	16	F9	1
A121	CH02g04	ttt tac ctt ttt acg tac ttg agc g	agg caa aac tct gca agt cc	Di	17	F1	1
A126	CH01b09b	tta tag cag caa cag gag cg	tat tcg gga ggc atg gta tg	Di	4	P2	1
A130	CH01c09	tca tct ttc tcg cct gcc	tcc atc aaa acc aag ttt tcg	Di	X	P1	1
A133	CH01d03	cca ctt ggc aat gac tcc tc	acc tta ccg cca atg tga ag	Di	X	*	1
A135	CH01e09b	cca tcc aac tac tgc ctt tcc	ttt gat gaa ccc ctt ctt cc	Di	10	P1	1
A136	CH01e121	aaa ctg aag cca tga ggg c	ttc caa ttc aca tga ggc tg	Di	8	*	1
A139	CH02a04	gaa aca ggc gcc att att tg	aaa gga gac gtt gca agt gg	Di	2/4/5/7	P8	1
A140	CH02a08	gag gag ctg aag cag cag ag	atg cca aca aaa gca tag cc	Di	10	*	1
A145	CH02g01	gat gac gtc ggc agg taa ag	caa cca aca gct ctg caa tc	Di	11	P2	1
A162	CH04f04	gtc ggt aca aac tca gga cc	cga cgt tcg atc ttc ctc tc	Di	5	P2	1
A165	CH04g09	ttg tcg cac aag cca gtt ta	gaa gac tca tgg gtg cca tt	Di	5/10	P4	1
A184	CN489175	agc cct ctc caa tac caa cc	ttt cct gga aga gat tga cg	Tri	X	*	3
A188	CN490740	agg atc ctt cct cga ttt gc	ggc att gag gtt ctt gat cc	Tri	X	F11	3
A227	CN493171	tct tca ttc gtc ggt ggt gga cc	tgt gtg gct att acc tga gg	Tetra	X	*	3
A253	CO540769	tcct agg gtc gga gag cag	ctc aag aat cac caa caa tgc	Tri	X	N5	3
A402	AT000420	gtt gga cca att atc tct gc	ata tac tgg gga ggt tga gg	Di	X	*	3
A428	CO902639	ctc ctt tat ctc ttt cct ccc	ttg tcg tcc caa atc aag cc	Di	X	*	3
P5	AB027617F	gct gac tgt tca tct cgt ttc c	tga gtt cat caa aag caa ggg	Tri	X	*	3

Published primer sequences by Liebhard *et al*., 2002[1]; Liebhard *et al*., 2003; Guilford *et al*., 1997[2] and predicted primers sequences from GenBank (Khashief and Daleen[3], unpublished data). X = linkage group not known, and * = not in the multiplex

Table 5.1a. Fragment sizes, chi squared values and segregation codes used for each primer in linkage map construction. (χ^2) Values over seven are significant.

Primer	Northern Spy allele fragment sizes	Cox Orange Pippin allele Fragment sizes	Segregation types	Chi squared (χ^2) values
A10	229:238	217	<lm×ll>	4
A14	100:103	105:107	<ab×cd>	5
A15	100	105:108	<nn×np>	14
A29	95	87:95	<nn×np>	26
A32	177	177:180	<nn×np>	9
A37	229:250	240	<lm×ll>	11
A38	161	163:172	<nn×np>	4
A41	113	109:113	<nn×np>	1.0
A42	194:221	213:217	<ab×cd>	4
A43	197	189:197	<nn×np>	1
A44	122:219	122:144	<ef×eg>	15
A49	131:137	120	<lm×ll>	3
A52	210	210:224	<nn×np>	12
A53	160:164	168	<lm×ll>	4
A57	143:150	141:171	<ab×cd>	5
A59	130:136	128:159	<ab×cd>	17
A60	124	136:140	<nn×np>	6
A61	106:110	106	<lm×ll>	2
A62	160:184	172:176	<ab×cd>	21
A64	129:142	144:148	<ab×cd>	13
A65	238	203:248	<nn×np>	4
A67	232:246	232:257	<ab×cd>	13
A71	103:129	118:129	<ef×eg>	96
A73	147	148:151	<nn×np>	6
A74	141:152	139	<lm×ll>	1
A75	92:94	89:96	<ab×cd>	22
A77	90:118	91:116	<ab×cd>	21
A78	242:246	231	<lm×ll>	0.3
A79	235:237	230	<lm×ll>	11
A80	168	170:174	<nn×np>	6

Table 5.1a. Continued

Primer	Northern Spy allele fragment sizes	Cox Orange Pippin allele Fragment sizes	Segregation types	Chi squared (χ^2) values
A81	156:164	153:182	<ab×cd>	21
A84	142	147:149	<nn×np>	0.2
A85	102:106	96	<lm×ll>	5
A86	179	170:180	<nn×np>	84
A88	118:126	116:126	<ef×eg>	40
A89	155:157	145:147	<ab×cd>	151
A90	138	124:138	<nn×np>	2
A91	118	102:117	<nn×np>	1.1
A92	127:134	127:129	<ef×eg>	11
A93	174	164:182	<nn×np>	3
A94	109:118	118	<lm×ll>	11
A96	88:96	95	<lm×ll>	4
A98	175:197	191:197	<ef×eg>	78
A101	131:137	129:137	<ab×cd>	5
A102	147:160	147	<lm×ll>	4
A103	173:177	176:177	<ef×eg>	3
A104	146	146:153	<nn×np>	9
A106	121:123	123	<lm×ll>	28
A107	117:119	119	<lm×ll>	0.4
A109	167:173	169	<lm×ll>	6
A111	81:87	81:109	<ef×eg>	12
A113	255	241:251	<nn×np>	4
A115	96:118	117:122	<ab×cd>	21
A120	146:157	150:162	<ab×cd>	15
A121	78:84	80	<lm×ll>	1
A126	171:176	173	<lm×ll>	5
A130	87:89	95	<lm×ll>	0.2
A133	144	136:144	<nn×np>	0.2
A135	118:126	119	<lm×ll>	0.1
A136	255:262	253	<lm×ll>	3

Table 5.1a. Continued.

Primer	Northern Spy allele fragment sizes	Cox Orange Pippin allele Fragment sizes	Segregation types	Chi squared (χ^2) values
A139	107	95:107	<nn×np>	6
A140	136:145	145:152	<ef×eg>	2
A145	220	199:226	<nn×np>	1
A162	165:169	151	<lm×ll>	6
A165	146:155	146	<lm×ll>	5
A184	239	233:239	<nn×np>	2
A188	195:211	189:194	<ab×cd>	14
A227	344:385	337:345	<ef×eg>	6
A253	280	280:282	<nn×np>	0.0
A402	170:174	162:170	<ef×eg>	21
A428	342:346	343	<lm×ll>	16
P5	261:267	259	<lm×ll>	0.2

Fragment sizes obtained from two apple cultivars Northern Spy × Cox Orange Pippin. The segregation types on the left hand of the cross (×) were from Northern Spy and the ones on the right were from Cox Orange Pippin. The same analysis was used in converting fragment sizes into JoinMap® codes.

Chi square (χ^2) = $\frac{(\text{Observed} - \text{Expected})^2}{\text{Expected}}$

Table 5.1b. Allele ratios found, degree of freedom, significant difference and segregation types classes

		Allele ratios														
Locus	**Seg.type**	**ac**	**ad**	**bc**	**bd**	**ee**	**ef**	**eg**	**fg**	**ll**	**lm**	**nn**	**np**	**Df**	**Signif**	**Classes**
A10	<lm×ll>	0	0	0	0	0	0	0	0	**29**	**47**	0	0	1	**	[ll:lm]
A14	<ab×cd>	**23**	**16**	**12**	**23**	0	0	0	0	0	0	0	0	3	-	[ac:ad:bc:bd]
A15	<nn×np>	0	0	0	0	0	0	0	0	0	0	**28**	**64**	1	*****	[nn:np]
A29	<nn×np>	0	0	0	0	0	0	0	0	0	0	**63**	**18**	1	*****	[nn:np]
A32	<nn×np>	0	0	0	0	0	0	0	0	0	0	**60**	**31**	1	****	[nn:np]
A37	<lm×ll>	0	0	0	0	0	0	0	0	**24**	**53**	0	0	1	*****	[ll:lm]
A38	<nn×np>	0	0	0	0	0	0	0	0	0	0	**32**	**50**	1	**	[nn:np]
A41	<nn×np>	0	0	0	0	0	0	0	0	0	0	**47**	**38**	1	-	[nn:np]
A42	<ab×cd>	**27**	**16**	**21**	**16**	0	0	0	0	0	0	0	0	3	-	[ac:ad:bc:bd]
A43	<nn×np>	0	0	0	0	0	0	0	0	0	0	**43**	**52**	1	-	[nn:np]
A44	<ef×eg>	0	0	0	0	**15**	**7**	**24**	**29**	0	0	0	0	3	****	[ee:ef:eg:fg]
A49	<lm×ll>	0	0	0	0	0	0	0	0	**32**	**47**	0	0	1	*	[ll:lm]
A52	<nn×np>	0	0	0	0	0	0	0	0	0	0	**23**	**53**	1	*****	[nn:np]
A53	<lm×ll>	0	0	0	0	0	0	0	0	**33**	**50**	0	0	1	*	[ll:lm]
A57	<ab×cd>	**21**	**25**	**26**	**13**	0	0	0	0	0	0	0	0	3	-	[ac:ad:bc:bd]
A59	<ab×cd>	**11**	**38**	**21**	**19**	0	0	0	0	0	0	0	0	3	****	[ac:ad:bc:bd]
A60	<nn×np>	0	0	0	0	0	0	0	0	0	0	**57**	**33**	1	**	[nn:np]
A61	<lm×ll>	0	0	0	0	0	0	0	0	**44**	**33**	0	0	1	-	[ll:lm]
A62	<ab×cd>	**16**	**40**	**17**	**14**	0	0	0	0	0	0	0	0	3	*****	[ac:ad:bc:bd]
A64	<ab×cd>	**27**	**10**	**16**	**30**	0	0	0	0	0	0	0	0	3	***	[ac:ad:bc:bd]

Table 5.1b. Continued

		Allele ratios														
Locus	**Seg.type**	**ac**	**ad**	**bc**	**bd**	**ee**	**ef**	**eg**	**fg**	**ll**	**lm**	**nn**	**np**	**Df**	**Signif**	**Classes**
A65	<nn×np>	0	0	0	0	0	0	0	0	0	0	**52**	**34**	1	*	[nn:np]
A67	<ab×cd>	**13**	**33**	**16**	**16**	0	0	0	0	0	0	0	0	3	***	[ac:ad:bc:bd]
A71	<ef×eg>	0	0	0	0	**55**	**3**	**17**	**2**	0	0	0	0	3	*****	[ee:ef:eg:fg]
A73	<nn×np>	0	0	0	0	0	0	0	0	0	0	**29**	**50**	1	**	[nn:np]
A74	<lm×ll>	0	0	0	0	0	0	0	0	**42**	**36**	0	0	1	-	[ll:lm]
A75	<ab×cd>	**15**	**12**	**23**	**41**	0	0	0	0	0	0	0	0	3	*****	[ac:ad:bc:bd]
A77	<ab×cd>	**13**	**27**	**14**	**40**	0	0	0	0	0	0	0	0	3	*****	[ac:ad:bc:bd]
A78	<lm×ll>	0	0	0	0	0	0	0	0	**42**	**47**	0	0	1	-	[ll:lm]
A79	<lm×ll>	0	0	0	0	0	0	0	0	**29**	**60**	0	0	1	****	[ll:lm]
A80	<nn×np>	0	0	0	0	0	0	0	0	0	0	**53**	**13**	1	**	[nn:np]
A81	<ab×cd>	**11**	**37**	**27**	**13**	0	0	0	0	0	0	0	0	3	*****	[ac:ad:bc:bd]
A84	<nn×np>	0	0	0	0	0	0	0	0	0	0	**38**	**42**	1	-	[nn:np]
A85	<lm×ll>	0	0	0	0	0	0	0	0	**33**	**54**	0	0	1	**	[ll:lm]
A86	<nn×np>	0	0	0	0	0	0	0	0	0	0	**2**	**90**	1	*****	[nn:np]
A88	<ef×eg>	0	0	0	0	**13**	**8**	**26**	**48**	0	0	0	0	3	*****	[ee:ef:eg:fg]
A89	<ab×cd>	**1**	**9**	**4**	**69**	0	0	0	0	0	0	0	0	3	*****	[ac:ad:bc:bd]
A90	<nn×np>	0	0	0	0	0	0	0	0	0	0	**45**	**33**	1	-	[nn:np]
A91	<nn×np>	0	0	0	0	0	0	0	0	0	0	**41**	**51**	1	-	[nn:np]
A92	<ef×eg>	0	0	0	0	**34**	**22**	**27**	**12**	0	0	0	0	3	**	[ee:ef:eg:fg]
A93	<nn×np>	0	0	0	0	0	0	0	0	0	0	**53**	**37**	1	*	[nn:np]

Table 5.1b. Continued

		Allele ratios														
Locus	**Seg.type**	**ac**	**ad**	**bc**	**bd**	**ee**	**ef**	**eg**	**fg**	**ll**	**lm**	**nn**	**np**	**Df**	**Signif**	**Classes**
A94	<lm×ll>	0	0	0	0	0	0	0	0	**63**	**31**	0	0	1	*****	[ll:lm]
A96	<lm×ll>	0	0	0	0	0	0	0	0	**54**	**36**	0	0	1	*	[ll:lm]
A98	<ef×eg>	0	0	0	0	**11**	**27**	**57**	**0**	0	0	0	0	3	*****	[ee:ef:eg:fg]
A101	<ab×cd>	**25**	**26**	**25**	**13**	0	0	0	0	0	0	0	0	3	-	[ac:ad:bc:bd]
A102	<lm×ll>	0	0	0	0	0	0	0	0	**38**	**56**	0	0	1	*	[ll:lm]
A103	<ef×eg>	0	0	0	0	**25**	**28**	**18**	**21**	0	0	0	0	3	-	[ee:ef:eg:fg]
A104	<nn×np>	0	0	0	0	0	0	0	0	0	0	**58**	**30**	1	****	[nn:np]
A106	<lm×ll>	0	0	0	0	0	0	0	0	**65**	**17**	0	0	1	*****	[ll:lm]
A107	<lm×ll>	0	0	0	0	0	0	0	0	**50**	**44**	0	0	1	-	[ll:lm]
A109	<lm×ll>	0	0	0	0	0	0	0	0	**54**	**31**	0	0	1	**	[ll:lm]
A111	<ef×eg>	0	0	0	0	**21**	**14**	**36**	**20**	0	0	0	0	3	***	[ee:ef:eg:fg]
A113	<nn×np>	0	0	0	0	0	0	0	0	0	0	**34**	**52**	1	*	[nn:np]
A115	<ab×cd>	**29**	**3**	**26**	**25**	0	0	0	0	0	0	0	0	3	*****	[ac:ad:bc:bd]
A120	<ab×cd>	**12**	**34**	**14**	**22**	0	0	0	0	0	0	0	0	3	****	[ac:ad:bc:bd]
A121	<lm×ll>	0	0	0	0	0	0	0	0	**40**	**47**	0	0	1	-	[lm:lm]
A126	<lm×ll>	0	0	0	0	0	0	0	0	**49**	**29**	0	0	1	**	[ll:lm]
A130	<lm×ll>	0	0	0	0	0	0	0	0	**39**	**43**	0	0	1	-	[ll:lm]
A133	<nn×np>	0	0	0	0	0	0	0	0	0	0	**37**	**41**	1	-	[nn:np]
A135	<lm×ll>	0	0	0	0	0	0	0	0	**39**	**42**	0	0	1	-	[ll:lm]
A136	<lm×ll>	0	0	0	0	0	0	0	0	**33**	**47**	0	0	1	-	[ll:lm]

Table 5.1b. Continued

		Allele ratios														
Locus	**Seg.type**	**ac**	**ad**	**bc**	**bd**	**ee**	**ef**	**eg**	**fg**	**ll**	**lm**	**nn**	**np**	**Df**	**Signif**	**Classes**
A139	<nn×np>	0	0	0	0	0	0	0	0	0	0	**50**	**28**	1	**	[nn:np]
A140	<ef×eg>	0	0	0	0	**16**	**14**	**22**	**16**	0	0	0	0	3	-	[ee:ef:eg:fg]
A145	<nn×np>	0	0	0	0	0	0	0	0	0	0	**43**	**37**	1	-	[nn:np]
A162	<lm×ll>	0	0	0	0	0	0	0	0	**28**	**50**	0	0	1	**	[ll:lm]
A165	<lm×ll>	0	0	0	0	0	0	0	0	**32**	**52**	0	0	1	**	[ll:lm]
A184	<nn×np>	0	0	0	0	0	0	0	0	0	0	**46**	**35**	1	-	[nn:np]
A188	<ab×cd>	**19**	**9**	**31**	**16**	0	0	0	0	0	0	0	0	2	****	[ac:ad:bc:bd]
A227	<ef×eg>	0	0	0	0	**17**	**7**	**11**	**17**	0	0	0	0	3	-	[ee:ef:eg:fg]
A253	<nn×np>	0	0	0	0	0	0	0	0	0	0	**42**	**42**	1	-	[nn:np]
A402	<ef×eg>	0	0	0	0	**17**	**1**	**21**	**25**	0	0	0	0	3	*****	[ee:ef:eg:fg]
A428	<lm×ll>	0	0	0	0	0	0	0	0	**17**	**30**	0	0	1	*****	[ll:lm]
P5	<lm×ll>	0	0	0	0	0	0	0	0	**41**	**45**	0	0	1	-	[ll:lm]

In bold numbers: are allele ratios for each segregation type classes, zeros means no segregation types, Df = degree of freedom and significant different shown as, - (dash) and *. Dash means no significant different between allele ratios found and one means little significant different and more stars mean high significant different. For example $\chi^2 > 7.8147$ then those numbers are significantly different if degree of freedom (df) = 3.

Table 5.2. Markers unlinked in linkage map construction.

Primer	Linkage group (LG)	Missing genotypes data (%)
A32	1	5
A86	2	4
A41	4	11
A88	4	1
A89	4	13
A29	5	15
A91	5	4
A93	5	6
A165	5	12
A44	5	21
A162	5	18
A14	6	22
A94	6	2
A96	8	6
A84	8	16
A136	8	16
A49	9	17
A73	10	17
A75	10	5
A77	10	2
A78	10	7
A79	10	7
A140	10	28
A52	11	20
A53	11	13
A98	11	1
A101	11	7
A102	11	2
A103	11	4
A145	11	16
A15	12	4
A104	12	8
A106	12	14
A111	13	5
A61	14	19
A64	14	15
A10	15	20
A67	15	18
A115	15	13
A113	15	10
A120	16	14
A71	17	19

Table 5.2. Continued

Primer	Linkage group	Missing data (%)
A121	17	9
A133	x	19
A130	x	14
A184	x	15
A227	x	44
A253	x	12
A402	x	32
A428	x	30
P5	x	10

x = represent published markers unlinked to any published linkage group on the framework map or predicted markers.

The missing data determines the accuracy of genotyping and the quality of data.