

Genetic diversity of root-infesting woolly apple aphid *Eriosoma lanigerum* (Hausmann) (Hemiptera:  
Aphididae) populations in the Western Cape

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## Declaration

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

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**ABSTRACT**

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Characterizing the genetic structure of a pest population can provide an understanding of the factors influencing its evolution and assist in its ultimate control. The aim of the present study was to characterize the genetic structure of woolly apple aphid *Eriosoma lanigerum* (Hausmann) populations in the Western Cape Province in South Africa. Since this economically important apple pest has not previously been characterized at molecular level, it was necessary to evaluate methods for determining the genetic structure of *E. lanigerum* populations. Two different molecular techniques were evaluated *viz.* random amplification of polymorphic DNA (RAPD) and amplified fragment length polymorphism (AFLP). This study represents the first application of the latter technique to members of the Aphididae.

Aphids were sampled from four regions in the Western Cape in South Africa *viz.* Elgin, Ceres, Vyeboom and Villiersdorp. A spatially nested sampling design was used to establish the distribution of the genetic variance of aphids. A total of 192 individuals from 13 farms were analysed. Ten RAPD primers were chosen for analysis from an initial assay of 25 after fragment reproducibility had been confirmed. For AFLP analysis three different rare-cutting restriction enzymes were evaluated for AFLP analysis, *viz.* *EcoRI*, *SseI* and *MluI*. The latter yielded the best results in combination with the frequent-cutting enzyme *MseI*. Twenty-five AFLP selective primer pairs were evaluated, out of which five were chosen for analysis of the total population.

Two hundred and fifty AFLP fragments and 47 RAPD fragments were scored for analysis. Both analyses indicated that a low level of genetic variation was apparent in *E. lanigerum* populations and that no differentiation resulted from geographic isolation. From RAPD analyses it was deduced that all variation could be attributed to differences between individuals. AFLP analysis indicated that, whereas genetic differences in *E. lanigerum* populations between orchards were negligible, a significant portion of genetic variation could be attributed to differences between farms and individuals within farms.

Therefore, AFLP analysis allowed for finer discrimination of the genetic structure of *E. lanigerum* populations than RAPD analysis and is recommended for studies of other aphid species. The fact that most of the genetic variation present in *E. lanigerum* populations could be found on small spatial scales indicated that sampling individuals over a wide geographic area was an ineffective way of detecting the genetic diversity present in *E. lanigerum* populations. The low level of variation in populations is most likely due to the exclusive occurrence of parthenogenetic reproduction, founder effects (including distribution of infested plant material from a limited source) and selective factors such as the use of resistant rootstocks or pesticides. Furthermore, the low level of variation found indicated that the possibility of controlling *E. lanigerum* in the Western Cape using host plant resistance is favourable. Thus, plant breeders developing

resistance to *E. lanigerum* can expect plant entries to be exposed to most of the genetic diversity present in Western Cape populations, regardless of location.

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## OPSOMMING

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Die bepaling van die genetiese struktuur van 'n landboukundige plaagpopulasie kan lei tot begrip van die faktore wat die populasie beïnvloed en kan uiteindelijke beheer vergemaklik. Die doel van die huidige studie was om die genetiese struktuur van die appelbloedluis *Eriosoma lanigerum* (Hausmann) in die Wes-Kaap Provinsie van Suid-Afrika te bepaal. Aangesien hierdie belangrike appelplaag nie van tevore op molekulêre vlak bestudeer is nie, was dit nodig om metodes vir die bepaling van die genetiese struktuur van *E. lanigerum* populasies te evalueer. Twee molekulêre tegnieke is geëvalueer, nl. lukraak geamplifiseerde polimorfiese DNS (RAPD) en geamplifiseerde fragment-lengte polimorfismes (AFLP). Hierdie studie is die eerste om laasgenoemde tegniek te gebruik om lede van die Aphididae te bestudeer.

Plantluse is verkry van vier verskillende gebiede in die Wes-Kaap Provinsie van Suid-Afrika nl. Elgin, Ceres, Vyeboom en Villiersdorp. 'n Hierargiese sisteem is gebruik om die verspreiding van die genetiese variasie van plantluse te bepaal. In totaal is 192 individue van 13 plase geanaliseer. Tien RAPD inleiers is gekies uit 'n analise van 25 verskillende inleiers nadat fragment reproduseerbaarheid bevestig is. Drie verskillende restriksie ensieme is geëvalueer vir AFLP analise nl. *EcoRI*, *SseI* en *MluI*. Die beste resultate is verkry toe *MluI* saam met *MseI* gebruik is. Vyf-en-twintig AFLP selektiewe inleier pare is geëvalueer waarvan vyf gekies is vir analise van die totale populasie.

Twee-honderd-en-vyftig AFLP fragmente en 47 RAPD fragmente is gedokumenteer vir analise. Beide RAPD en AFLP analyses het getoon dat daar 'n lae vlak van genetiese variasie in *E. lanigerum* populasies is en dat geen differensiasie as gevolg van geografiese isolasie ontstaan het nie. Uit RAPD analise is daar afgelei dat al die variasie toegeskryf kon word aan verskille tussen individue. AFLP het aangetoon dat alhoewel verskille in *E. lanigerum* populasies tussen boorde laag was, kon 'n hoë persentasie van die variasie toegeskryf word aan verskille tussen plase en individue binne plase.

AFLP analise het meer insig in die genetiese struktuur van *E. lanigerum* populasies verskaf, en word dus aanbeveel vir studies van ander plantluse. Omdat meeste van die genetiese variasie oor klein geografiese afstande verkry word, is steekproefneming oor groot gebiede 'n ondoeltreffende manier om die genetiese variasie binne 'n monster te meet. Die lae vlak van genetiese variasie is waarskynlik te wyte aan partenogenetiese vermeerdering, stigter gevolge (insluitend verspreiding van geïnfesteerde plantmateriaal vanaf 'n beperkte bron), sowel as selektiewe faktore soos die gebruik van bestande onderstokke en insekdoders. Verder dui die lae vlak van variasie aan dat die moontlikheid vir beheer deur gasheerplantbestandheid goed is in die Wes-Kaap. Planttelers kan verseker wees dat hulle plante blootgestel sal wees aan meeste van die genetiese variasie in die Wes-Kaap appelbloedluis populasies ongeag hulle ligging.

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**LIST OF ABBREVIATIONS AND SYMBOLS**

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$\lambda$	Lambda
$\gamma$	Gamma
$^{\circ}\text{C}$	Degrees centigrade
$\mu\text{g}$	Microgram
$\mu\text{l}$	Microlitre
$\mu\text{M}$	Micromolar
AFLP	Amplified fragment length polymorphism
AMOVA	Analysis of molecular variance
ATP	Adenosine triphosphate
bp	Base pair (s)
BSA	Bovine serum albumin
CTAB	N-cetyl-N,N,N-trimethyl-ammonium bromide
ddH <sub>2</sub> O	Double-distilled water
DNA	Deoxyribonucleic acid
dNTP	Dinucleotide triphosphate
EDTA	Ethylenediaminetetraacetic acid
HCL	Hydrochloric acid
kb	Kilo base (s)
M	Molar
MgCl <sub>2</sub>	Magnesium chloride
ml	Millilitre
mM	Millimolar
mtDNA	Mitochondrial DNA
NaCl	Sodium chloride
NH <sub>4</sub> Ac	Ammonium acetate
ng	Nanogram
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
Pmol (es)	Picomole (s)
RAPD	Random amplified polymorphic DNA
RFLP	Restriction fragment length polymorphism
SDS	Sodium dodecyl sulphate
TBE buffer	Tris borate EDTA buffer
TE buffer	Tris EDTA buffer

TEN buffer	Tris EDTA NaCl buffer
TRIS	2-amino-2(hydroxymethyl)-1,3-propanediol
UPGMA	Unweighted pair group method algorithm
V	Volts
W	Watts

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**TABLE OF CONTENTS**


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DECLARATION.....	I
ABSTRACT.....	II
OPSOMMING.....	IV
ACKNOWLEDGEMENTS.....	V
LIST OF ABBREVIATIONS AND SYMBOLS.....	VI
CHAPTER 1: INTRODUCTION.....	1
1.1 Aims of study.....	1
1.2 References.....	2
CHAPTER 2: LITERATURE REVIEW .....	4
2.1 The woolly apple aphid.....	4
2.1.1 Origin .....	4
2.1.2 Life and seasonal cycle.....	4
2.1.3 Reproduction .....	5
2.1.4 Damage.....	5
2.1.5 Dissemination.....	6
2.1.6 Control.....	6
2.1.7 <i>E. lanigerum</i> biotypes.....	6
2.2 Genetic structure of aphid populations .....	7
2.2.1 Parthenogenesis and its effect on genetic variation.....	8
2.2.2 Processes generating genetic variation .....	9
2.2.3 Factors responsible for decreased variation .....	10
2.2.4 Genetic structure and pest management.....	11
2.2.5 Observed patterns of aphid genetic structure .....	12
2.3 Molecular techniques and aphid genetic structure .....	13
2.3.1 Allozyme analysis .....	13

2.3.2 Mitochondrial DNA Analysis.....	14
2.3.3 Microsatellite analysis.....	14
2.3.4 Random amplification of polymorphic DNA.....	15
2.3.5 Amplified fragment length polymorphism.....	16
2.4 References.....	20
CHAPTER 3: MATERIAL AND METHODS.....	30
3.1 Aphid material.....	30
3.1.1 Sampling procedures.....	30
3.1.2 Establishment of parthenogenetic lines.....	30
3.2 Random amplification of polymorphic DNA.....	36
3.2.1 DNA isolation.....	36
3.2.2 DNA amplification.....	36
3.2.3 Gel electrophoresis.....	37
3.3 Amplified fragment length polymorphism.....	38
3.3.1 DNA isolation.....	38
3.3.2 DNA restriction and ligation of adaptors.....	38
3.3.3 Preselective amplification.....	39
3.3.4 Primer labelling.....	39
3.3.5 Selective amplification.....	39
3.3.6 Gel electrophoresis.....	40
3.4 Data analysis.....	41
3.4.1 Data scoring.....	41
3.4.2 Level of genetic variation.....	41
3.4.3 Individual genetic distance matrices ...	42
3.4.4. Distribution of genetic variation.....	42
3.4.5 Population structure.....	42
3.5 References.....	43

CHAPTER 4: RESULTS .....	44
4.1 Random amplification of polymorphic DNA. ....	44
4.1.1 DNA isolation.....	44
4.1.2 Reproducibility .....	44
4.1.3 Level of genetic variation.....	44
4.1.4 Distribution of genetic variation.....	45
4.2 Amplified fragment length polymorphism.....	45
4.2.1 DNA isolation.....	45
4.2.2 Reproducibility .....	45
4.2.3 Level of genetic variation.....	45
4.2.4 Distribution of genetic variation.....	47
4.2.5 Population structure.....	48
CHAPTER 5: DISCUSSION.....	52
5.1 Methodology .....	52
5.1.1 Random amplification of polymorphic DNA.....	52
5.1.2 Amplified fragment length polymorphism.....	53
5.2 Genetic structure .....	55
5.2.1 Level of genetic diversity .....	55
5.2.2 Distribution of genetic variation.....	56
5.3 Inferences from genetic structure.....	59
5.3.1 Distribution of genetic variation.....	59
5.3.2 Level of genetic variation.....	60
5.3.3 Implications of decreased genetic diversity.....	63
5.3.4 Genetic structure and pest management .....	63
5.4 References.....	64
CHAPTER 6: CONCLUSION.....	69

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CHAPTER 1  
INTRODUCTION

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A multidisciplinary approach is necessary to understand pest biology and to develop control strategies. One aspect of this approach is the growing awareness that ecological principles can and should be applied to agricultural systems. Of particular importance to agriculture is the fact that ecological understanding is inseparable from information about the genetic structure of the pest population(s) in the system being evaluated. Genetic structure in an ecological sense refers to the level and distribution of genetic variation found within a population (Roderick 1996). The significance of genetic structure is related to the importance of variation as a distinguishing trait in biology, as it is the basis of any evolutionary process. The need to understand the nature of variation between and within pest populations has been demonstrated many times for many different taxa. The accumulation of literature on the subject has been accelerated by the growing number of molecular techniques that can be used to determine genetic variation, as classical morphological techniques often cannot provide the resolution required (Cenis *et al.* 1993). Entomologists are starting to realise that knowledge of the genetic variation of an insect pest population can aid in the understanding of its biology and ultimate control in an integrated pest management system (Loxdale & Lushai 1998). These principles are especially important for aphids, given their complex biology and the difficulty with which they are controlled. An ecological approach, and in particular the determination of genetic variation, should be applied to understand the woolly apple aphid, *Eriosoma lanigerum* (Hausmann) (Hemiptera: Aphididae), one of the most damaging pests of apple trees in the Western Cape Province in South Africa. Damavandian (1999) and Heunis (2001) have studied the biology of underground and aboveground populations of this pest respectively. However, control is still erratic. The characterisation of the genetic structure of *E. lanigerum* may therefore prove invaluable.

### 1.1 AIMS OF STUDY

In view of the fact that the knowledge of ecological principles and genetic variation can aid in the understanding of pest biology and the implementation of control measures, the aims of this study were to:

1. Evaluate and optimise means of determining the genetic structure of *E. lanigerum* populations.

No methods have been described as yet for determining the genetic structure of *E. lanigerum* populations. Recently, studies analysing the genetic variation in various insect species have relied increasingly on the use of molecular techniques (Loxdale & Lushai 1998). Various molecular techniques have been evaluated as tools for characterising aphid populations with varying degrees of success (Hales *et al.* 1997). Therefore, the aim of the present study was to evaluate and optimise two molecular techniques for determining the genetic structure of *E. lanigerum* populations *viz.* random amplification of polymorphic

DNA (RAPD) and amplified fragment length polymorphism (AFLP). RAPD has been used successfully in a number of studies of aphid species, other than the woolly apple aphid, to examine genetic structure. However, AFLP is a relatively novel technique for insect studies (McMichael & Prowell 1999, Reineke *et al.* 1999, Yan *et al.* 1999, Parsons & Shaw 2001) that has never been applied to aphids. The evaluation and comparison of these techniques for use in studies of *E. lanigerum* was therefore a critical objective.

## 2. Characterize the genetic structure of *E. lanigerum* populations.

No information exists regarding the genetic structure of *E. lanigerum* populations in any country where this insect is found. It is not known how much genetic variation this pest possesses or how this variation is distributed. Previous studies characterising the genetic structure of populations of other aphid species have led to unique insights regarding various aspects of aphid biology. It was therefore an important objective to determine the level of genetic variation in *E. lanigerum* populations and establish the distribution of this variation. The study was confined to subterranean populations of the aphid in the main apple-growing regions of the Western Cape Province.

## 3. Make inferences concerning the genetic structure of *E. lanigerum* populations.

Information of the genetic structure of a pest population allows inferences to be made regarding the factors responsible for the amount and distribution of genetic variation. This may lead to an understanding of the processes that shape the genetic structure and its effect on the population. It can also aid in making recommendations about the future control measures against *E. lanigerum*.

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CHAPTER TWO  
LITERATURE REVIEW

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It is necessary to review three distinct, but pertinent, subjects in order to place the study of the genetic structure of *Eriosoma lanigerum* populations in the Western Cape Province in context. First, aspects of woolly apple aphid biology and control in the Western Cape will be described. Next, the rapidly growing field of aphid population structure is considered. Finally, molecular markers that can be used to evaluate aphid genetic variation are assessed.

## 2.1 THE WOOLLY APPLE APHID

### 2.1.1 Origin

The woolly apple aphid, *E. lanigerum*, has its origin in eastern North America where it has been known as a pest for almost 200 years (Hoyt & Madsen 1960, Donald & Brown 1988). From North America, *E. lanigerum* was distributed on nursery material virtually worldwide (Marlatt 1897, Baker 1915, Marcovitch 1934, Schoene & Underhill 1935, Greenslade 1936). This insect is now regarded as a pest wherever apple, *Malus domestica* (Borkh), is grown commercially (Walker *et al.* 1988). In South Africa, *E. lanigerum* was first recorded in 1895 by Lounsbury (Myburgh *et al.* 1973) and it has since spread throughout the Western Cape Province, the principal area of apple production in South Africa.

### 2.1.2 Life and seasonal cycle

In North America *E. lanigerum* is holocyclic on American elm, *Ulmus americana* L. (Baker 1915, Greenslade 1936, Crane *et al.* 1936, Annecke & Moran 1982, Welty & Murphy 2000). Aphid eggs hatch in spring to produce stem mothers or fundatrices. These aphids produce two spring generations parthenogenetically that feed on the American elm. A third winged generation appears early in summer and flies to apple trees or related plants, where they settle on the aerial parts of the trees. A portion of these aphids moves down the tree trunk to the roots where they may multiply. In autumn, a few nymphs develop into wingless males that mate with wingless females. Each mated female lays an egg nearly the size of her body. These eggs are able to withstand harsh climatic conditions by overwintering in cracks and crevices of elm bark or underground on the roots of apple trees.

In South Africa and other parts of the world where the American elm is absent, the life cycle of *E. lanigerum* is restricted to apple trees and is anholocyclic. Alate forms are produced exclusively during

autumn, possibly as a response to overcrowding, but these do not reproduce (Heunis 2001). Colonies of *E. lanigerum* are found underground throughout the year, where they feed on the roots of apple trees. These colonies are usually the source of aerial infestations. In spring and summer, a portion of the crawlers (first instar nymphs) from root populations migrate up the trunks of trees and remain in large numbers on the aerial parts of the tree until autumn (Marlatt 1897, Schoene & Underhill 1935, Hoyt & Madsen 1960, Damavandian 1999, Heunis 2001). Above the ground, aphids settle in leaf axils or in injured bark and pruning wounds where they complete their development. There are four nymphal stages. In the Western Cape Province, subterranean *E. lanigerum* populations have approximately eighteen generations per year (Damavandian 1999).

### 2.1.3 Reproduction

The woolly apple aphid is heteroecious and requires the American elm as primary host and *Malus* spp. as secondary host in order to display a holocyclic mode of development (Marlatt 1897, Crane *et al.* 1936). In North America, where extreme climatic conditions are frequent during winter and the American elm is widespread, this mode of reproduction is common, with sexual reproduction alternating with parthenogenesis. In South Africa, where climatic conditions during winter are mild and the primary host absent, the woolly apple aphid reproduces anholocyclically by apomictic parthenogenesis. The egg stage has never been observed in South Africa (Heunis 2001).

### 2.1.4 Damage

The biology of *E. lanigerum* is such that apple trees are damaged both above and below the ground. Aboveground damage by the woolly apple aphid includes the destruction of developing buds in leaf axils and a reduction in tree vigour due to feeding on wounds and pruning cuts (Annecke & Moran 1982, Welty & Murphy 2000). The woolly apple aphid can also act as a direct pest when it infests the fruit cores of some cultivars (Essig 1942, Welty & Murphy 2000). In addition to feeding aboveground, woolly apple aphid may be found throughout the year on apple roots. Belowground, feeding by *E. lanigerum* causes large galls on the roots, resulting in decreased uptake of water and nutrients (Brown *et al.* 1991, Damavandian 1999). Root damage also weakens the tree, which reduces tree health and prevents wounds from healing (Welty & Murphy 2000). Subterranean colonies often remain undetected and uncontrolled because of their cryptic situation. They can cause significant damage to a tree before colonies become visibly apparent.

### **2.1.7.2 *E. lanigerum* biotypes in South Africa**

Northern Spy and related rootstocks were developed to provide resistance against *E. lanigerum*. These rootstocks provided a large measure of control wherever they were used (Knight *et al.* 1962). However, in 1968 there were reports that a biotype of *E. lanigerum* had overcome this resistance factor when these rootstocks became severely infested with the aphid (Giliomee *et al.* 1968). The infestation occurred in the Elgin area. Aphids were taken from this region and also from Pniel, an area 30 miles away and used to inoculate Northern Spy rootstocks. The first group of aphids became established but the other failed to do so. This indicated that a biotype able to overcome the Northern Spy resistance factor had evolved in Elgin.

*E. lanigerum* biotypes were distinguished once again in Elgin in 1994, based on insecticide resistance (Pringle *et al.* 1994). Woolly apple aphid populations that were being controlled satisfactorily with vamidothion were compared with populations suspected of having developed tolerance to this chemical. From this study it was concluded that distinct biotypes of the woolly apple aphid exist that are tolerant to vamidothion.

### **2.1.7.3 *E. lanigerum* biotypes worldwide**

Two cases of *E. lanigerum* biotype development were reported outside South Africa. These were both based on the differential ability to colonise apple varieties, indicating the importance of host plant–aphid interactions as a selective factor in woolly apple aphid biology. In South Australia, two biotypes of the woolly apple aphid were reported (Sen Gupta & Miles 1975). The so-called Blackwood strain was able to attack varieties of apple resistant to the other strain, known as the Clare strain. Both strains also displayed clear differences in the ease with which they colonised different varieties of apple and different parts of any one variety. In western North Carolina, a woolly apple aphid biotype capable of colonising cultivars with Northern Spy type resistance was found (Young *et al.* 1982). In all other areas in the USA, Northern Spy still provides a large measure of resistance.

## **2.2 GENETIC STRUCTURE OF APHID POPULATIONS**

Genetic structure is an important concept in ecology as levels of genetic variation can reflect both the evolutionary history of a population and its potential to evolve (Berry 1989, McDonald 1997). Ecological data indicate that virtually every population is unique (Berry 1989). The levels of variation that occur in different populations have important implications.

Genetic variability is the basis of any evolutionary process since it provides the raw genetic material for individuals to adapt to a given situation. The existence of variation in populations therefore determines its

### 2.1.5 Dissemination

In South Africa, the woolly apple aphid has a limited potential for dispersal, as it occurs in the apterous form during most of the year (Heunis 2001). Adult aphids and nymphs other than crawlers largely remain sessile, except in extreme environmental conditions (Asante *et al.* 1993). Crawlers are therefore responsible for most of the local movement of the aphid and within-tree dispersal of young nymphs is very high (Asante *et al.* 1993). However, between-tree dispersal is low. Long distance dispersion of crawlers can take place by wind or distribution by other insects and birds (Heunis 2001). These crawlers can give rise to new infestations in the aerial parts of trees as well as on the roots if they are able to gain access through cracks in the soil or on exposed roots (Heunis 2001). Woolly apple aphid can also be distributed on nursery trees.

### 2.1.6 Control

Three main methods are used to control *E. lanigerum* populations in South Africa *viz.* biological control, chemical sprays and the use of resistant rootstocks. The parasitic wasp *Aphelinus mali* (Haldeman) can aid in the control of *E. lanigerum* colonies in aerial parts of the tree. However, this parasitoid appears too late in the season to prevent colony formation and bud damage (Heunis 2001). Spray applications are still considered necessary in most commercial orchards. At present, the chemicals used most commonly against the woolly apple aphid are vamidothion, endosulfan and chlorpyrifos and more recently, imidacloprid (Pringle *et al.* 1994, 1998). These chemicals have a short residual action and are often unable to penetrate the waxy filaments covering aphid colonies, leading to inadequate control. The exception is imidacloprid, which is applied to the soil and can control populations for up to three years (Pringle 1998). Plant resistance provided by the Northern Spy and certain Merton and Malling-Merton rootstocks played an important role in woolly apple aphid control in former years (Knight *et al.* 1962). However, resistance has been overcome by a resistance-breaking biotype in the Western Cape Province (Giliomee *et al.* 1968).

### 2.1.7 *E. lanigerum* biotypes

#### 2.1.7.1 *Biotype definition*

The term biotype can be defined as a population or an individual distinguished from other populations or individuals of its species by a nonmorphological trait (Eastop 1973, Diehl & Bush 1984, Panda & Khush 1995). In agriculture biotypes usually develop as a result of adaptation to or development on a particular host or exposure to selective factors such as insecticides. Biotypes resistant to insecticides or able to attack previously resistant plant varieties are common in agriculture (Shufran *et al.* 2000). The occurrence of aphid biotypes threatens the durability of control measures and the failure to recognise distinct populations can thus have costly and frustrating consequences for pest control (Tomiuk & Wohrmann 1980).

success under changing conditions (Armstrong & Wratten 1996). It is generally assumed that species exhibiting high levels of genetic variation are more persistent, as they have the ability to respond to both biotic and abiotic changes. In populations with high levels of genetic variation biotype development is common and can allow adaptation to a variety of changes in the environment (Hales *et al.* 1997). However, the survival of a species with low genetic variability is threatened by these changes (Maynard Smith 1978, Blackman 1979). Therefore, it is clear that knowledge of the genetic structure of a pest population can provide invaluable information on which to construct and modify pest management and forecasting systems.

The genetic structure of aphid populations has received increased interest over the past two decades (Hales *et al.* 1997). This is due to two factors. First, the complicated life cycle and biology of different aphid species have resulted in unique patterns of genetic variation, which is of theoretical interest. Second, many agricultural crops serve as host plants for aphid species, whose feeding can have catastrophic effects on production. Knowledge of the genetic structure of aphid pest populations is proving invaluable in aiding their control. An explosion of new findings in classical genetics, molecular biology and population genetics is taking place regarding this intrinsically fascinating and economically significant group.

In this section, various aspects of genetic structure in aphid populations are considered. Since *E. lanigerum* reproduces by parthenogenesis, only literature relevant to this mode of reproduction and its influence on genetic structure will be reviewed. The section starts with a summary of the effect of parthenogenesis on genetic variation. Next, factors responsible for generating genetic diversity in parthenogenetic populations are described as well as the factors that can decrease variability. The section concludes with a review of the effect of genetic structure of populations on pest management.

### **2.2.1 Parthenogenesis and its effect on genetic variation**

In parthenogenetic aphids, reproduction takes place solely by apomixis. In aphids displaying this type of reproduction, egg maturation is ameiotic with the chromosomes undergoing what is essentially a mitotic division (Blackman 1979, 1980, 1981). This results in the formation of true clones, where offspring are genetically identical to the mother, barring mutations, chromosomal rearrangements and perhaps rare mitotic recombination events (Blackman 1979, 1980, Hales *et al.* 1997). This mode of reproduction can have a significant effect on the genetic structure of aphid populations.

Sexual reproduction is known to promote genetic variability and provide an adaptive potential for populations to evolve. In contrast, asexual species are often assumed to be less responsive to changing environmental conditions and synonymous with an evolutionary dead-end (Blackman 1981). In asexual populations a low rate of genetic variation is expected, with a resulting lack of genetic flexibility when faced with changing external conditions coupled with a high rate of extinction (Glesener & Tilman 1978,

Jaenike 1978, Maynard Smith 1978). However, in recent years electrophoretic investigations have disproved the theory that asexual species have less genetic variability than sexual species (Lynch 1984, Tomiuk 1990). In certain aphid species parthenogenetic lineages may be more specialised than their sexual counterparts (Dixon 1985, Hales *et al.* 1997). Many parthenogenetic species are geographically more widely distributed and ecologically more diverse than their sexual relatives (Lynch 1984). This is apparent from various agricultural systems, where parthenogenetic reproduction in aphids allowed adaptation to new selective agents such as insecticides (Sunnucks *et al.* 1996, Hales *et al.* 1997).

## **2.2.2 Processes generating genetic variation**

Parthenogenetic populations rely on mechanisms such as mutation, chromosome rearrangement and perhaps mitotic recombination to increase genetic variation. These processes also occur in sexually reproducing populations but it has been suggested that certain parthenogenetic species may display elevated rates of these processes to compensate for low recombinational rates of production of genetic variance (Blackman 1981, Lynch 1984, Hales *et al.* 1997).

### **2.2.2.1 Mutation**

A mutation in the germ-line of a parthenogenetic aphid will cause one or more of its progeny to differ in genotype from their mother and thus have the potential to found a new clone (Blackman 1979). Mutations are usually regarded as rare events. However, they become matters of certainty rather than chance if the population size is large, as parthenogenetic aphid populations usually are (Blackman 1980, Dixon 1985, Hales *et al.* 1997). In parthenogenetic aphids, where each aphid develops from a single cell, the likelihood of mutation influencing development at the organ level is greatly increased (Dixon 1985). Therefore, parthenogenesis increases both the chances of mutations being incorporated into genotypes as well as propagating these mutations.

### **2.2.2.2 Chromosome rearrangement**

Chromosomal rearrangements are relatively common in aphids, since hemipteran chromosomes have no localised centromere (Blackman 1979, 1980, Sunnucks *et al.* 1996, Hales *et al.* 1997). Gross genetic changes involving dissociation or rearrangement of chromosomes alter the relative positions of genes and may affect their expression (Blackman 1979). Several aphid chromosomal rearrangements are associated with radical new biotypes associated with new environmental tolerances (Sunnucks *et al.* 1996, Hales *et al.* 1997).

### **2.2.2.3 Mitotic recombination**

Mitotic recombination, the process whereby crossing over occurs and heterozygous marker genes segregate in somatic cells, is an abnormal genetic event in aphids (Cognetti 1961, Blackman 1980, Tomiuk & Wohrmann 1982, Tomiuk 1990). However, like mutation, its potential significance could increase when it is considered in the context of the large size of aphid field populations. Even occasional recombination could affect levels of genetic variation considerably (Hales *et al.* 1997).

## **2.2.3 Factors responsible for decreased variation**

In aphid populations, various factors can result in decreased genetic variation. These include selective factors and founder effects.

### **2.2.3.1 Selective factors**

Selective factors often result in population bottlenecks, which in turn may play an important role in aphid biology. A population bottleneck occurs when a population undergoes a severe temporary reduction in size (Hartl & Clark 1989). In aphid populations severe bottlenecks due to selective factors such as insecticide treatment or unfavourable climatic conditions have been recorded (Fuller *et al.* 1999). Another example is to be found in host-alternating aphids where the switch from the primary to secondary hosts might be associated with population bottlenecks leading to the loss of genetic diversity (Simon & Hebert 1995).

### **2.2.3.2 Founder effects and introduced species**

Founder effects are a severe form of a population bottleneck that occurs when a small group of emigrants from an established subpopulation founds a new subpopulation (Hartl & Clark 1989). Parthenogenesis has the advantage of allowing a single individual to initiate a new population (Lynch 1984), which may lead to strong founder effects. This is especially apparent in aphid populations, which display a high rate of increase due to high fecundity and overlapping generations. Aphids have the potential to quickly colonise the surrounding plants, leading to very high-density infestations, which may all have originated from a single individual (Fuller *et al.* 1999). Consequently, genetic variation in these populations is minimal.

The most damaging insect pests in South Africa have often been introduced unwittingly and as such may exhibit strong founder effects. Mostly, the origin and genetic structure of these pests are unknown, as in the case of *E. lanigerum*. The analysis of genetic variation may help to interpret basic ideas concerning the population dynamics of introduced pests. It may also provide data that are critical to understand the factors affecting rates and adaptive potential of genetic change in exotic pests, which may provide an explanation for the successes or failures of invading pest populations (Armstrong & Wratten 1996). The genetic

structure of an invading species and its subsequent ability to adapt depends, among other factors, on the structure of the initial founding population (Nicol *et al.* 1998).

#### **2.2.4 Genetic structure and pest management**

Knowledge of the genetic structure of pest populations can assist in their control. In particular, the three control measures that are implicated are plant resistance, use of insecticides and biological control.

##### **2.2.4.1 Plant resistance**

Aphid populations become genetically diverse mainly as result of the nature of the association with their host plants (Tomiuk 1990). The measurement of genetic variation is therefore crucial for understanding herbivore-plant interactions. The greater the variation within pest populations, the faster populations will evolve in response to changing conditions such as a change in plant variety (Tomiuk 1990). When new genes are deployed for plant resistance, the development of damaging resistance breaking biotypes is a common occurrence, especially in populations where the initial level of genetic variation was high (Puterka & Peters 1989, Shufran *et al.* 2000).

Plant breeders can benefit from information about the clonal diversity of the pest against which they are trying to develop resistance. Geographic differences or narrow genetic variability within pest populations will give false results during plant resistance performance trials and will undoubtedly lead to field resistance being short-lived (Shufran *et al.* 2000). To avoid this, plants should be exposed to as much of the diversity of the pest as possible during plant screenings in order to make meaningful selections. It is therefore advisable that levels of genetic diversity are determined before varieties that are resistant to a pest are identified and used.

##### **2.2.4.2 Insecticide resistance**

Knowledge of clonal diversity offers essential data on aphid biology that may provide an understanding of problems such as the development and spread of pesticide resistance in aphid populations. The genetic ability of an insect biotype to overcome the suppressive effect of an insecticide may be so great that the value of the effect is nullified before the insecticide is used on a widespread geographic basis (Shufran *et al.* 1997).

##### **2.2.4.3 Biological control**

Biological control may be complicated in aphid populations showing increased levels of genetic diversity since an almost infinite number of biotypes may be found in terms of behaviour and physiology. Eastop (1973) postulated that different biotypes of an aphid species are likely to differ in habitat and that their life

processes may be confined by different parameters. For example, one biotype may develop at a temperature below the threshold of activity for a predator while another will not. Information about population structure can therefore help predict the success or failure of a biological control system.

### **2.2.5 Observed patterns of aphid genetic structure**

The genetic structure of most aphid species, in contrast to many animals, is very flexible in both time and space (Hales *et al.* 1997). This is evidenced by previous studies of aphid populations, which have produced varied results regarding the level of variation present as well as the distribution of this variance.

#### **2.2.5.1 Level of variation**

Aphid species may exhibit low levels of genetic variation. Low levels of genetic variation were found in populations of the aphids *Melaphis rhois* (Hebert *et al.* 1991), *Aphis gossypii* (Martinez-Torres *et al.* 1997a), *Rhopalosiphum maidis* (Simon *et al.* 1995) and *Sitobion avenae* (Wilson *et al.* 1999). However, high levels of genetic variation have also been reported in studies of other aphids. In these aphid populations biotype formation is common. This was evidenced in studies of the aphids *Schizaphis graminum* (Powers *et al.* 1989, Black *et al.* 1992, Shufran *et al.*, 2000), *Therioaphis trifolii* (Sunnucks *et al.* 1997) and *A. pisum* (Buornoville *et al.* 2000). Molecular techniques were found to confirm biotype identification based on morphological techniques and identify previously undetected virulent biotypes.

#### **2.2.5.2 Distribution of genetic variation**

In aphids, it is not unusual for a high proportion of the variation to be found in extremely small areas - usually individual fields or plants (Hales *et al.* 1997). The most striking example of this is to be found in the pea aphid *Acyrtosiphon pisum*. When individuals from different galls on the same twig were compared, genetic variation was high enough to discriminate individuals from different galls (Fukatshu & Ishikawa 1994). Pea aphids from a single field also displayed a high level of diversity (Barrette *et al.* 1994). However, a study of pea aphids sampled using an intensive sampling scale encompassing numerous countries, revealed an overall low level of variation (Birkle & Douglas 1999). This pattern of greater amounts of variation on a small scale was also found in populations of *S. graminum* (Shufran *et al.* 1991, Shufran & Wilde 1994). Genetic variation on larger spatial scales has also been found in aphid populations. Regional variation as a result of geographic isolation was evident in anholocyclic populations of the aphid *Elatobium abietinum*. Individuals of this species from New Zealand lacked detectable levels of variation (Nicol *et al.* 1997a, 1998) whereas those from Iceland possessed sufficiently high levels for discrimination of eastern and western populations, with high levels of variation within these two populations (Sigurdsson *et al.* 1999).

## 2.3 MOLECULAR TECHNIQUES AND APHID GENETIC STRUCTURE

The need to understand aphid pest ecology and genetic diversity is clear. To fulfill this need, it is essential that all techniques that may aid this multidisciplinary problem should be used. In the past, conventional methods used to provide insight into aphid population genetic structure were limited in their ability to resolve ecological questions, as they were unable to distinguish between morphologically identical clones (Cenis *et al.* 1993). However, molecular techniques offer great promise in the field of aphid population ecology and in particular the determination of population genetic structure. Recent advances in molecular techniques guarantee detailed understanding of the genetics of species and may provide insight into problems we may only have guessed at in the past.

Presently, a number of molecular markers exist that can be used to determine the level of genetic variation within aphid species. The markers that have been used most often are allozyme analysis, mitochondrial DNA (mtDNA) analysis, microsatellites and random amplification of polymorphic DNA (RAPD). These marker techniques will be described and compared briefly and their applications in molecular aphidology discussed. More complete reviews will be presented for RAPD analysis as well as for amplified fragment length polymorphism (AFLP), a technique that holds great promise for studies of aphid population genetics, since these two marker systems were chosen for the analysis of *E. lanigerum* population genetic structure. A summary of the main characteristics of the markers mentioned can be found in Table 2.1.

### 2.3.1 Allozyme analysis

Allozyme analysis was the first attempt to determine aphid genetic diversity using molecular markers (Hoy, 1994). This technique relies on the detection of variant proteins produced by allelic forms of the same locus (Hames & Rickwood 1981). Allozyme analysis was used to study the levels of genetic variation of the aphids *Macrosiphum euphorbiae*, *M. funestum*, *Aphid sambuci*, *Wahlgreniella nervata* (Tomiuk & Wohrmann 1980), *Melaphis rhois* (Hebert *et al.* 1991), *Diuraphis noxia* (Puterka *et al.* 1992), *Rhopalosiphum padi* (Simon *et al.* 1995, Simon *et al.* 1996), *R. maidis* (Simon *et al.* 1995), and *A. pisum* (Tomiuk & Wohrmann 1980, Buornoville *et al.* 2000). In all these studies, allozyme analysis detected surprisingly low levels of genetic variation and polymorphisms were detected at only a few loci. Allozyme analysis indicated that aphids showed average heterozygosities of only 1.5 %, whereas most other invertebrates show an average heterozygosity of 10 % (Barrette *et al.* 1994, Simon & Hebert 1995). It was therefore clear that allozyme analysis was unable to detect useful levels of genetic variation in aphid populations. This prompted a search for markers with increased resolution for determining population genetic structure. Recently, many of the applications of allozyme and protein analysis have been superseded and expanded upon using DNA-based techniques (Loxdale & Lushai 1998). As a consequence,

greater resolution has been achieved at the population level allowing accurate analysis of the genetic structure of populations of organisms with little detectable protein variation such as aphids.

### 2.3.2 Mitochondrial DNA analysis

Mitochondrial DNA (mtDNA) variation in aphids has been studied mainly through comparison of restriction site maps (restriction fragment length polymorphisms or RFLP) or by size variation of the mtDNA molecule. MtDNA is a valuable marker used to indicate maternal gene flow, as it is predominantly transmitted through maternal lines (Awise *et al.* 1987, Loxdale & Lushai 1998). A second major advantage of mtDNA analysis is that the technique can usually differentiate to an infraspecific level since mtDNA mutates approximately 20 times faster than nuclear DNA (Loxdale & Lushai 1998). MtDNA polymorphisms have been shown to be fairly common in the aphid species *A. pisum* (Barrette *et al.* 1994), *Sitobion* spp. (Sunnucks *et al.* 1996, Figueroa *et al.* 1999), *R. padi* (Martinez *et al.* 1992, Martinez-Torres *et al.* 1996, 1997a, 1997b), *S. graminum* (Powers *et al.* 1989, Shufran *et al.* 2000) and *T. trifolii* (Sunnucks *et al.* 1997), but less so in *R. maidis* (Simon *et al.* 1995). Despite the success of these studies, it is possible that the use of mtDNA may be limited in aphid population genetics as a result of unusual barriers such as heteroplasmy and nuclear transpositions (Hales *et al.* 1997). A further confounding factor, noted for aphids, is the presence of multiple copies of certain mtDNA genes (Loxdale & Lushai 1998). Also, while many conserved mtDNA primers may be used in aphids and much is known about the evolutionary rate of different mtDNA regions, it may still be difficult to find primers that amplify regions of appropriate variability for individuals that are closely related (Hales *et al.* 1997).

### 2.3.3 Microsatellite analysis

Microsatellites are referred to by a variety of names, including simple sequence repeats (SSR) and variable number of tandem repeats (VNTR). Microsatellite repeats consist of arrays of mono-, di-, tri-, tetra- and penta-nucleotide units widely dispersed throughout the genome (Cregan & Quigley 1997). They usually display high levels of genetic variation based on differences in the number of tandemly repeated units at a locus. The regions flanking the microsatellites are generally conserved among genotypes of the same taxon and are therefore used for PCR primer design. Microsatellite loci are relatively abundant in the insect genome and they mutate at a relatively high rate. These characteristics of microsatellite markers thus render them appropriate for studies of population genetics, including those of aphids. Microsatellite sequences have been cloned and used successfully for genetic diversity studies in two aphid taxa *viz.* *Sitobion* spp (Sunnucks *et al.* 1996) and *A. gossypii* (Fuller *et al.* 1999). Although the technique shows promise for use in aphid population genetics, its widespread use is limited by the time and effort required to identify microsatellites and their flanking nucleotide sequences to design primers.

### 2.3.4 Random amplification of polymorphic DNA

#### 2.3.4.1 Description

Random amplification of polymorphic DNA (RAPD) uses a single, randomly constructed nucleotide sequence consisting of ten bases as a primer in a PCR reaction (Williams *et al.* 1990, Welsh & McClelland 1990). This primer can be designed without any knowledge of nucleotide sequence information for the organism being analysed. As only a single primer is used, amplification is constrained to occur only in regions of the genome where the primer sequence and its inverse are found between 0.1 and 3 kb apart (Haymer 1994, Loxdale & Lushai 1998). At less stringent conditions than is normally used in a PCR reaction (*viz.* short primer, low annealing temperature), the primer hybridises to and amplifies arbitrary regions of a genome. RAPD allows the simultaneous synthesis of multiple amplification products. Polymorphisms between the genomes of individuals can usually be detected when mutations influence the base sequence of primer binding sites or the length of the amplified region (Williams *et al.* 1990). The first phase of a study utilising RAPD is the determination of the conditions that allow amplification. Subsequent to this, primers are screened in order to select one or more sequences suitable both in terms of revealing polymorphisms and producing a few well-defined, reproducible bands (Loxdale *et al.* 1996).

#### 2.3.4.2 Advantages

RAPD has been used in a large number of studies as a result of a number of advantages. Relative to other markers, RAPD is technically less demanding, fast and requires no prior sequence information for primer design. It has been found to reveal high levels of polymorphisms and is therefore especially suited for differentiating between closely related individuals (Rafalski 1997). It was suggested that this technique is best used for clonal organisms because variation detected will be more likely to be related to clonal genotypes rather than sexual recombination (Loxdale & Lushai 1998). RAPD can therefore be a useful tool for studying the genetic structure of parthenogenetic aphids such as *E. lanigerum*.

#### 2.3.4.3 Disadvantages

In addition to a host of advantages, RAPD analysis suffers from several drawbacks. RAPD banding profiles show dominance in heterozygotes i.e. they cannot be used to distinguish between homozygotes and heterozygotes (Carlson *et al.* 1991, Hadrys *et al.* 1992). Additional techniques are therefore required for useful Mendelian data. It is also difficult to discern whether bands of a similar size are primed at the same locus. Therefore it is not possible to determine whether fragments between unrelated individuals are homologous without the application of additional techniques such as Southern blotting (Pillay & Kenny 1995, Rafalski 1997). A further cause for concern is that numerous factors can affect the reproducibility and standardisation of reactions e.g. DNA quality and quantity, magnesium concentrations, and primer

quality and concentration (Rafalski 1997, Loxdale & Lushai 1998). However, the lack of reproducibility with RAPD is usually solved when reaction conditions have been accurately determined (Rafalski 1997, Loxdale *et al.* 1996).

#### **2.3.4.4 Applications**

Since its introduction, RAPD has been used to reveal polymorphisms in a wide variety of insects. It has also been used to determine the genetic structure of a number of aphid species including: *S. graminum*, *D. noxia*, *A. pisum*, *Uroleucon ambrosiae* (Black *et al.* 1992), *Sitobion* spp. (Figueroa *et al.* 1999), *R. padi*, *A. gossypii*, *Myzus persicae* (Martinez-Torres *et al.* 1997a), *Metopolophium dirhodum* (Nicol *et al.* 1997a, Nicol *et al.* 1997b), *E. abietinum* (Nicol *et al.* 1997a; Nicol *et al.* 1998) and *T. trifolii* (Sunnucks *et al.* 1997). The success of these studies and the positive aspects of RAPD, despite its drawbacks, are the main reasons this technique was chosen to analyse the genetic structure of *E. lanigerum*.

### **2.3.5 Amplified fragment length polymorphism**

#### **2.3.5.1 Description**

The amplified fragment length polymorphism (AFLP) technique was first described by Vos *et al.* (1995). Using this technique, genomic DNA is restricted using a frequent and rare-site cutter e.g. the 6 base recognition restriction enzyme *EcoRI* and the 4 base recognition restriction enzyme *MseI*. These fragments are ligated to so-called adaptors, which are oligonucleotide sequences consisting of two parts, *viz.* an enzyme-specific sequence and a core sequence. Subsequent to ligation reactions, a PCR reaction is performed that is made stringent using a high annealing temperature. These non-selective reactions make use of primers consisting of an enzyme-adaptor specific sequence and a core sequence. A second, selective radioactive PCR is performed to limit the number of amplified fragments to a number suitable for resolution on high resolution polyacrylamide gels. Primers are used that are identical to those of the first, non-selective PCR but contain an additional selective extension consisting of 1 - 3 arbitrary nucleotides at the 3' side of the primer. Typically, detection of AFLP fragments requires radioisotope labelled primers during the second amplification. However, non-radioisotopic detection of AFLP bands can be based on the use of silver staining techniques (Chalhoub *et al.* 1997), chemiluminescence (Lin *et al.* 1999), fluorescent-labelled primers (Roman *et al.* 1999) or infrared dye-labelled primers (Myburg *et al.* 2001).

#### **2.3.5.2 Advantages**

The advantages of AFLP analysis are threefold. First, sets of restriction fragments may be analysed by PCR without knowledge of the nucleotide sequence of the genome being studied. Second, AFLP analysis allows co-amplification of high numbers of restriction fragments, usually between 50 and 100, which allows for greater resolution for the determination of genetic variation (Vos *et al.* 1995). AFLP analysis

also has the added advantage in that it is robust and fragments have a high level of reproducibility since stringent reaction conditions are used (Vos & Kuiper 1997).

### **2.3.5.3 Disadvantages**

Even though a small amount of marker development work is needed, the AFLP process can be time-consuming, laborious and technically demanding (Vos & Kuiper 1997). Also, depending on the species, DNA quality may be a limiting factor for AFLP analysis since high quality DNA is needed to ensure complete restriction enzyme digestion (Reineke *et al.* 1998). A further serious disadvantage of AFLP analysis is that special instrumentation and software is required to discriminate between homozygotes and heterozygotes. Using radioactive assays, AFLP fragments are generally scored as dominant marker systems unless the facilities are available for ascertaining co-dominance by band quantification (Geerlings *et al.* 1999). However, codominant allelic determination is more convenient using chemiluminescence, fluorescence or infrared-labelled primers but these AFLP detection methods require significant capital investment and proprietary technology and are still somewhat limited by inadequate software (Jansen *et al.* 2001, Myburg *et al.* 2001).

### **2.3.5.4 Applications**

AFLP analysis has so far found few applications in entomology since the technique is relatively novel and requires a level of optimisation for different insect taxa. In insect populations, AFLP analysis has been used to detect variation in gypsy moth *Lymantria dispar* populations (Reineke *et al.* 1999), analyse strain-specific differences in the fall armyworm *Spodoptera frugiperda* (McMichael & Prowell 1999), investigate the population genetics of the yellow fever mosquito *Aedes aegypti* (Yan *et al.* 1999) and determine species boundaries of crickets of the genus *Laupala* (Parsons & Shaw 2001). In other phyla, AFLP analysis has been found to produce excellent results, especially in parthenogenetic populations. For example, in plants, the technique has been used to determine the clonal genetic structure of dandelion populations *Taraxacum* spp. (Mes *et al.* 2002), dwarf bamboo *Sasa senanensis* (Suyama *et al.* 2000) and the evergreen shrub *Rhododendron ferrugineum* (Escaravage *et al.* 1998). The genetic structure of a parthenogenetic nematode *Meloidogyne* spp. was also determined successfully using AFLP analysis (Semblat *et al.* 1998). The excellent results obtained with the aforementioned parthenogenetic species using AFLP indicate that this technique may be useful for determining the genetic structure of anholocyclic aphid populations such as *E. lanigerum*.

Table 2.1: Overview of the genetic markers used for determination of aphid genetic structure (adapted from von Treuren 2000).

	<b>Abundance</b>	<b>Level of polymorphism</b>	<b>Locus specificity</b>	<b>Codominance of alleles</b>	<b>Reproducibility</b>	<b>Labour-intensity</b>	<b>Technical demands</b>	<b>Operational costs</b>	<b>Development costs</b>	<b>Quantity of DNA required</b>
<b>Allozymes</b>	Low	Low	Yes	Yes	High	Low	Low	Low	Low	N/A
<b>MtDNA (RFLP)</b>	High	Medium	Yes	Yes	High	High	High	High	Medium-high	High
<b>Microsatellites</b>	High	High	Yes	Yes	High	Low	Low-medium	Low-medium	High	Low
<b>RAPD</b>	High	Medium	No	No	Low	Low	Low	Low	Low	Low
<b>AFLP</b>	High	High	No	No	High	High	High	High	Low	Medium

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CHAPTER THREE

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**MATERIAL AND METHODS**

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### 3.1 APHID MATERIAL

#### 3.1.1 Sampling procedures

In order to assess the genetic structure of *E. lanigerum* populations on different spatial scales, a hierarchical sampling design was used. Sampling levels were regions, farms within regions, orchards within farms and trees within orchards. *E. lanigerum* individuals were sampled from the Western Cape from seven farms in Elgin, three in Ceres, two in Vyeboom and one in Villiersdorp (Fig. 3.1). Where possible, aphids were sampled from four or five orchards per farm and two trees per orchard. Two aphids were analysed per tree. Trees with varying combinations of cultivar and rootstock from orchards planted at a range of dates were sampled for aphid material. These variables are listed in Table 3.1.

Aphids were sampled only from the roots of apple trees. Infested roots were removed and transported to the laboratory in a cool bag. Only adult aphids that were free from parasitism by *Aphelinus mali* were used for analysis. Individual aphids were removed by hand and subsequently washed in 70 % ethanol in order to remove wax layers and surrounding debris. All aphids were stored at  $-20\text{ }^{\circ}\text{C}$  for up to a week before DNA isolation.

#### 3.1.2 Establishment of parthenogenetic lines

*E. lanigerum* parthenogenetic lines were established from ten individuals obtained from Oak Valley and eight from Molteno Middeldrug farm in Elgin. Aphids were cloned by placing a single adult aphid on the exposed root of an apple seedling on M793 rootstock. Trees were kept in a growth chamber at approximately  $20\text{ }^{\circ}\text{C}$  and a photoperiod of 12 hours. Contamination of clones from adjacent trees was prevented by placing masking tape painted with Plantex<sup>®</sup>, which traps crawlers, around the base of the tree trunk and around the tops of the plant pots. Individuals from each parthenogenetic line were removed at least one month after clone initiation and frozen at  $-20\text{ }^{\circ}\text{C}$  prior to DNA isolation.

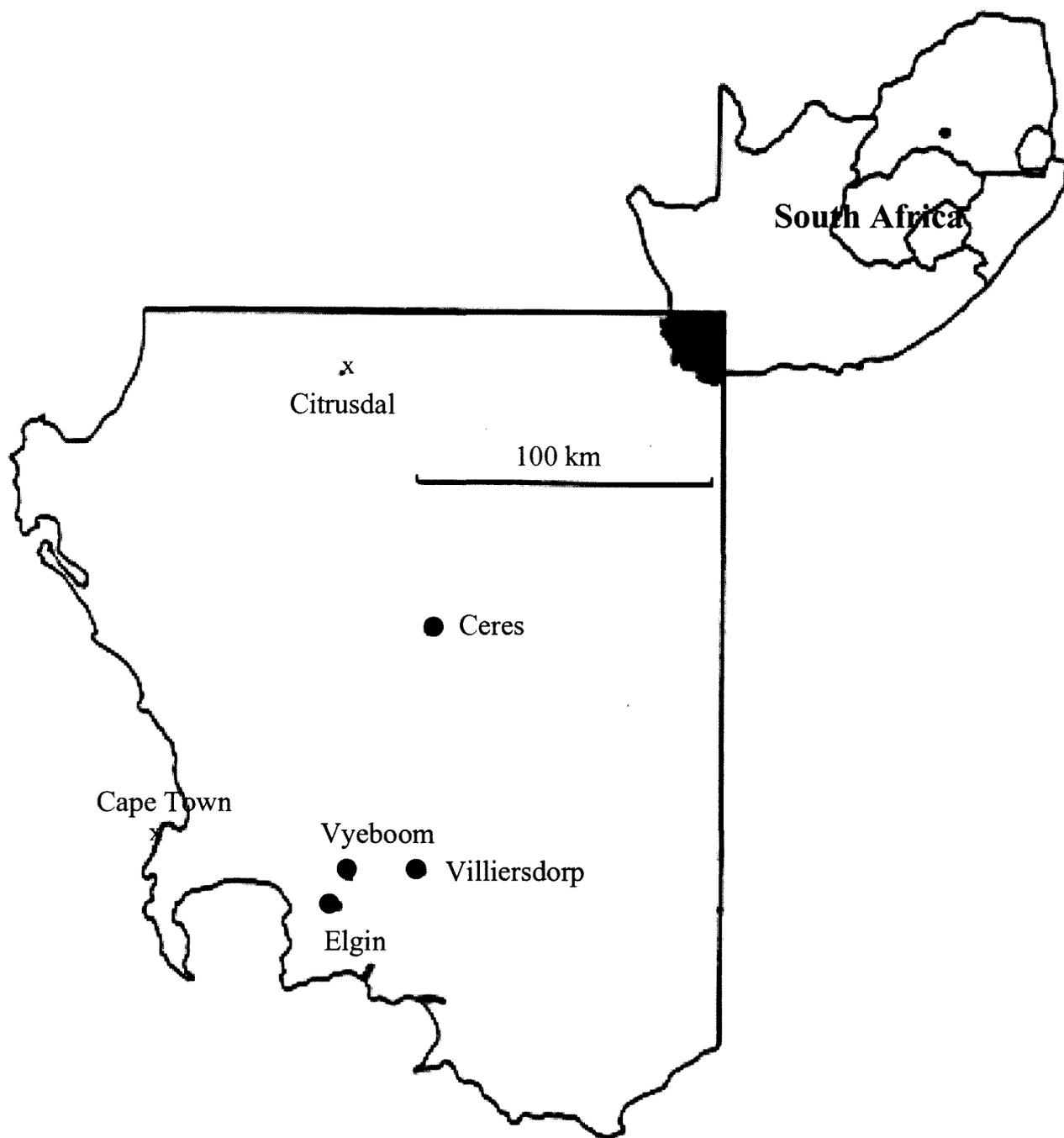


Fig. 3.1 Locality map of the Western Cape. Circles indicate location of *E. lanigerum* sample collection sites.

Table 3.1. Sampling variables for *E. lanigerum* collection.

Geographic location	Farm name	Geographic co-ordinates*	Orchard code	Host plant		Orchard planting date	Number of aphids
				Cultivar	Rootstock		
Elgin	Oak Valley	34.16S 19.05E	G7	Granny Smith	Seedling <sup>S</sup>	1978	10
	Molteno Middeldrug	34.18S 19.06E	1	Granny Smith, Starking	M793 <sup>R</sup>	1970	8
	Molteno Grogans	34.19S 19.04E	79A	Golden Delicious	M793 <sup>R</sup>	1989	4
			79C	Royal Gala	M793 <sup>R</sup>	1989	4
			80A	Granny Smith	M793 <sup>R</sup>	1975	4
			89A	Granny Smith	M793 <sup>R</sup>	1986	4
			Molteno Geelbos	34.15S 19.05E	33A	Granny Smith	M793 <sup>R</sup>
	33B	Granny Smith			M793 <sup>R</sup>	1970	4
	33C	Starking			M793 <sup>R</sup>	1970	4
	35B	Golden Delicious			M793 <sup>R</sup>	1971	4
	Jakkalsrivier	34.17S 19.09E			2B1	Royal Gala	M793 <sup>R</sup>
			2B2	Royal Gala	Seedling <sup>S</sup>	1987	4
			4B1	Golden Delicious	Seedling <sup>S</sup>	1988	4
			4B2	Golden Delicious	Seedling <sup>S</sup>	1992	4
			8B1	Royal Gala	Seedling <sup>S</sup>	1993	4

\* Geographic co-ordinates are given in decimal degrees

R = Formerly resistant rootstock; S = susceptible rootstock

Table 3.1. cont. Sampling variables for *E. lanigerum* collection.

Geographic location	Farm name	Geographic co-ordinates*	Orchard code	Host plant		Orchard planting date	Number of aphids
				Cultivar	Rootstock		
Elgin	Grabouw Farms	34.19S 19.03E	102	Golden Delicious	Seedling <sup>S</sup>	1956	4
			103	Granny Smith	Seedling <sup>S</sup>	1988	4
			112	Golden Delicious	Seedling <sup>S</sup>	1956	4
			115	Golden Delicious	Seedling <sup>S</sup>	1956	4
	Braeburn	34.24S 19.08E	5	Golden Delicious	Seedling <sup>S</sup>	1974	4
			9	Golden Delicious	Seedling <sup>S</sup>	1972	4
			14	Granny Smith	M109 <sup>S</sup>	1997	4
			15	Golden Delicious	Seedling <sup>S</sup>	1997	4
			16	Granny Smith	Seedling <sup>S</sup>	1997	4
Ceres	Lindeshof		V13	Granny Smith, Starking	M 793 <sup>R</sup>	1966	4
			V1	Golden Delicious, Granny Smith	Seedling <sup>S</sup>	1959	4
			A3	Golden Delicious, Starking	Seedling <sup>S</sup>	1971	4

\* Geographic co-ordinates are given in decimal degrees

R = Formerly resistant rootstock; S = susceptible rootstock

Table 3.1 cont. Sampling variables for *E. lanigerum* collection.

Geographic location	Farm name	Geographic co-ordinates*	Orchard code	Host plant		Orchard planting date	Number of aphids
				Cultivar	Rootstock		
Ceres	Lindeshof	33.05S 19.32E	P2A	Oregon Spur	Seedling <sup>S</sup>	1988	4
			A1	Starking, Granny Smith	M793 <sup>R</sup>	1962	4
	Nooitgedacht		K26	Starking	Seedling <sup>S</sup>	1969	4
			L33	Granny Smith	Seedling <sup>S</sup>	1932	4
			K25	Oregon Spur	Seedling <sup>S</sup>	1989	4
			SO3	Granny Smith	Seedling <sup>S</sup>	1960	4
			TO1	Starking	Seedling <sup>S</sup>	1955	4
			Langrivier	32	Granny Smith	Seedling <sup>S</sup>	1989
	33A			Granny Smith	Seedling <sup>S</sup>	1968	4
	34			Granny Smith	Seedling <sup>S</sup>	1980	4
	37			Granny Smith	Seedling <sup>S</sup>	1967	4
	39			Star Crimson	Seedling <sup>S</sup>	1976	4
	Villiersdorp		Theewaterskloof	34.00S 19.29E	4	Granny Smith, Golden Delicious	Seedling <sup>S</sup>

\* Geographic co-ordinates are given in decimal degrees

R = Formerly resistant rootstock; S = susceptible rootstock

Table 3.1. cont. Sampling variables for *E. lanigerum* collection.

Geographic location	Farm name	Geographic co-ordinates *	Orchard code	Host plant		Orchard planting date	Number of aphids
				Cultivar	Rootstock		
Vyeboom	Leccino	34.07S 19.09E	2	Golden Delicious, Starking	Seedling <sup>S</sup>	1976	4
			5	Golden Delicious, Early Red	Seedling <sup>S</sup>	1989	2
	Damar	34.07S 19.11E	4C	Golden Delicious	Seedling <sup>S</sup>	1985	2
			7	Top Red	Seedling <sup>S</sup>	1985	2
			9A1	Braeburn	M793 <sup>R</sup>	1988	4
			9A2	Pink Lady	M25 <sup>S</sup>	1988	2
			18	Braeburn	M793 <sup>R</sup>	1985	4
<b>Total</b>							<b>196</b>

\* Geographic co-ordinates are given in decimal degrees

R = Formerly resistant rootstock; S = susceptible rootstock

## 3.2 RAPD ANALYSIS

### 3.2.1 DNA isolation

Total genomic DNA was extracted from live adult *E. lanigerum* individuals. The salting-out extraction protocol described by Sunnucks *et al.* (1996) for the aphid *Sitobion avenae* was used. Briefly, this entailed the incubation of macerated individuals at 55 °C for a minimum of three hours in an extraction buffer consisting of 30 µl 20mM proteinase K and 300 µl of a 9:1 solution containing TEN buffer (10 mM Tris-HCl pH 8.0, 2 mM EDTA pH 8.0, 0.4 M NaCl) and 2 % (w/v) SDS. Subsequent to this, DNA was precipitated and washed three times with ethanol. DNA quality and quantity was examined by comparison with λ DNA standards (Promega) on 0.8 % (w/v) agarose gels (Seakem) to which ethidium bromide (1 µg/ml) was added for visualisation. Gels were electrophoresed at 60 V in 1 x TBE buffer (100 mM Tris, 100 mM boric acid, 2 mM EDTA, pH 8.3) and visualised under a Geldoc<sup>TM</sup> 1000 system (Biorad).

### 3.2.2 DNA Amplification

#### 3.2.2.1 Primer selection and reproducibility assays

In a preliminary analysis, DNA from the eighteen established *E. lanigerum* parthenogenetic lines was subjected to PCR amplification using 25 different random oligonucleotide primers (Operon Technologies Inc., Alameda, USA). All primers were examined for reproducibility using two different tests. Firstly, three individuals from the same clonal line were tested separately with the same primer at least three times. Secondly, primers were tested with DNA extractions performed on different occasions. Reactions were considered to be reproducible if the same banding profile was produced in all cases. Of the 25 primers examined, 10 primers were chosen for analysis based on these reproducibility tests as well as the information content obtained (Table 3.2).

#### 3.2.2.2 Reaction conditions

All RAPD amplifications were performed in a total volume of 10 µl. Each reaction contained approximately 10 ng DNA, 2 - 3 mM MgCl<sub>2</sub>, 1 µl 10X reaction buffer (Promega), 200 µM of each dNTP, 5 pmol primer and 1 unit of *Taq* polymerase (Promega). Thermal cycles were performed in the GeneAmp PCR Instrument System 9600 DNA thermocycler (Applied Biosystems). These cycles consisted of an initial denaturation step of 94 °C for 3 minutes, followed by 40 cycles consisting of 1 minute at 92 °C, 1.5 minutes at 35 °C and 2 minutes at 72 °C. A final extension period of 10 minutes at 72 °C completed the reaction.

Table 3.2. Primer sequences used for RAPD analysis.

Primer name	Primer sequence 5'-3'
OPA 1	CAG GCC CTT C
OPA 2	TGC CGA GCT G
OPA 3 *	AGT CAG CCA C
OPA 4	AAT CGG GCT G
OPA 6	GGT CCC TGA C
OPA 10	GTG ATC GCA G
OPA 13 *	CAG CAC CCA C
OPA 15	TTC CGA ACC A
OPA 18 *	AGG TGA CCG T
OPC 20 *	ACT TCG CCA C
OPK 1	CAT TCG AGC C
OPK 11 *	AAT GCC CCA G
OPK 17 *	CCC AGC TGT G
OPM 2	ACA ACG CCT C
OPM 5 *	GGG AAC GTG T
OPM 6	CTG GGC AAC T
OPM 8	TCT GTT CCC C
OPM 9	GTC TTG CGG A
OPM 10 *	TCT GGC GCA C
OPM 11	GTC CAC TGT G
OPM 13	GGT GGT CAA G
OPM 14	AGG GTC GTT C
OPM 15	GAC CTA CCA C
OPM 16 *	GTA ACC AGC C
OPM 18 *	CAC CAT CCG T

\* Primers selected for analysis

### 3.2.3 Gel electrophoresis

1.8 µl 6X loading dye (Promega) was added to 10 µl of the amplification product. Five µl of this mixture was loaded onto 1.8 % (w/v) agarose gels (Seakem), to which ethidium bromide (1 µg/ml) was added for visualisation of the fragments. Gels were electrophoresed at 60 V for three hours in 1 x TBE buffer (100

mM Tris, 100 mM boric acid, 2 mM EDTA, pH 8.3) and visualised under a Geldoc™ 1000 system. A 100 bp molecular weight ladder (Promega) was used for size determination of fragments.

### 3.3 AFLP ANALYSIS

#### 3.3.1 DNA isolation

Three DNA isolation protocols were evaluated for AFLP use *viz.* the high-salt extraction protocol used for RAPD analysis (Sunnucks *et al.* 1996), the CTAB protocol described by Reineke *et al.* (1998) and a modification of this protocol. The latter protocol was used in the main analysis: Individual adult aphids were macerated in 500 µl lysis buffer containing 0.1 M Tris (pH 8), 10 mM EDTA, 2 % SDS (w/v) and 0.2 mg/ml proteinase K. This reaction was incubated for one hour at 58 °C, after which 1/10 volume CTAB and 140 µl of a 5 mM NaCl solution were added. Incubation was then continued for ten minutes at 65 °C. DNA was extracted once with chloroform/isoamyl alcohol (24:1, v/v) and polysaccharides removed by the addition of 225 µl of a 5 M NH<sub>4</sub>Ac solution. DNA was precipitated using 0.25 volume 30 % (v/v) PEG. After centrifugation for 20 minutes at 4 °C, the precipitate was washed three times with 70 % ethanol and vacuum dried. Finally, the precipitate was resuspended in 20 µl TE buffer (10 mM Tris HCl, 0.1 mM Na<sub>2</sub>EDTA, pH 8.0). DNA concentration and quality was estimated by comparison with standard λ DNA concentrations (Promega) on 0.8 % (w/v) agarose gels (Seakem) electrophoresed at 60 V.

#### 3.3.2. DNA restriction and ligation of adaptors

In a preliminary analysis using the eighteen established *E. lanigerum* clonal lines, three different restriction enzymes were evaluated as rare cutters *viz.* *MluI* (Roche), *EcoRI* (Promega) and *SseI* (New England Biolabs). Based on the results from this analysis the restriction enzyme *MluI* was used in the main analysis. Genomic DNA (200 ng) from each aphid was digested with five units each of the restriction enzymes *MseI* (New England Biolabs) and *MluI* for three hours at 37 °C in One-Phor-All buffer (Pharmacia) and 0.1 µg/ul BSA in a final volume of 40 µl. An adaptor ligation mixture of 10 µl containing 5 pmoles of *MluI* adaptor, 50 pmoles of *MseI* adaptor (Table 3.3), 1 unit of T4 DNA ligase (Promega), 1 mM ATP (Roche) in One-Phor-All reaction buffer was added to each of the digestion reactions. This was incubated overnight at 37 °C. Subsequently, reactions were diluted 1:10 in 1 x TE buffer and stored at -20 °C prior to preselective amplification.

### 3.3.3 Preselective amplification

Thirteen  $\mu\text{l}$  of the template resulting from restriction enzyme digestion and adaptor ligation were added to a mixture consisting of 75 ng of each *MluI* and *MseI* preselective primer (Table 3.3), 200  $\mu\text{M}$  of all four dNTPs, 1 unit of *Taq* polymerase (Promega) and 1.5 mM  $\text{MgCl}_2$  in a final volume of 50  $\mu\text{l}$ . Amplification was performed in the GeneAmp PCR Instrument System 9600 DNA thermocycler (Applied Biosystems). The following cycle program was used: 5 minutes at 72 °C followed by 30 cycles with the cycle profile 30 seconds at 94 °C, 1 minute at 56 °C and 1 minute at 72 °C. The reaction was completed with a final extension period of 72 °C for 5 minutes and stored at 4 °C. Successful amplification was determined by agarose gel electrophoresis of 5  $\mu\text{l}$  of the preamplification product. The remaining 45  $\mu\text{l}$  of the amplification product was diluted tenfold in 1 x TE buffer and stored at -20 °C.

### 3.3.4 Primer labelling

*MluI* selective primers (Table 3.3) were end-labelled prior to amplification. This was performed using 5 ng *MluI* selective primer, 0.25  $\mu\text{Ci}$  [ $\gamma$ - $^{32}\text{P}$ ] ATP (Easytides, NEN) and 0.05 units T4 polynucleotide kinase (Promega) in One-Phor-All buffer in a total volume of 0.5  $\mu\text{l}$  per reaction. The reaction was incubated for two hours at 37 °C and terminated by keeping the mixture at 65 °C for 10 minutes.

### 3.3.5 Selective amplification

#### 3.3.5.1 Primer selection and reproducibility assays

Initially, 25 primer combinations (Table 3.3) were examined for selective amplification using the eighteen cloned *E. lanigerum* individuals. From this pilot study, five primer pairs that were identified as the most informative and reliable, based on band number and clarity, were chosen for analysis. Tests similar to those performed to evaluate the reproducibility of RAPD fragments were used to evaluate AFLP fragments with different selective primer pairs. In addition, reproducibility was confirmed using different adaptor ligation and preselective amplification reactions of at least three individuals and testing these for fragment reproducibility with the various selective primers.

#### 3.3.5.2 Reaction conditions

Selective amplification was carried out in a standard PCR reaction cocktail using 2.5  $\mu\text{l}$  diluted pre-amplified DNA, 200  $\mu\text{M}$  of each dNTP, 0.025 units *Taq* in storage buffer A (Promega), 1.5 mM  $\text{MgCl}_2$  and 15 ng *MseI* primer (Table 3.3) and 0.5  $\mu\text{l}$  of the labelling reaction in a final volume of 10  $\mu\text{l}$ . The cycle

profile was as follows: 30 seconds at 94 °C, 30 seconds at 65 °C, and 1 minute at 72 °C. The annealing temperature was reduced by 0.7 °C for the next 12 cycles and continued at 56 °C for 24 cycles.

### 3.3.6 Gel electrophoresis

Amplification products were mixed with 3 µl formamide dye (98 % formamide, 10 mM EDTA and 0.05 % each of bromophenol blue and xylene cyanol FF) and denatured at 90 °C for 5 minutes. Four µl of the mixture was loaded onto a 6 % (w/v) denaturing polyacrylamide gel (40 % acrylamide stock solution (Promega), 6M urea, 1 x TBE) and electrophoresed in 1 X TBE buffer (100 mM Tris, 100 mM boric acid, 10 mM EDTA, pH 8.3) at 80 W for 2 - 3 hours. Gels were dried on Whatmann 3 mm paper and exposed to Kodak Biomax X-ray films for up to ten days.

Table 3.3. Primer and adaptor sequences (5'-3') (Integrated DNA technologies) used for AFLP analysis.

Primer / primer pair	<i>Mlu</i> I Primer	<i>Mse</i> I Primer
<b>Adaptors</b>		
<i>Mlu</i> I adaptor	CTC GTA GAC TGC GTA AC CT GAC GCA TTG GCG C	
<i>Mse</i> I adaptor		GAC GAT GAG TCC TGA G TA CTC AGG ACT CAT
<b>Non-selective primers</b>		
<i>Mlu</i> I	GAC TGC GTA ACC GCG T	
<i>Mse</i> I		GAT GAG TCC TGA GTA A
<b>Selective primers</b>		
Primer pair 1 *	GAC TGC GTA ACC GCG T / <sup>1</sup> GC	GAT GAG TCC TGA GTA A / <sup>1</sup> CAT
Primer pair 2 *	GAC TGC GTA ACC GCG T / GC	GAT GAG TCC TGA GTA A / AG
Primer pair 3 *	GAC TGC GTA ACC GCG T / AA	GAT GAG TCC TGA GTA A / GC
Primer pair 4 *	GAC TGC GTA ACC GCG T / AA	GAT GAG TCC TGA GTA A / CA
Primer pair 5 *	GAC TGC GTA ACC GCG T / AA	GAT GAG TCC TGA GTA A / GG
Primer pair 6	GAC TGC GTA ACC GCG T / GC	GAT GAG TCC TGA GTA A / AA
Primer pair 7	GAC TGC GTA ACC GCG T / GC	GAT GAG TCC TGA GTA A / AC

\* Primers chosen for selective amplification for analysis of all *E. lanigerum* individuals

<sup>1</sup>Selective nucleotides

Table 3.3 cont. Primer and adaptor sequences (5'-3') used for AFLP analysis.

Primer pair 8	GAC TGC GTA ACC GCG T / <sup>1</sup> GC	GAT GAG TCC TGA GTA A / <sup>1</sup> AT
Primer pair 9	GAC TGC GTA ACC GCG T / GC	GAT GAG TCC TGA GTA A / CA
Primer pair 10	GAC TGC GTA ACC GCG T / GC	GAT GAG TCC TGA GTA A / GC
Primer pair 11	GAC TGC GTA ACC GCG T / GC	GAT GAG TCC TGA GTA A / GG
Primer pair 12	GAC TGC GTA ACC GCG T / GC	GAT GAG TCC TGA GTA A / GT
Primer pair 13	GAC TGC GTA ACC GCG T / GC	GAT GAG TCC TGA GTA A / TA
Primer pair 14	GAC TGC GTA ACC GCG T / GC	GAT GAG TCC TGA GTA A / CAA
Primer pair 15	GAC TGC GTA ACC GCG T / GC	GAT GAG TCC TGA GTA A / CAC
Primer pair 16	GAC TGC GTA ACC GCG T / AA	GAT GAG TCC TGA GTA A / AA
Primer pair 17	GAC TGC GTA ACC GCG T / AA	GAT GAG TCC TGA GTA A / AG
Primer pair 18	GAC TGC GTA ACC GCG T / AA	GAT GAG TCC TGA GTA A / AT
Primer pair 19	GAC TGC GTA ACC GCG T / AA	GAT GAG TCC TGA GTA A / CC
Primer pair 20	GAC TGC GTA ACC GCG T / AA	GAT GAG TCC TGA GTA A / GC
Primer pair 21	GAC TGC GTA ACC GCG T / AA	GAT GAG TCC TGA GTA A / TA
Primer pair 22	GAC TGC GTA ACC GCG T / AA	GAT GAG TCC TGA GTA A / TC
Primer pair 23	GAC TGC GTA ACC GCG T / AA	GAT GAG TCC TGA GTA A / TG
Primer pair 24	GAC TGC GTA ACC GCG T / AA	GAT GAG TCC TGA GTA A / CAA
Primer pair 25	GAC TGC GTA ACC GCG T / AA	GAT GAG TCC TGA GTA A / CAT

<sup>1</sup>Selective nucleotides

### 3.4 DATA ANALYSIS

#### 3.4.1 Data scoring

For dominant RAPD and AFLP markers, only two states were distinguished at each band position, *viz.* band presence or absence. Band presence represented a dominant homozygote (AA) or heterozygote (Aa) and absence denoted a recessive homozygote (aa). Fragments were scored by hand for band presence (1) or absence (0) to create binary matrices. Fragments of the same size in two individuals were assumed to represent homologous DNA sequences.

#### 3.4.2 Level of genetic variation

The percentage of polymorphic bands was used as an index of diversity, as was done, for example, by Semblat *et al.* (1998), Cardoso *et al.* (2000) and Parsons & Shaw (2001). To calculate this index, the total number of polymorphic bands was divided by the total number of bands assayed in the population. A

polymorphic band was defined as a band either present or absent in at least two individuals. Diversity indices were calculated for each of the four regions as well as for each individual farm.

### 3.4.3 Individual genetic distance matrices

Estimates of genetic distance were calculated from pairwise fingerprint comparison. The software GenAlEx, developed by Peakall & Smouse (2001) was used for all statistical analyses except where indicated otherwise. The calculation of genetic distances for the data set followed the method of Peakall *et al.* (1995) as:

$$E = n[1 - (2nxy/2n)],$$

where  $n$  is the total number of polymorphic bands and  $2nxy$  is the number of bands shared by two individuals. This is a true Euclidean metric as required for the subsequent analysis of molecular variance. Genetic distance matrices were calculated for RAPD and AFLP data sets as well as for each individual AFLP primer pair.

### 3.4.4. Distribution of genetic variation

The genetic distance matrix was used to perform a hierarchical analysis of molecular variance (AMOVA). The AMOVA procedure as calculated in GenAlEx following the methods of Excoffier *et al.* (1992) and Huff *et al.* (1993). AMOVA analysis was performed for the total population as well as for each of the four regions. Total genetic variation was partitioned into four levels: among regions, among farms within regions, among orchards within farms and among individuals. Variation was summarised as the percentage of the total variance present. Statistical significance was tested by random permutation, with the number of permutations set to 999.

### 3.4.5 Population structure

Cluster analysis was performed based on genetic similarity matrices using the unweighted pair group method algorithm (UPGMA) (Sokal & Sneath 1963). The software PAUP was used to construct a dendrogram. Midpoint rooting was used since no suitable outgroup was available. Bootstrap values of greater than 70 were considered to be significant.

### 3.5 REFERENCES

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**CHAPTER 4****RESULTS**

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**4.1 RANDOM AMPLIFICATION OF POLYMORPHIC DNA****4.1.1 DNA isolation**

The high-salt DNA extraction protocol yielded DNA of a high quality. No observable DNA degradation or impurities were visible on agarose gels. Each individual aphid yielded a total of 500 – 800 ng DNA.

**4.1.2 Reproducibility**

Fragments were tested for reproducibility using DNA extracted from individuals of the eighteen cloned *E. lanigerum* lines from Oak Valley and Molteno farms in Elgin. Since *E. lanigerum* reproduces by apomictic parthenogenesis, all offspring produced by an individual should be genetically identical. Each of the ten primers used was able to consistently produce bands identical both in size and intensity when used to amplify DNA from related individuals. Therefore, all RAPD fragments used in this study were reproducible.

**4.1.3 Level of genetic variation**

RAPD analysis of 196 *E. lanigerum* individuals with ten different primers provided a total of 47 fragments. The number of observed bands per primer varied from two to nine, with a mean of 4.7 bands. Only well amplified fragments ranging in size from 100 to 1000 bp were used for analysis. For space considerations, only the products amplified by primers OPK 17 (Fig. 4.1) and OPA 13 (Fig. 4.2) are shown.

RAPD banding profiles were similar for all *E. lanigerum* individuals throughout the four geographic regions sampled. Little variation in presence and intensity of bands was detected between aphids of any geographic origin. All aphids shared most of the bright, well-amplified bands. Polymorphic bands formed 8.5 % of the total number of bands. RAPD analysis therefore suggested that a low level of genetic variation was present among populations and individuals of *E. lanigerum*.

#### 4.1.4 Distribution of genetic variation

Analysis of molecular variance showed that all variation could be attributed to variation between individuals (AMOVA,  $P < 0.001$ ). There was no detectable variation as result of regional, farm or orchard differences.

### 4.2 AMPLIFIED FRAGMENT LENGTH POLYMORPHISM

#### 4.2.1 DNA isolation

All three DNA extraction protocols evaluated for AFLP analysis provided DNA of a high quality without observable impurities. However, poor fragment reproducibility was detected with aphid DNA obtained using the high salt DNA extraction protocol that was used for RAPD analysis. The best results were obtained using a modified CTAB DNA extraction protocol. Using this protocol, approximately 350 – 800 ng DNA was obtained per aphid.

#### 4.2.2 Reproducibility

Reproducibility of AFLP markers was evaluated using individuals from the eighteen parthenogenetic *E. lanigerum* lines that had been established. The same tests used to evaluate the reproducibility of RAPD markers were used for AFLP markers. Using the CTAB DNA extraction protocol, identical, reproducible banding patterns were produced for individuals from the same parthenogenetic line. The use of this DNA extraction protocol ensured that all AFLP fragments evaluated in this study were reproducible.

#### 4.2.3 Level of genetic variation

AFLP analysis of 192 *E. lanigerum* individuals from four localities in the Western Cape provided a total of 250 fragments using five primer pairs. AFLP fragment patterns were identical in most individuals examined with a high proportion of bands appearing as continuous lines across the gels. Of the 250 fragments analysed, 77 (30.8 %) were polymorphic. Many of these polymorphic bands were only encountered at a low frequency, with 56 (72.73 %) of the total polymorphic bands occurring at a frequency of less than 0.1. The remaining 21 (27.27 %) were found at a frequency of greater than 0.9. An example of the amplification products is shown in Fig. 4.3.

#### 4.2.3.1 Primer differences

The five primer pairs used detected an average of 50 fragments per primer pair. Primer pairs differed in their ability to detect polymorphisms within populations. The differences between the number of bands and the percentage polymorphic fragments detected by each primer pair are shown in Table 4.1.

Table 4.1. Percentage polymorphic bands detected by each of the five AFLP primer pairs for *E. lanigerum* populations from Elgin, Ceres, Vyeboom and Grabouw.

Primer pair	No. Bands	No. Polymorphic bands	% Polymorphic bands
1	20**	7**	35**
2	52**	16**	30.77**
3	45**	12**	26.67**
4	64**	18**	28.13**
5	69**	24**	34.78**

\*P < 0.001; \*\*P > 0.01

#### 4.2.3.2 Diversity indices

##### 4.2.3.2.1 Regional diversity indices

AFLP markers displayed variation within and among regions. These numbers of polymorphic bands were used as an index of genetic diversity to determine differences between aphid populations in Elgin, Ceres, Vyeboom and Villiersdorp (Table 4.2).

Table 4.2. Diversity indices for *E. lanigerum* populations from Elgin, Ceres, Vyeboom and Villiersdorp calculated using the proportion of polymorphic bands per region.

Region	No. Polymorphic bands	No. Bands assayed	% Polymorphic bands
Elgin	53	209	25.36
Ceres	35	211	16.59
Vyeboom	7	171	4.09
Villiersdorp	0	163	No variation

##### 4.2.3.2.2 Farm diversity indices

The number of polymorphic bands was also used to determine diversity indices per farm. These results are shown in Table 4.3.

Table 4.3. Diversity indices for *E. lanigerum* populations from farms in Elgin, Ceres, Vyeboom and Villiersdorp calculated using the proportion of polymorphic bands per farm.

Region	Farm	No. Polymorphic bands	No. Bands assayed	% Polymorphic bands
Elgin	Oak Valley	28	172	16.28
	Molteno	22	180	12.22
	Jakkalsrivier	2	173	1.16
	Grabouw Farms	12	171	7.02
	Geelbos	No variation	163	No variation
	Grogans	No variation	163	No variation
	Braeburn	No variation	163	No variation
Ceres	Lindeshof	11	178	6.18
	Nooitgedacht	28	200	14.00
	Langrivier	11	173	6.36
Vyeboom	Leccino	7	170	4.12
	Damar	7	170	4.12
Villiersdorp	Theewaterskloof	No variation	163	No variation

#### 4.2.4 Distribution of genetic variation

The results of analysis of molecular variance (AMOVA) for the total AFLP data set are summarised in Table 4.4. Equal amounts of genetic variation were attributed to variation among farms per region and individuals within farms. No regional variation was detected. However, there were differences between farms within regions and between individuals within farms. There were no differences between aphids collected from formerly resistant and susceptible rootstocks.

Table 4.4. Results of AMOVA for the AFLP data set showing the percentage of variation apportioned among regions, farms and individuals.

	Source of variation (%)	df	SS	MSS
<i>n</i> Bands	250			
Among regions	0**	3	23.163	7.721
Among farms / region	50*	9	212.998	23.666
Individuals within farms	50*	179	260.048	1.453

\*P < 0.001; \*\*P > 0.01

Molecular variance was also analysed for *E. lanigerum* populations in each of the four regions (Table 4.5). Elgin was the only region in which a significant amount of the variation (63 %) could be attributed to differences among farms. In Ceres, Vyeboom and Villiersdorp the major component of variation was as result of variation between individuals within orchards.

Table 4.5. Results of AMOVA for Elgin, Ceres, Vyeboom and Villiersdorp showing the percentage of variation apportioned among regions, farms and individuals.

	Elgin	Ceres	Vyeboom	Villiersdorp
<i>n</i> Samples	106	60	20	6
<i>Source of variation</i>				
Among farms	63*	7**	0**	No variation
Among orchards / farm	0**	13**	14**	No variation
Individuals within orchards	37*	80*	86*	No variation

\*P < 0.001; \*\*P > 0.01

#### 4.2.5 Population structure

Cluster analysis using UPGMA grouped the three populations (assuming a midpoint root) into 3 distinct clusters (Fig. 4.4). Seven individuals from Oak Valley farm formed a distinct cluster, with 100% bootstrap support. Within this cluster, one individual showed slight differences. Two individuals from Molteno Middelrug also formed a cluster more distant from the remaining populations, with 100% bootstrap support. All other samples from each of the four regions formed one large cluster. Within this cluster, five groups, with a bootstrap support of greater than 70, were found in which the individuals were more closely related. These were formed by the remaining Oak Valley and Molteno Middelrug samples, three individuals from Grabouw Farms (Elgin), two from Lindeshof (Ceres), two from Nooitgedacht (Ceres) and finally a group consisting of an individual each from Leccino and Damar (Vyeboom). Wherever a distinct cluster was formed the individuals comprising it were never obtained from the same tree.

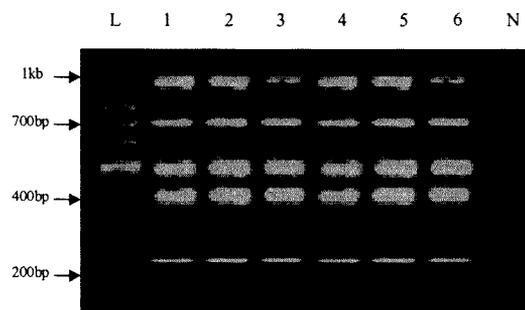


Fig 4.1. Example of RAPD profiles of *E. lanigerum* individuals to demonstrate the reproducibility of fragments generated using primer OPK 17. L = 100 bp ladder (Promega); lanes 1 - 3 = RAPD profiles from 3 different PCR reactions of progeny cloned from a single individual from Oak Valley; lanes 4 - 6 = RAPD profiles of progeny cloned from a single individual from Oak Valley using DNA extracted on 3 different occasions; N = negative control.

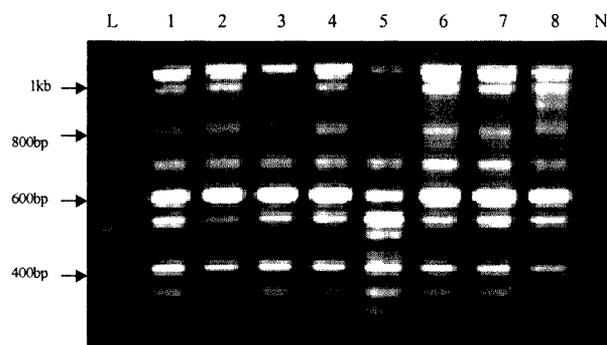


Fig. 4.2. Example of a RAPD profile of *E. lanigerum* individuals generated using primer OPA 13 showing one of the few samples in which variation was detected in between individuals (lane 5 = Molteno Middelrug). L = 100 bp ladder (Promega); lanes 1 - 4 = individuals from Oak Valley; lanes 5 - 8 = individuals from Molteno (Middelrug); N = negative control.

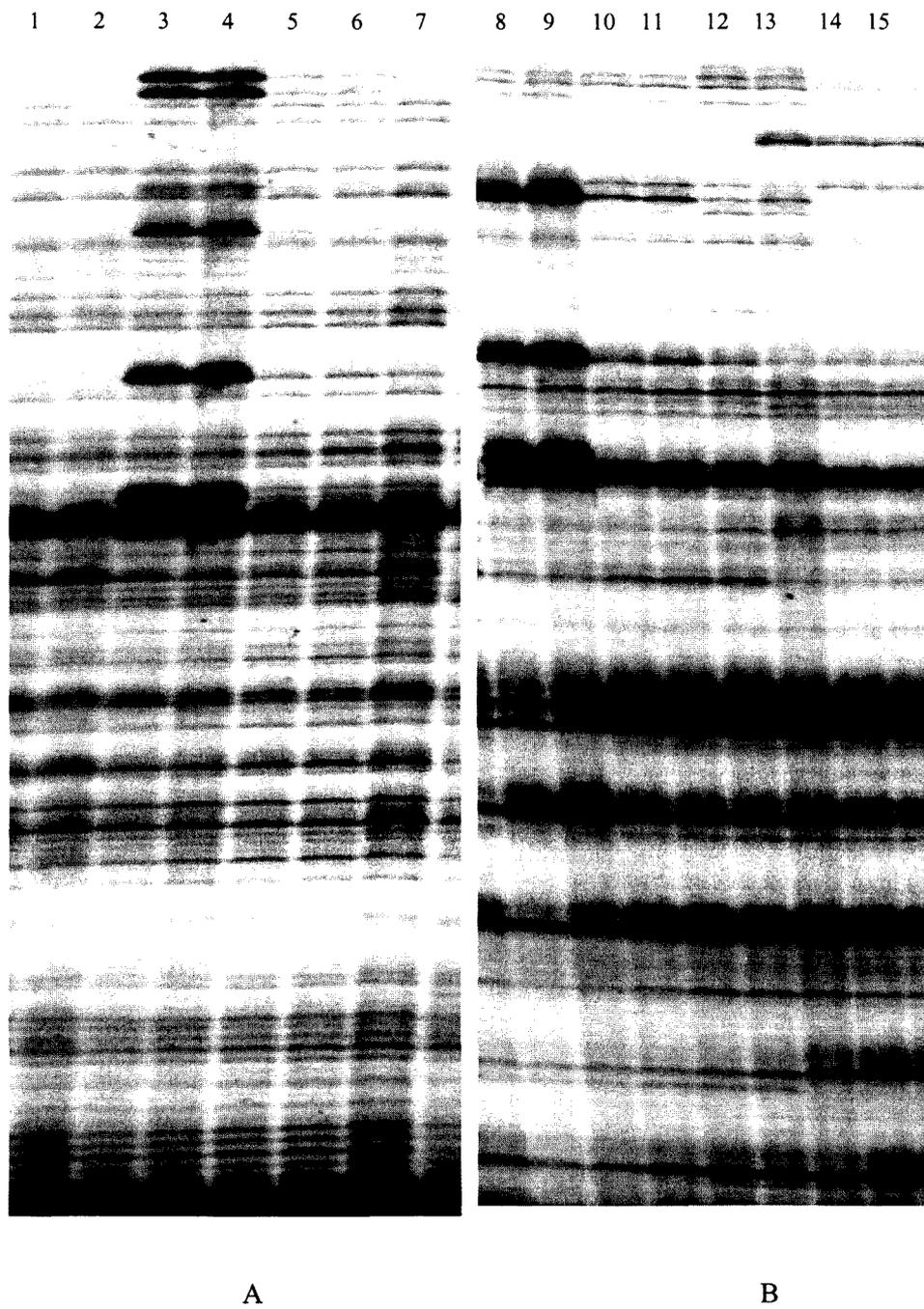


Fig. 4.4. Subset of AFLP products of *E. lanigerum* individuals generated using (A) primer pair 2 and (B) primer pair 4 (Table 3.3). Lanes 1, 2, 8 – 12 = individuals from Oak Valley; lanes 3 – 7, 13 – 15 = individuals from Molteno Middelrug.

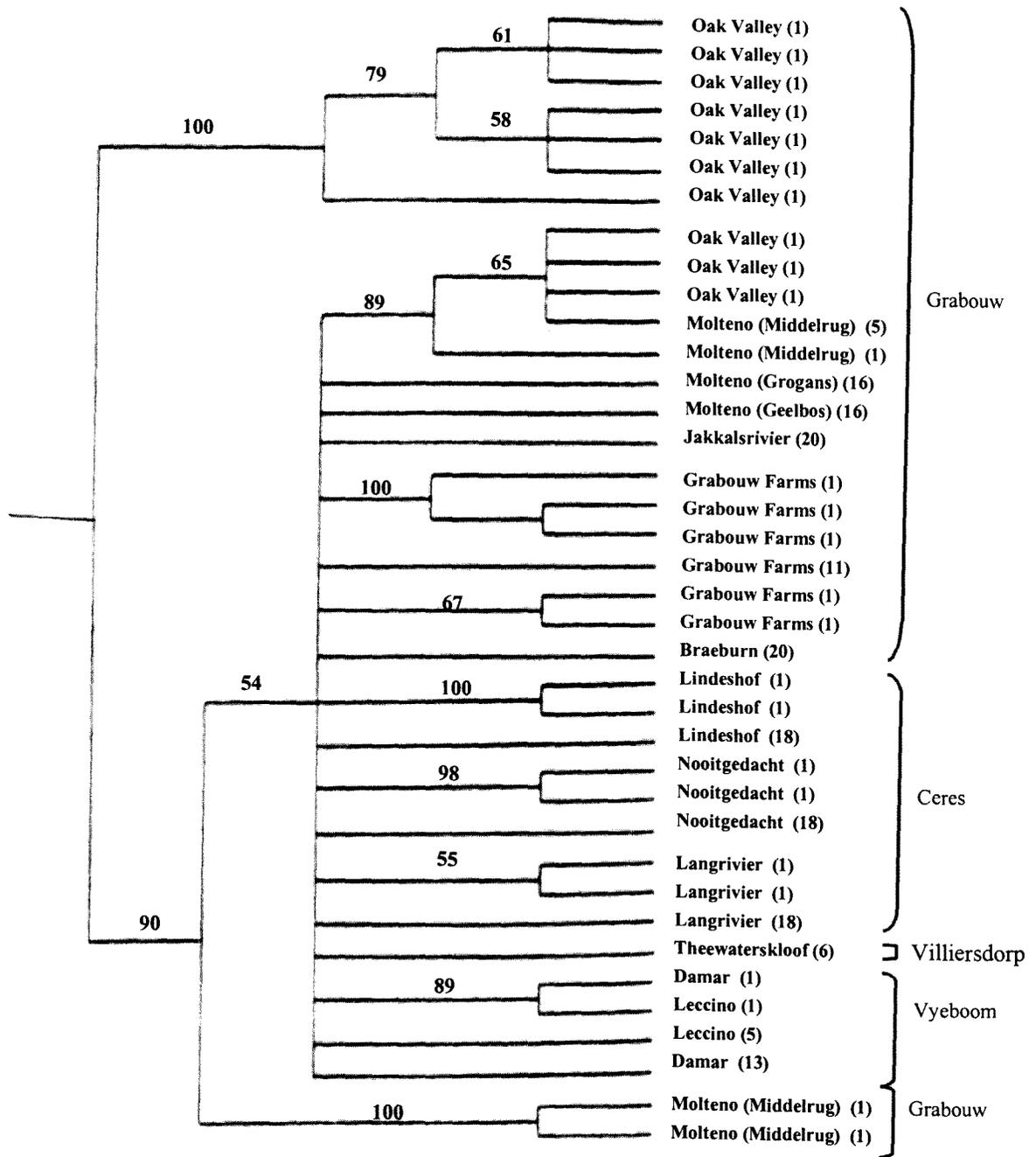


Fig. 4.3. UPGMA dendrogram with accompanying bootstrap values showing the relationships between 192 *E. lanigerum* populations sampled from 13 farms in the Western Cape. Numbers in parentheses indicate the number of individuals in each group.

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CHAPTER 5  
DISCUSSION

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### 5.1 METHODOLOGY

The methodology evaluated and optimised for the analysis of *E. lanigerum* genetic structure is of both practical and theoretical interest since this insect has not been characterized at molecular level prior to this study. For aphid research, the use of DNA fingerprinting techniques for the analysis of genetic structure has become an important tool. There is an ongoing investigation to determine which molecular techniques are better suited to assess the genetic structure of populations of different aphid species. The present study was conducted in part to evaluate and optimise RAPD and AFLP as methods of DNA fingerprinting for parthenogenetic *E. lanigerum* populations. RAPD has been applied successfully to other aphid species. Therefore its ability to characterize the genetic structure of *E. lanigerum* populations was assessed. AFLP has been used for the molecular characterisation of numerous plant species and a few insects, but has not been applied for the analysis of aphid populations. Therefore, assessing the use of AFLP for determining the genetic structure of *E. lanigerum* is of special importance not only for this species, but may also provide an indication of the success with which it may be applied to other aphid species.

#### 5.1.1 Random amplification of polymorphic DNA

##### 5.1.1.1 DNA isolation

The DNA obtained using a high-salt extraction protocol was of a good quality, which allowed well-amplified fragments to be obtained. The DNA quantity obtained per aphid ranged between 500 and 800 ng. Since RAPD analysis requires only 10 ng DNA per reaction, this quantity is sufficient for approximately 50 reactions. This large amount of RAPD reactions that can be performed using the DNA from a single individual is useful, since it allows numerous RAPD reactions to be performed, each with different primers. This can be used to increase the information content of a study.

##### 5.1.1.2 Reproducibility

A major problem associated with RAPD markers is poor reproducibility (Rafalski 1997, Loxdale & Lushai 1998). Reproducibility tests were performed on cloned individuals from Oak Valley and Molteno farms in Elgin. Since *E. lanigerum* reproduces by apomictic parthenogenesis, cloned individuals are supposed to be genetically identical and should therefore provide identical fingerprints when analysed with RAPD. All

RAPD primers used in this study were able to consistently amplify an identical set of bands in each individual from the same parthenogenetic line. Therefore a high level of fragment reproducibility was maintained in this study, which lends credibility to the results obtained.

#### **5.1.1.3 Characterisation of genetic structure**

RAPD analysis indicated that low levels of genetic diversity were present in *E. lanigerum* populations from four regions in the Western Cape. From the 47 RAPD fragments scored, only 8.5 % were found to be polymorphic. RAPD fingerprints showed that all aphids were identical with only minimal differences and that all variation was as a result of variation between individuals. No variation could be detected as a result of differences between regions, farms, orchards or trees. However, it is known that although RAPD can provide an accurate indication of genetic variation, the resolution of the technique for determining precise genetic structure is limited (Barker *et al.* 1999).

#### **5.1.1.4 Marker evaluation**

RAPD has proved extremely useful for identification of genetic polymorphisms in aphids (Black *et al.* 1992, Martinez-Torres *et al.* 1997, Nicol *et al.* 1997a, b, Sunnucks *et al.* 1997, Figueroa *et al.* 1999). It is a relatively quick, inexpensive means of obtaining an estimate of the amount of variation present in a population. To this end RAPD has been used successfully in this study of *E. lanigerum* by highlighting low levels of genetic variation. However, to increase the resolution of the results and obtain a clearer idea of how levels of diversity are distributed in *E. lanigerum*, aphids from the same sample used for RAPD analysis were subjected to AFLP analysis.

### **5.1.2 Amplified fragment length polymorphism**

#### **5.1.2.1 DNA isolation**

One of the technical difficulties that was encountered during AFLP analysis involved DNA quality and subsequent fragment reproducibility. For AFLP fragments to be reproducible, complete DNA digestion by restriction endonucleases is required (Reineke *et al.* 1998). An incomplete restriction digestion is a major technical problem, since even a small fraction of partially digested fragments may result in detectable bands after amplification. This will be interpreted as false polymorphisms. Partial DNA digestion may result from contamination of DNA with inhibitors such as negatively charged polysaccharides and phenols, which are known to occur in aphid DNA (Reineke *et al.* 1998, Sunnucks *et al.* 1996). These inhibitors could not be removed using the high-salt DNA extraction protocol that was used for RAPD analysis. However, using a modification of the CTAB DNA extraction protocol described by Reineke *et al.* (1998) DNA free from

inhibitors was produced, which resulted in a high level of reproducibility. This protocol produced approximately 350 - 800 ng of DNA per aphid, which ensured that the DNA from a single individual was sufficient for AFLP analysis. This avoided the need for laborious aphid cloning procedures. Therefore, the CTAB DNA extraction protocol is suited to AFLP analysis of *E. lanigerum*. This protocol may also be useful for AFLP analysis of other aphid species.

#### **5.1.2.2 Reproducibility**

One of the advantages of AFLP analysis is a high level of reproducibility (Vos & Kuiper 1997). This was also true for this study provided that a DNA extraction protocol was used that was able to remove inhibitors that could block restriction sites. All AFLP fragments generated from DNA using a CTAB extraction protocol were reproducible when subjected to stringent reproducibility assays.

#### **5.1.2.3 Genetic structure**

As expected, AFLP confirmed the results obtained using RAPD by indicating that low levels of variation were apparent in *E. lanigerum* populations in the Western Cape. However, unlike RAPD, AFLP was able to discriminate between different variance factors and clearly distinguished individuals from Oak Valley and Molteno farms from all other populations. A total of 250 AFLP fragments were produced using five primer combinations. Of these bands 30.8 % were polymorphic, which allowed for a more accurate discrimination of genetic structure than RAPD.

Five AFLP primer pairs were used to characterize the genetic structure of *E. lanigerum* populations. Each primer pair detected a different proportion of polymorphic bands and indicated different results regarding the distribution of variance (Table 4.1). However, these differences were not significant. This supported the results by indicating that screening populations with additional primer pairs would not have resulted in a greater degree of resolution. The 250 fragments screened were therefore sufficient for obtaining an accurate estimate of genetic variation.

#### **5.1.2.4 Marker evaluation**

AFLP, like RAPD, highlighted low levels of variation and an absence of geographic differentiation in *E. lanigerum* samples. However, AFLP allowed for finer discrimination of the population genetic structure than RAPD, with up to 30 times more bands detected per analysis. AFLP detected variation in *E. lanigerum* populations due to sources other than differences between individuals and clearly distinguished individuals from Oak Valley and Molteno Middelrug farms in Elgin from all other individuals. This was not the case when RAPD was used. Therefore the use of AFLP allowed for a greater degree of resolution for determining genetic structure. The superiority of AFLP over RAPD for determining genetic structure

corroborated the results of similar studies conducted to characterize the plant taxa *Lens* (Sharma *et al.* 1996), *Brassica* (Das *et al.* 1999) and *Salix* (Barker *et al.* 1999).

Numerous studies have confirmed that the AFLP technique is informative, reliable and reproducible (Vos *et al.* 1995, Vos & Kuiper 1997). AFLP holds a further advantage for use in aphid studies. A limiting factor for the use of certain molecular techniques for studies of aphids is that large amounts of DNA are required. This is often problematic since aphids are small. AFLP requires minimal amounts of DNA for analysis and the DNA from a single aphid is sufficient for an assay, as demonstrated in this study. This bypasses the need for cloning procedures, which are often labour intensive. However, a great disadvantage of AFLP is that fragments cannot be scored as codominant markers without the use of special instrumentation and heavy capital investment. Nonetheless, even when AFLP fragments are scored as dominant markers (as in this study), the technique compares favourably with other molecular markers that have been used to analyse aphid populations e.g. allozyme, mitochondrial DNA, microsatellite analysis and RAPD when the large information content of these markers is considered.

AFLP was originally developed for genetic mapping in plants (Vos *et al.* 1995) and has since been widely applied by plant researchers to measure genetic diversity. Recent molecular analyses of the moth *Lymantria dispar* (Reineke *et al.* 1999), the fall armyworm *Spodoptera frugiperda* (McMichael & Prowell 1999), the yellow fever mosquito *Aedes aegypti* (Yan *et al.* 1999) and crickets of the genus *Laupala* (Parsons & Shaw 2001) showed that this technique can also be applied successfully to insects. To our knowledge the present study was the first attempt to analyse the genome of an aphid species using AFLP. The results show that AFLP can be used successfully to determine the genetic structure of parthenogenetic populations of the aphid *E. lanigerum* even though low levels of genetic variation are apparent in this species locally. AFLP analysis therefore represents a powerful tool for detecting infraspecific variation in aphids and may be particularly useful for analysis of anholocyclic aphid populations in which low levels of genetic variation are often found.

## 5.2 GENETIC STRUCTURE

### 5.2.1 Level of genetic diversity

*E. lanigerum* populations were sampled from four different regions and collected from trees with different combinations of rootstock and cultivar. Therefore, since variation in geographic location and host plant differences are typically amongst the most important variables in aphid population genetics (Hales *et al.* 1997), it is likely that much of the genetic diversity of *E. lanigerum* was sampled in the Western Cape Province.

Genetic variation in *E. lanigerum* populations was minimal throughout the four regions sampled, as indicated by both RAPD and AFLP analyses. The proportion of polymorphic bands detected by both techniques was low. RAPD and AFLP detected a total of 8.5 % and 30.8 % polymorphic bands respectively. All polymorphic bands occurred at frequencies of either less than 0.1 or greater than 0.9. This indicated that polymorphic bands were either absent or present in one or a few individuals and not restricted to specific groups of aphids. RAPD analysis of other aphid species detected as many as 48 % polymorphic bands in the aphid *A. pisum* (Buornoville *et al.* 2000). However, using RAPD, the low level of variation detected in *E. lanigerum* populations was also found in populations of the aphids *R. padi*, *A. gossypi*, *M. persicae* (Martinez-Torres *et al.* 1997) and *E. abietinum* (Nicol *et al.* 1997a). Since there is no record of AFLP analysis of other aphid species, analogous comparisons cannot be made. However, analysis of other insects using AFLP detected a far greater proportion of polymorphic bands than that detected for *E. lanigerum*. For example, in crickets of the genus *Laupala* 43 % of the bands were polymorphic (Parsons & Shaw 2001) whereas in the moth *L. dispar* as many as 89 % of the bands were polymorphic (Reineke *et al.* 1999). In apomictic plant species similarly high levels of genetic variation were detected using AFLP (Escaravage *et al.* 1998, Mes *et al.* 2002, Suyama *et al.* 2000). The results of these studies confirm that an impoverished level of genetic variation is found in *E. lanigerum* populations in the Western Cape Province.

## 5.2.2 Distribution of genetic variation

Despite the decreased variation found in *E. lanigerum* populations, AFLP allowed for the discrimination of variance factors based on diversity indices (proportion polymorphic bands), genetic variation using AMOVA and population structure based on UPGMA analysis.

### 5.2.2.1 Variation between regions

*E. lanigerum* populations analysed showed no apparent differences as result of geographic isolation. Individuals from Elgin, Grabouw, Vyeboom and Villiersdorp displayed similar fingerprints created using both RAPD and AFLP analyses. No particular genotype was found that was peculiar to a region, with the possible exception of individuals from Oak Valley and Molteno farms in Elgin. AMOVA analysis based on AFLP data (Table 4.5) indicated that all variation could be attributed to variation between farms and individuals within farms, rather than differences between regions. This pattern was also apparent from cluster analysis (Fig. 4.4), where individuals from all four regions formed a single cluster. Therefore, greater variation in *E. lanigerum* populations was apparent on relatively small geographic scales. This pattern of distribution of variance on small scales was also described for the aphids *S. graminum* (Shufran *et al.* 1991, Shufran & Wilde 1994) and *A. pisum* (Barrette *et al.* 1994, Fukatshu & Ishikawa 1994, Birkle & Douglas 1999). Thus, the distribution of genetic variation in *E. lanigerum* populations in the Western Cape

Province appear to follow a typical pattern for that of aphids, with most of the genetic variation being present over small geographic areas.

#### **5.2.2.2 Variation within regions**

##### **Elgin**

Elgin was the only region in which populations showed significant proportions of variation as result of differences between farms (63 %) (Table 4.5) and less as result of variation between individuals (37 %). *E. lanigerum* populations from Elgin were the most diverse populations from the four regions analysed. The diversity index based on the percentage of polymorphic bands was 25.36 (Table 4.2), which was significantly higher than those of Ceres, Vyeboom and Villiersdorp.

##### **Ceres**

Populations from Ceres were the second most diverse of the four regions from which samples were taken, with a diversity index of 16.59 (Table 4.2). From the AMOVA analysis, a large proportion of the variation (80 %) (Table 4.5) could be attributed to variation between individuals in this region.

##### **Vyeboom**

Vyeboom *E. lanigerum* populations had a diversity index of 4.09 (Table 4.2), which was lower than indices from both Elgin and Ceres. According to AMOVA analysis (Table 4.5) it could be deduced that all variation in aphid populations from this region could be attributed to differences among individuals within farms.

##### **Villiersdorp**

Villiersdorp populations showed no variation, which was most likely due to the small sample size, and will therefore not be further discussed.

#### **5.2.2.3 Variation within farms**

AMOVA analysis for the combined data set from the four regions indicated that 50 % (Table 4.4) of the variation was due to differences between farms. However, this value differed between regions.

##### **Elgin**

Variation within farms in Elgin was also apparent from AMOVA analysis, which indicated that *E. lanigerum* populations from this region were the only populations where a significant proportion of the

variation (63 %) (Table 4.5) resulted from differences between farms. This pattern was also apparent from UPGMA analysis (Fig. 4.4), where Oak Valley and Molteno Middelrug farms in Elgin formed clusters distinct from other populations. Diversity indices based on the proportion of polymorphic bands showed significantly different results for populations from different farms in Elgin (Table 4.3). These indices form amongst the highest and lowest diversity values for *E. lanigerum* populations from all farms. Populations from Oak Valley and Molteno Middelrug have amongst the highest diversity indices of populations of all farms analysed *viz.* 16.28 and 12.22 respectively. Other farms in Elgin had much lower levels of diversity. Jakkalsrivier and Grabouw Farms showed low levels of variation, with diversity indexes of 1.16 and 7.02 respectively. Populations from Geelbos, Grogans and Braeburn were invariant.

### **Ceres**

Only 7 % of the variation in *E. lanigerum* populations from Ceres could be attributed to differences between farms, as revealed by AMOVA analysis (Table 4.5). Aphid populations were sampled from three farms in Ceres and these populations all showed intermediate to high levels of diversity. Aphids from Nooitgedacht were the most genetically diverse in Ceres and the second most diverse of all in this study, with a diversity index of 14.00 (Table 4.3). Langrivier and Lindeshof farms had intermediate levels of diversity, with diversity indices of 6.18 and 6.36 respectively. Cluster analysis showed that two individuals each from Lindeshof and Nooitgedacht were more closely related than other individuals.

### **Vyeboom**

AMOVA analysis indicated that there was no difference between the two *E. lanigerum* populations from Leccino and Damar farms in Vyeboom. Both farms had intermediate diversity indices of 4.12 (Table 4.3). Cluster analysis grouped one individual from each farm within the main cluster (Fig. 4.4).

#### **5.2.2.4 Variation within orchards**

Only *E. lanigerum* populations from Ceres and Vyeboom showed any degree of variation within orchards, but according to AMOVA analysis these levels are not significant (Table 4.5). There were no differences in aphids collected from orchards planted with formerly resistant or susceptible rootstocks. Orchard differences (including differences between rootstocks) were therefore negligible.

#### **5.2.2.5 Variation within trees**

Two aphids from every tree were analysed. It may be important to note that wherever cluster analysis grouped individuals, these never consisted of aphids sampled from the same tree. This indicated that there was a degree of variation to be found between aphids on the same tree.

### 5.3 INFERENCES FROM GENETIC STRUCTURE

Characterisation of the genetic structure of a population enables inferences to be made regarding the role of various factors responsible for its nature. This study has established that levels of genetic variation of *E. lanigerum* populations in the Western Cape were low and that, like many other aphid species, most of the variation occurred over small spatial scales. These facts can be used to gain insight into the history and biology of the population and make recommendations for its control.

#### 5.3.1 Distribution of genetic variation

*E. lanigerum* populations in the Western Cape show no differentiation as result of regional isolation. Therefore, most of the variation in *E. lanigerum* populations can be found on relatively small spatial scales. Most of the variation in *E. lanigerum* populations could be equally attributed to differences between farms or individuals (Tables 4.4 and 4.5) whereas orchard differences including rootstock differences (within the same farms) were negligible. Therefore, between farm differences may be more important than within farm differences. These factors may include management decisions such as the use of particular agricultural practices or systems of control as well as local rather than regional biotic factors. Aphid variation due to differences between farms was greater in Elgin than in the other regions. This was not surprising since more farms were sampled from this region than in any other region. Greater differences in local biotic conditions and management practices may have been apparent in Elgin since samples were taken over a wider geographic area.

At Oak Valley and Molteno farms in Elgin, a proportion of the individuals displayed genotypes distinct from those found in other populations. Since only certain individuals were found with distinct genotypes, variables such as rootstock and climate, which are the same throughout both farms, could not have influenced genotype development. However, it is possible that different selection factors may have acted on only certain individuals. These selection factors may include the presence of pathogenic fungi which are known to occur locally on these farms (Damavandian 1999). It is also possible that the factors that generate genetic diversity such as mutation, chromosome rearrangement and mitotic recombination occur at a higher rate in these isolated groups of individuals. Therefore, it may be possible that in future these may react differently to selection pressures such as resistant rootstocks or pesticides. However, it is probable that these groups of individuals are too few and too isolated for this to have a significant impact on the entire *E. lanigerum* population.

### 5.3.2 Level of genetic variation

#### 5.3.2.1 Parthenogenesis

*E. lanigerum* in the Western Cape reproduces exclusively by apomictic parthenogenesis. Therefore, all genetic variation in this population is generated by mutation, chromosome rearrangement or mitotic recombination rather than meiotic (sexual) recombination. These limited means of increasing diversity may have had an important influence on the genetic structure of the woolly apple aphid populations in this region.

Parthenogenesis may limit the accumulation of genetic variability in certain populations and thereby reduce their adaptive potential (Blackman 1981). However, in the case of *E. lanigerum* this is unlikely. The pest shows significant adaptive potential in the Western Cape since it was able to flourish here since its introduction. It is more likely that parthenogenesis allowed the conservation of the fittest *E. lanigerum* genotype for the environment in which it was found. It is known that once aphid clones in an agricultural system have the genetic constitution appropriate to the environmental conditions, they proliferate and little variation is needed to cope with the changes from year to year (Hales *et al.* 1997). Generally, selection in aphids by the environment acts largely at the level of the whole genome and favours those clones with the most broadly adapted genotypes (Wilson *et al.* 1999). Since no distinct *E. lanigerum* genotypes were found in this study even though this pest has established itself successfully, clonal selection may have ensured the maintenance of the most broadly adapted genotype for the environment. The single clone found in the Western Cape might be broadly adapted to conditions throughout the four regions from which aphids were sampled.

#### 5.3.2.2 Founder effects and dissemination

*E. lanigerum* populations in the Western Cape most likely share a common centre of origin, which is illustrated by the fact that populations that are very similar at the genome level were found in widely separated areas. Founder effects are fairly common in aphid populations due to their high rate of increase as result of increased fecundity and overlapping generations (Fuller *et al.* 1999). It is possible that these effects may also have played a role in shaping the genetic structure of *E. lanigerum* populations in the Western Cape.

##### 5.3.2.2.1 Initial introduction into the Western Cape

*E. lanigerum* was introduced into the Western Cape from the USA on infested plant material and the earliest record of *E. lanigerum* in the Western Cape was documented approximately 150 years ago (Myburgh *et al.* 1973). This evolutionarily short time span may not have allowed sufficient opportunity for the insect to

diversify. Also, the present lack of diversity might be the result of introduction of only a few genotypes with limited genetic variation. Since *E. lanigerum* reproduces only by apomictic parthenogenesis in South Africa, the introduced individuals could have rapidly accumulated offspring with identical genetic material barring mutations, chromosomal rearrangements or mitotic recombination. This would have maintained impoverished levels of genetic diversity.

#### **5.3.2.2.1 Dissemination in the Western Cape**

It appears as if the origin of the *E. lanigerum* infestation in the Western Cape lies in Elgin. It has been established that the origin of a population generally lies in the area where the greatest level of diversity occurs. Since populations from Elgin are the most diverse, it is most likely the founding population of the Western Cape *E. lanigerum* infestation.

It is probable that *E. lanigerum* spread from Elgin to other apple-growing regions in the Western Cape on a limited source of infested nursery material. Prior to 1975, only three or four apple nurseries were in existence in the Western Cape from which planting material could be obtained (pers. comm. D.K. Strydom, D. Cunningham). In 1975, the South African Plant Improvement Organization (SAPO) was formed with the aim of making virus-free planting material available to the South African deciduous fruit industry (van Niekerk 1975). Plant material from two nurseries in Elgin were used to establish SAPO mother blocks (pers. comm. D.K. Strydom, D. Cunningham). Subsequent to the establishment of SAPO, a nursery certification scheme was laid down by the Plant Improvement Act (Act No. 56 of 1976), which stated that all nurseries qualified under the state certification scheme were obliged to purchase plant material exclusively from SAPO (Anon. 1976). The first state-certified supergrade propagation material was released by SAPO in 1976. Therefore, from 1976 onwards, all planting material used to establish new orchards originated from SAPO and in turn this material could be traced to two nurseries in Elgin. It is therefore possible that one or a few *E. lanigerum* strains could have spread throughout the Western Cape from these limited plant material distribution points. The only other means by which the insect could have spread from Elgin was by wind or crawler movement. However, since large geographic distances and mountain barriers separate the four regions, this is unlikely.

Future efforts can be directed toward a comparison of genetic diversity in the Western Cape and North America, from where the insect originated, to confirm whether the lack of genetic variation in the Western Cape has arisen from founder effects or if this species as a whole (including holocyclic populations) shows decreased levels of diversity.

### 5.3.2.3 Selective factors

The utilisation of cultivars of recent origin by *E. lanigerum* and their heavy exposure to pesticides could have selected for particular genotypes and in turn caused population bottlenecks. This would account for the impoverished diversity found in populations in the Western Cape.

#### 5.3.2.3.1 Host plant selection

Host plant–aphid interactions are important selective factors in the biology of *E. lanigerum*, as evidenced by the fact that three of the four instances of reported biotype development occurred as a result of exposure to resistant plant varieties. The use of Northern Spy and related rootstocks in the Western Cape selected for a strain of *E. lanigerum* that was able to overcome resistance (Giliomee *et al.* 1968). This selective factor may have resulted in decreased levels of genetic variation. However, the use of resistant rootstocks alone could not have selected for a single genotype as susceptible rootstocks were still in use when resistant rootstocks were introduced. Also, this study confirmed that there were no differences in aphids collected from formerly resistant and susceptible rootstocks. Selection for a single genotype could only have occurred if the resistance-breaking biotype was given a selective advantage on both resistant and susceptible rootstocks. However, this is unlikely since Service & Lenksi (1982) pointed out that no aphid clone is most fit on all host phenotypes.

It is possible that the resistance breaking biotype spread from Elgin to Ceres, Vyeboom and Villiersdorp, where *E. lanigerum* occurs on both susceptible and formerly resistant rootstocks. If this is so, it may be significant to note that most of the orchards from which samples were obtained in Ceres, Vyeboom and Villiersdorp were planted after 1968, which was when biotype development due to host selection was reported in Elgin (Giliomee *et al.* 1968). If the resistance-breaking biotype was present on the nursery material of the four nurseries present in Elgin (including those nurseries from which SAPO mother blocks were established) it could have spread throughout the Western Cape in this manner.

#### 5.3.2.3.2 Pesticide selection

Throughout its history in the Western Cape *E. lanigerum* has been exposed to a wide variety of pesticides, both for the control of this pest and the control of other pests in the same agricultural system. Any one or a combination of these chemicals could have selected for a particular genotype, which would have resulted in a population bottleneck and subsequently reduced genetic variation. A pesticide resistant *E. lanigerum* biotype has been described in the Western Cape based on resistance to the chemical vamidothion (Pringle *et al.* 1994). However, vamidothion does not have an effect on underground woolly apple aphid populations. The insecticide imidacloprid is the first registered chemical known to affect underground *E. lanigerum* populations. However, this chemical was not used on at all the farms from which aphids were sampled (e.g.

Molteno Middelrug farm). Therefore it is unlikely that this chemical alone selected for a specific genotype across the Western Cape.

### **5.3.3 Implications of decreased genetic diversity**

This study has shown that *E. lanigerum* populations in the Western Cape Province generally possess low levels of genetic variation, which has certain important implications. Species with low levels of genetic variability generally lack the ability to adapt to changes in either the biotic or abiotic environment since they possess only one or a few genotypes on which natural selection can act (Armstrong & Wratten 1996). In populations of many different taxa, low levels of genetic diversity have been known to adversely affect their persistence (Armstrong & Wratten 1996, Nicol *et al.* 1997a). Decreased genetic variation affects the adaptability and viability of populations by altering characteristics such as growth rate, population size or extinction probability (Lande 1994, Dudash & Fenster 2000, Sherwin & Moritz 2000). This occurrence may also be associated with increased susceptibility to pathogens and pests (Frankham 1995). Low levels of genetic variation may have important implications in a pest management system.

### **5.3.4 Genetic structure and pest management**

The low level of genetic variation found in *E. lanigerum* populations in the Western Cape Province has implications for both the development of control strategies and prospect for successful future control.

#### ***5.3.4.1 Development of control strategies***

The development of control strategies against *E. lanigerum* may be assisted by knowledge of the impoverished levels of genetic variation that were found as well as the distribution of this variation. This information is of special use to researchers conducting experiments to evaluate control practices against *E. lanigerum*, for example pesticide field trials. These experiments require evaluation of these practices with a sample population of *E. lanigerum* that is representative of the total genetic diversity of the pest population present in the Western Cape Province. If such a genetically representative sample is not used, geographic differences or narrow genetic variability within the pest population will give false results during experimental trials and could lead to poor control in the field (Shufran *et al.* 2000). To avoid this, control practices should be exposed to as much of the diversity of the pest as possible to obtain meaningful results. This study confirmed that the genetic structure of *E. lanigerum* is such that collecting individuals from additional regions is an ineffective method of increasing the diversity of aphid samples. Samples should rather be taken from additional farms and trees within farms to get a more representative sample of *E. lanigerum*.

Furthermore, the results obtained may be of particular value to breeders developing host plant resistance to *E. lanigerum*. When evaluating field apple varieties, plant breeders should not be unduly concerned with the genetic diversity of Western Cape *E. lanigerum* samples. They can expect their plant entries to be exposed to most of the genetic diversity in *E. lanigerum* populations regardless of location since geographic differentiation is lacking in these populations in the Western Cape Province.

#### **5.3.4.2 Prospect for future control**

Reduced genetic variation can severely decrease the ability of a population to adapt to control strategies (Armstrong & Wratten 1996). In these populations biotype development, which can disrupt control practices, is rare (Shufran *et al.* 2000). It may therefore be likely that the durability of certain control methods can be maintained to a larger extent in *E. lanigerum* populations in the Western Cape Province. The possibility for the success of future control programs in this area is therefore encouraging.

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CHAPTER 6  
CONCLUSION

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The characterization of the genetic structure of an insect pest may prove invaluable for understanding its biology and assisting its control in an integrated system. This study was conducted to apply these ecological principles to *E. lanigerum* populations in the Western Cape. The objectives were threefold, all of which were met successfully.

The first objective was to evaluate and optimise means of determining the genetic structure of *E. lanigerum*. Two molecular techniques were evaluated for this purpose *viz.* random amplification of polymorphic DNA (RAPD) and amplified fragment length polymorphism (AFLP). Both techniques were applied with success, without major technical problems. Similar levels of genetic structure were detected using both marker systems. However, AFLP analysis allowed for finer discrimination of the population structure. These results are important since the technique has not been applied before to studies of aphids. AFLP analysis provided a high number of fragments per assay, making them suitable for discriminating population structure in species where genetic variation was potentially low such as those of parthenogenetic aphid populations. Furthermore, AFLP is reliable and robust, can be applied to any species. The technique overcomes problems associated with development time, information content and reproducibility that can plague other marker systems. This technique is therefore recommended for future studies of *E. lanigerum* as well as for those of other aphids.

The second objective of this study was to characterize the genetic structure of root-infesting *E. lanigerum* populations in the Western Cape. Results revealed that aphid populations from four regions in the Western Cape generally possessed low levels of genetic diversity. Analysis of the spatial distribution of genetic variation showed that most of the variation was on a small scale. Most of the variation was due to differences between farms and individuals within farms rather than regional differences. Individuals from two farms in Elgin, *viz.* Oak Valley and Molteno Middelrug, showed levels of variation significantly higher than those from other farms and formed distinct groups when analysed by cluster analysis.

The final objective was to make inferences regarding the genetic structure of *E. lanigerum* in order to gain insight into its biology and the possibility for control. Analysis of the spatial distribution of variation revealed that individuals were similar throughout the four regions, which indicated that they most likely shared a recent common ancestor. The low amounts of genetic variation found in *E. lanigerum* populations could also be due to the exclusive occurrence of parthenogenesis as a reproductive mechanism as well as selective factors such as the use of resistant rootstocks and pesticides. The possibility for future control of

*E. lanigerum* seems encouraging since this insect does not possess sufficiently high levels of variation to adapt to changes in the environment.

The results from this study are of both practical and theoretical importance. They indicate that the application of ecological principles and in particular the characterisation of genetic structure is a powerful approach to understand fundamental aspects of *E. lanigerum* biology and control.