Population genetics of *Phragmidium violaceum*

Don R. Gomez
B. Ag. Sc. (Hons), The University of Adelaide

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I dedicate this thesis to my parents,
Rosalio and Rosalie for their untiring love and support

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Abstract

European blackberry (*Rubus fruticosus* agg.) is a genetically diverse 'Weed of National Significance' in Australia. The blackberry rust fungus, *Phragmidium violaceum* (Uredinales), has been present in Australia since 1984. Strains of *P. violaceum* were released for biological control of European blackberry in 1991 (strain F15) and 2004 (eight additional strains). DNA markers for 'selective amplification of microsatellite polymorphic loci' (SAMPL) were developed and applied for investigating the population structure, mating system and gene flow of *P. violaceum* in Australia. Variation among strains of *P. violaceum* for infection efficiency as a function of leaf age was also investigated.

Genetic diversity in *P. violaceum* was examined among isolates from Europe (n=19), the likely centre of diversity of this pathogen, and among isolates from Australia (n=18) and New Zealand (n=7). Amplified fragment length polymorphisms (AFLPs) revealed few to no polymorphisms among the 44 isolates from Europe and Australasia. Markers for SAMPL revealed more genetic variation than did AFLPs. The generation of 51 loci using SAMPL primer pairs $(GACA)_4$ + H-G and R1 + H-G revealed that European isolates were more diverse than Australasian isolates, with 37 and 22% of loci polymorphic, respectively. Cluster analysis revealed geographic clades, with Australasian isolates forming one cluster separated from two clusters comprising the European isolates. However, low bootstrap support at these clades suggested that Australian isolates had not differentiated significantly from European isolates since the first record of *P. violaceum* in Australia in 1984. In general, the results support two hypotheses. First, that the population of *P. violaceum* in Australia was founded from a subset of individuals originating from Europe. Second, that *P. violaceum* in New Zealand originated from the Australian population of *P. violaceum*, possibly by wind dispersal of urediniospores across the Tasman Sea.

SAMPL was applied to 87 isolates of *P. violaceum* sampled from four populations of *P. violaceum* occurring on naturalised infestations of European blackberry in Victoria during the spring of 2003. SAMPL revealed considerable genetic variation, with 91% of individuals representing unique electrophoretic phenotypes within three of the populations studied. The fourth population, from Korumburra, had a clonal structure with 19 of 20 isolates represented by one SAMPL phenotype. Analysis of molecular variance (AMOVA) revealed considerable genotypic differentiation among all populations sampled in 2003 (Φ=0.4019, P<0.0001), indicating that gene flow was infrequent among these populations at the time of sampling. The apparent lack of correlation between the level of differentiation and distance between all populations suggested little or no genetic isolation by distance, which is consistent with the stochastic nature of aerial spore movement and establishment (gene flow) over large areas. The population at Whitfield, sampled in the spring of 2002 and 2003, did not share any SAMPL phenotypes between sampling periods (Φ=0.2838, P<0.0001). This observation indicated that genetic drift may play an important role in population change over time.

Marked asynchrony in life history with respect to random mating was also observed among populations of *P. violaceum*. Calculation of the Index of Association (I_A) from clone-corrected data revealed that the occurrence of random mating varied among populations in space and time. In 2003 only one population, from Wangerrip (I_A=-0.088, P=0.715), was panmictic, as was the population sampled in 2002 from Whitfield (I_A=-0.101, P=0.83). However, in 2003, isolates recovered from the Whitfield infestation were at multilocus disequilibrium (I_A=1.117, P<0.05). Although recombination plays an important role in maintaining genotypic variation within populations of the rust fungus, these results suggested that stochastic events, such as random genetic drift and migration, may also be important in maintaining genotypic variation within populations.

Isolates V1, V2 and strain F15 of *P. violaceum* varied in infection efficiency as a function of leaf age. A significant inverse logistic relationship between uredinium density and leaf age was observed among the three isolates. Regression analyses revealed strain F15 as having a separate logistic regression to isolates V1 and V2, which shared the same model. Although strain F15 produced fewer uredinia than isolates V1 and V2 for leaves aged between 2 and 11 days, the rate at which pustule density declined was less for strain F15. As leaf age exceeded 11 days, uredinium density was greater for strain F15 than for isolates V1 and V2. This is the first report of differences among strains in terms of leaf age-mediated resistance.

This study supports the hypothesis that populations of *P. violaceum* in Australia reflect metapopulation structure. Insight into the population dynamics of *P. violaceum* in Australia provides a theoretical basis for observations of the evolution of pathogen strains following their release and establishment in a new environment. This knowledge can be used to build upon the theoretical and applied framework for the selection and dissemination of rust fungi on variable target weed populations for effective biological control in the long term.

Declaration

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text

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Publications and conference proceedings

The following publications and conference proceedings were produced during the Ph.D. candidature:

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Gomez, D. R., Evans, K. J., Scott, E. S., Harvey, P. R., Baker, J. and Morin, L. (2004) Genetic variation in the blackberry biocontrol agent *Phragmidium violaceum* (Schultz), in *Proceedings of the 14th Australian Weed Conference* (Eds, Sindel, B. M. and Johnson, R.), Weed Society of New South Wales, Sydney: 109.

Gomez, D. R., Evans, K. J., Harvey, P. R., Baker, J., Mahr, F. A. and Scott, E. S. (2005) The metapopulation structure of *Phragmidium violaceum*, in *Proceedings of the 15th Biennial Australasian Plant Pathology Society Conference*, Geelong, Victoria, IN PRESS.

Morin, L., Aveyard, R., Batchelor, K. L., Evans, K. J., Gomez, D. R., Hartley, D., Jourdan, M. and Scott, J. L. (2005) New strains of *Phragmidium violaceum* for the biological control of blackberry, in *Proceedings of the 15th Biennial Australasian Plant Pathology Society Conference*, Geelong, Victoria, IN PRESS.

Abbreviations

°C	degrees Celsius
AFLP	amplified fragment length polymorphism
AMOVA	analysis of molecular variance
ANOVA	analysis of variance
bp	base pair
km, cm, mm	kilometre, centimetre, millimetre
DDW	double distilled water
DNA	deoxyribonucleic acid
dNTP	2'-deoxynucleotide triphosphatases
EDTA	ethylenediamine *tetra* acetic acid
g, mg, µg, ng	gram, milligram, microgram, nanogram
g	gravitational force
h	hours
ISSR	inter-simple sequence repeat
ITS	internal transcribed spacer
ml, µl	millilitre, microlitre
M, mM	molar, millimolar
$MgCl_2$	magnesium chloride
min	minute
No.	number
$CaCl_2$	calcium chloride
PCR	polymerase chain reaction
PVP-360	polyvinylpyrrolidone, molecular weight 360,000
RAPD	random amplified polymorphic DNA
RFLP	restriction fragment length polymorphism
RO	reverse osmosis
s	seconds
SCAR	sequence characterised amplified region
TAE	Tris-acetate-EDTA
TBE	Tris-borate-EDTA
TE	Tris-EDTA
UPGMA	unweighted pair group method, arithmetic average
USA	United States of America
VNTR	variable number tandem repeat

Glossary of terms

Deme, local population	A geographic subpopulation of breeding individuals (species; Hartl, 1994) that may be coevolving and interbreeding with other subpopulations through migration events.
Isolate	An individual culture of a fungus.
Panmixis, panmixia, panmictic	A population that has a breeding system that involves random mating.
Pathogenicity	The capacity of an organism to cause disease (Andrivon, 1993).
Random genetic drift	The stochastic, undirected changes in allele frequency that occur in all populations and most noticeable in smaller populations (Hartl, 1994)
Strain	An isolates that has been characterised for the purpose of biological control.
Virulence	The degree of ability of an organism to cause a susceptible disease response in the host plant with reference to specific interactions between genes for virulence (pathogen) and resistance (host) (Andrivon, 1993).

1 Introduction

European blackberry, *Rubus fruticosus* L. aggregate, is a serious weed of Australian primary production and natural ecosystems, with at least 15 closely related species naturalised since early European settlement (Evans *et al.*, 2004b). Recognised as a 'Weed of National Significance' (Thorp & Lynch, 2000), European blackberry occupies 8.8 million hectares (James & Lockwood, 1998), an area greater than the total land area of Tasmania. The species is also considered an important weed in New Zealand, South Africa, North America and Chile (Amor *et al.*, 1998). It is estimated that 43% of the total blackberry infestation in Victoria, Australia, is inaccessible to integrated weed management (Mahr & Bruzzese, 1998). Biological control is the only feasible means of managing this weed in remote and ecologically sensitive areas.

Blackberry leaf rust is caused by the fungus *Phragmidium violaceum* (Schultz) Winter (Uredinales). *P. violaceum* was selected as a biological control agent for European blackberry in Australia because of its high level of host specificity. The pathogen is capable of causing severe disease in species of the *R. fruticosus* agg., whilst having no significant impact on native *Rubus* species and commercial *Rubus* production since the first report of *P. violaceum* in Australia in 1984 following its unauthorised introduction (Morin & Evans, 2003). The introduction of candidate biological control strains of *P. violaceum*, selected in Europe in the 1970s (Bruzzese & Hasan, 1986), was delayed as a result of the unauthorised introduction of the rust fungus. Despite the wide distribution and occurrence of unknown founding strains of the rust (Watson, 1991), strain F15 of *P. violaceum* from France was eventually authorised and released in 1991 and 1992 for biological control.

Since the introduction of *P. violaceum* to Australia, there has been a significant reduction in biomass of susceptible blackberry taxa growing in regions where the weather is conducive to the development of rust (Mahr & Bruzzese, 1998). However, in any given location it is unknown if descendants of strain F15 or founding strains of *P. violaceum* are causing disease. Furthermore, disease incidence and severity can vary greatly in time and space from small to large degrees (Evans *et al.*, 2005). Evans *et al.* (2005) identified resistance among eight taxa of *R. fruticosus* agg., to disease caused by *P. violaceum* strain SA1 isolated in Australia in 1998. Variation in disease resistance among blackberry taxa might explain why some blackberry taxa escape severe disease in environments conducive to disease development. In short, a significant area of blackberry is still escaping disease at levels required for effective biological control.

In 1996, the Cooperative Research Centre for Australian Weed Management revived the biological control program for European blackberry, including the selection of eight additional strains of *P. violaceum* in Europe that were released in Australia in 2004 (L. Morin, personal communication). The aim of the program was to improve the impact of rust over a broader geographical area and range of blackberry taxa. One major problem facing biological control researchers during selection of strains of *P. violaceum* has been an incomplete and inconsistent taxonomic treatment of exotic *Rubus* in Australia. The situation has improved following a recent revision of the taxonomy of exotic *Rubus* (Evans & Weber, 2003; Evans *et al.*, 2004b). With newly characterised plant material, strains of *P. violaceum* virulent to European blackberry biotypes naturalised in Australia were selected so that following their establishment in the Australian environment, populations of *P. violaceum* would have the potential to cause disease across the range of the *R. fruticosus* agg. In theory, the pathogen population would also have greater potential to evolve at a rate that would match the

evolution of rust resistant biotypes of the weed. However, there remains insufficient knowledge on how pathogen biology and evolution, especially reproductive mode and gene flow, affect establishment and persistence of introduced strains of *P. violaceum* and/or introgression of new genes into existing populations of the fungus. Understanding the mechanisms that drive evolution of *P. violaceum* and its host in Australia can help identify efficient means for selecting and releasing additional strains of *P. violaceum* for effective, long-term biological control.

This thesis is divided into the following sections: 1) a review of the literature, 2) general materials and methods, 3) experimental research and 4) a general discussion of the implications of the findings and future research directions. The literature review will examine the link between population biology of *P. violaceum* and the epidemiology of blackberry rust. The population genetics of plant pathogenic fungi, with particular reference to rust fungi and host-pathogen complexes of natural ecosystems, will then be reviewed in relation to the development of molecular markers for *P. violaceum*.

2 Literature review

2.1 Introduction

Population genetics is being applied increasingly to understand the dynamics of plant disease epidemics (Milgroom & Peever, 2003). The deliberate release of exotic plant pathogens, especially rust fungi (Uredinales), for the biological control of introduced weeds, relies on molecular techniques for identifying and monitoring the fate of the released pathogens with certainty and for investigating factors contributing to the evolution of pathogen populations (Evans & Gomez, 2004). Two factors likely to influence population change in species of Uredinales are gene flow and reproductive mode, which will be defined in this review. The use of DNA markers to elucidate population dynamics offers a means to confirm the presence and relative contribution of these driving forces to population change.

A preliminary survey of genetic diversity in *P. violaceum* in Australia was conducted prior to the release of eight additional strains in 2004. Thirteen genotypes of *P. violaceum* were identified among 18 single uredinium-derived isolates collected across mainland Australia between 1997 and 1999 (Evans *et al.*, 2000). The study revealed that 14 of the 18 single-uredinium isolates of *P. violaceum* did not share any *Hae*III restriction fragments of DNA with *P. violaceum* strain F15, suggesting that the F15 genotype was rare in the environment. Evans *et al.* (2000) questioned the success of establishment of strain F15 for biological control of European blackberry in Australia. However, the authors did not discount recombination events that may have occurred between strain F15 and founding strains of *P. violaceum*. Further studies were deemed necessary in order to characterise the fate of rust strains released for biological control and to improve understanding of how the host and pathogen are coevolving in Australia.

In this review of the literature, the science underlying biological control of European blackberry with *P. violaceum* will be evaluated in the context of the biology and ecology of the weed and its management. The population genetics of plant pathogens, especially Uredinales, and the molecular tools available to study these will be reviewed for determining the most suitable approach for elucidating genetic structure and population dynamics of *P. violaceum.*

2.2 The weed: *Rubus fruticosus* aggregate

2.2.1 History of introduction and spread

European blackberry is thought to have been introduced to Australia in the 1840s, possibly earlier, with multiple introductions of unknown origin (Parsons & Cuthbertson, 1992). Introduction was actively promoted by the first government-appointed botanist to Victoria, Baron Ferdinand Von Mueller, and the so-called acclimatization societies of the day. The plant was promoted as a food source, and was also grown to control erosion along waterways. Within 50 years of its introduction, blackberry was still being actively promoted despite gaining notoriety for colonising and tarnishing farmland (Amor & Richardson, 1980). Blackberry was declared a noxious weed in parts of Victoria in 1894 (Parsons & Cuthbertson, 1992).

2.2.2 Impact on Australian environment and economy

European blackberry consumes land used for agriculture, forestry and conservation and affects productivity, aesthetic value, ecosystem diversity and recreational activity (James & Lockwood, 1998). The cost associated with loss in primary production and weed control was

last estimated as A$41.5 million in 1984 (Field & Bruzzese, 1984). The loss of biodiversity in natural ecosystems and the impact on aesthetics and recreation is immeasurable.

2.2.3 Morphology and growth habit

European blackberry is a thorny shrub with an extensive perennial root system. Above ground, blackberry forms a dense canopy with first year canes (primocanes) emerging from buds in the crown, or occasionally from root segments, with some taxa capable of extending 2 – 3 m in height (Amor & Richardson, 1980; Amor *et al.*, 1998). Apices of primocanes arch and touch the soil in late autumn, forming roots and daughter plants that grow in the following spring. Primocanes die back to the crown in the winter, following the production of floricanes (inflorescence) in the second season (Amor *et al.*, 1998). Fruits may vary in shape, from round to oblong and are black when ripe.

2.2.4 Taxonomy

The closely related taxa of European blackberry are collectively referred to as the *Rubus fruticosus* L. aggregate. Matching virulent strains of *P. violaceum* to each taxon of European blackberry naturalised in Australia is dependent on the latter having a useful and reliable taxonomic treatment. A recent taxonomic revision of exotic *Rubus* in Australia has removed a number of inconsistencies in the naming of European blackberry taxa among the Australian states (Evans *et al.*, 2004b). Nevertheless, the systematics of *Rubus* remains problematic. The majority of European blackberry taxa are polyploid and faculatively apomictic (Nybom, 1988). Since apomixis is facultative in this aggregate, a small percentage of seed may be produced sexually through hybridisation between polyploid, apomictic taxa or between apomictic taxa and diploid sexual outcrossers. The resulting biotypes can differ

morphologically in small or large degrees. Distinguishing these taxa on morphological characteristics alone can be difficult.

DNA techniques are proving to be valuable tools in clarifying taxonomic problems of European blackberry in Australia and elsewhere (Evans *et al.*, 1998; Nybom, 1995). A robust procedure for the identification of *Rubus* clones involves the detection of restriction fragment length polymorphisms (RFLP) using M13 bacteriophage DNA as an RFLP probe (Rogstad *et al.*, 1988; Zimmerman *et al.*, 1989). Evans *et al.* (1998 and 2004b), identified 34 M13 DNA phenotypes that were linked to 15 taxa of the *R. fruticosus* agg., and one undetermined taxon. A further 16 DNA phenotypes were undetermined, based on morphology, or were of questionable identity. The undetermined DNA phenotypes are either new taxa, taxa that have not yet been recognized nor characterized in Europe, or taxa that no longer exist in Europe. Exotic *Rubus* species have had over 150 years to evolve in Australia, and it is very possible that new biotypes may have arisen by hybridization or somatic mutation.

The most widespread biotype of European blackberry in Australia, previously called *R. procerus*, *R. discolor* or *R.* affin. *armeniacus*, has been identified as *R. anglocandicans*, based on a match in M13 RFLP DNA profile with samples of morphologically similar specimens of *R. anglocandicans* from England (Evans & Weber, 2003). *R. anglocandicans* appears to exist as a clonal lineage in Australia, with greater than 97% of samples (n = 76) collected across Australia between 1996 and 2001, representing a single DNA phenotype. Other taxa, such as *R. leucostachys*, have a higher level of genetic diversity (Evans *et al.*, unpublished), and this type of knowledge must be taken into account when screening isolates of *P. violaceum* as candidate biological control strains for this genetically variable weed.

2.2.5 Distribution and ecology

The *R. fruticosus* agg., occurs in all states of Australia as well as in the Australian Capital Territory (Figure 2.1). Globally, the *R. fruticosus* agg., occurs in regions where the annual rainfall exceeds 760 mm and is generally restricted to latitudes 30° to 60°N and 28° to 40°S (Figure 2.2). The species aggregate is also known to occur in equatorial regions in areas of high elevation and in low rainfall climates, where it may be prevalent in locally wet areas, often growing along water courses such as irrigation channels (Amor & Richardson, 1980). Long distance dispersal of European blackberry is mainly caused by animal dispersal of seeds from ingested fruit. Seeds may also be transported by water along creeks and rivers. Germination of seeds generally occurs in spring, but seedlings rarely establish in areas with low light intensity (Amor *et al.*, 1998).

Figure 2.1 Distribution of *Rubus anglocandicans* in Australia. *R. anglocandicans* is the most widespread taxon of European blackberry in Australia (Evans & Weber, 2003). Distribution map provided by M. Whalen, Flinders University.

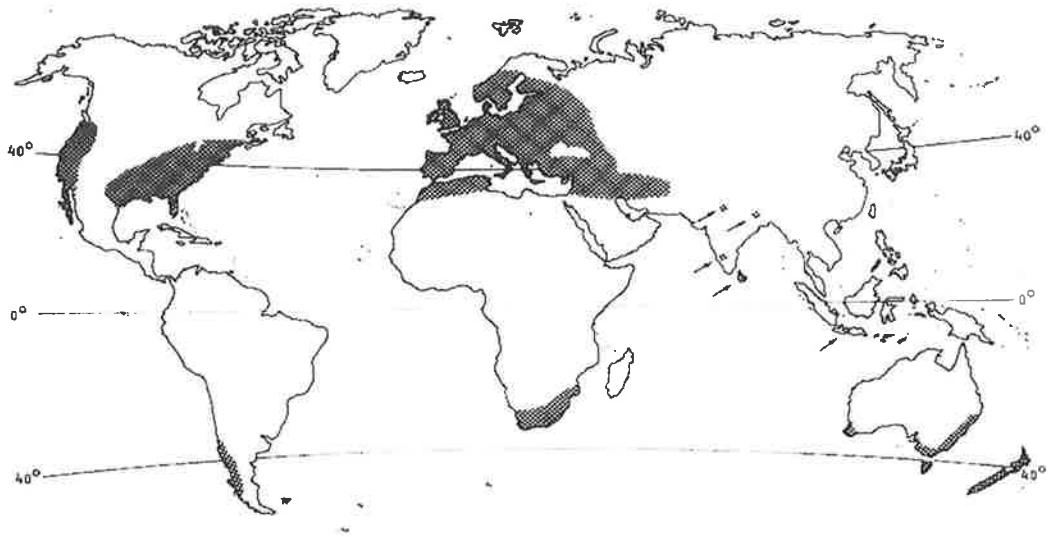

Figure 2.1 World distribution of *R. fruticosus* L. aggregate. Shaded areas indicate regions where European blackberry is naturalised (Amor & Richardson, 1980).

2.2.6 Integrated weed management

A number of herbicides are registered for use in controlling European blackberry in Australia (Bruzzese & Lane, 1996; Bruzzese *et al.*, 2000). The efficacy of a particular herbicide is dependent on several factors: the class of herbicide, the timing of application, the blackberry taxon being controlled, the age of blackberry thickets, and the use of additional cultural control methods such as fire, grazing, slashing, burning and mechanised weeding (Bruzzese & Lane, 1996). Nevertheless, chemical and cultural control can be highly effective when best-practice management is used (Bruzzese *et al.*, 2000). However, due to the perennial root mass present in established infestations, frequent applications of herbicide and cultural control are required over several seasons for effective suppression of the weed. Since conventional control has the potential to be a lengthy exercise, the cost of controlling established blackberry infestations can place great strain on operators, equipment and financial resources of the landholder (Waters, 1998). Where the land manager is responsible for millions of hectares of state or crown land, there is often insufficient resources for managing the weed conventionally and reliance on other options, such as biological control, become paramount.

The problem of control is compounded further when considering that 43% of total blackberry infestation in Victoria is inaccessible to integrated weed management because the infestation is remote and/or located in an ecologically sensitive environment (Mahr & Bruzzese, 1998). In this situation, biological control is the only management option currently available to the land manager. In other situations, biological control becomes the dominant weed management option For example, application of certain herbicides in riparian environments can lead to non-target impacts and deleterious effects on the environment (Bruzzese & Lane, 1996). A biological control agent that slows the spread of the weed from a river bank to adjacent pasture can give the land manager extra time to implement management measures based on

low impact herbicide or cultural control methods. Under these conditions, biological control has an important role to play as part of an integrated approach for management of European blackberry in Australia (Bruzzese *et al.*, 2000).

2.3 The biocontrol agent: *Phragmidium violaceum*

2.3.1 Host specificity and mode of action

The Uredinales represent an ancient group of obligate biotrophic plant pathogens which have coevolved intimately with their plant host (Savile, 1971). As such, species usually have a narrow host range and often have a high level of host specificity required for biological control. Species of *Phragmidium* have a host range restricted to species in the family Rosaceae (Cummins, 1959) with the genera *Rosa*, *Rubus* and *Potentilla* providing most of the hosts for *Phragmidium* species. *P. violaceum* completes its lifecycle on species of the *R. fruticosus* agg., but has occasionally been recorded on *R. caesius* (dewberry) and *R. ursinus* (loganberry) (Laundon & Rainbow, 1969).

P. violaceum is a suitable biological control agent due its high specificity to the *R. fruticosus* agg., (Morin & Evans, 2003), as well as its potential to cause severe disease on susceptible plants. Commercial varieties of *Rubus* such as Silvan and Thornfree blackberry have species of the *R. fruticosus* agg., in their complex breeding pedigrees (McGregor, 1998) and hence there has been concern about them being possible hosts for *P. violaceum*. However, there has been no impact of rust disease caused by *P. violaceum* on commercial berry production or on species of *Rubus* indigenous to Australia (Morin & Evans, 2003).

As a biological control agent and biotrophic pathogen, *P. violaceum* infects all green tissues and causes leaf death. Partial defoliation over several growing seasons weakens plants and reduces vegetative spread of the weed (Mahr & Bruzzese, 1998). Rust alone will not eradicate blackberry, rather, effective biological control reduces blackberry from being a dominant species to an understorey species that can coexist with the native species. This phenomenon has been observed in some regions of Victoria where the rust has reduced weed density to a level where it no longer represents an economic or environmental threat (E. Bruzzese, personal communication).

2.3.2 History of *P. violaceum* in Australia

Prior to the first record of *P. violaceum* in Australia (Marks *et al.*, 1984), the pathogen was showing promise as a biological control agent for blackberry in Chile (Oehrens & Gonzales, 1977). In 1978 the Victorian Department of Crown Lands and Survey (now the Victorian Department of Primary Industries) and CSIRO Division of Entomology tested a suite of *P. violaceum* isolates from Europe, for host specificity and pathogenicity towards the four most widespread taxa of European blackberry in Victoria (Bruzzese & Hasan, 1986). This research resulted in the selection and authorised releases of *P. violaceum* strain F15 in Australia in 1991 and 1992. Seven years before the authorised release of strain F15, blackberry-rust was found to exist in Victoria (Marks *et al.*, 1984). When strain F15 was released, it was recognised that the unauthorised strains had become widespread and well established in the Australian environment (Watson, 1991; Bruzzese & Lane, 1996). However, the establishment of strain F15 was not verified scientifically and little was known about the impact of the unauthorised strains on the establishment and efficacy of strain F15 in Australia. More recently, eight strains of *P. violaceum* from Europe were released in New South Wales,

Victoria and Western Australia for biological control in 2004 (L. Morin, personal communication).

2.3.3 Life history of *P. violaceum*

2.3.3.1 Uredinales rust fungi and their spore states

The Uredinales are basidiomycetes that represent a diverse group of pathogenic organisms. Rust fungi are typically obligate biotrophs, but some species have been grown *in vitro* on specialised culture media (Turel, 1973; Bhatti & Shattock, 1980; Bhatti, 1984a; Bhatti, 1984b; Rahabar-Bhatti, 1984; Melilan & Ojeda, 1985; Moricca *et al.*, 2000).

The rust fungi can either be autoecious, completing their lifecycle on the one host, or heteroecious, alternating between two different host species to complete their lifecycle (Scott & Chakravorty, 1982). Species selected for biological control are usually autoecious; otherwise the extent of host specificity testing might exceed available resources. The rust fungi can be classed further as being macrocyclic or microcyclic. Macrocyclic rust fungi are capable of producing five different spore states, each in specialised fruiting bodies (Table 2.1; Scott & Chakravorty, 1982; Agrios, 1997). These spore states are: spermatia, aeciospores, urediniospores, teliospores and basidiospores, with each spore type denoted by roman numerals 0, I, II, III and IV, respectively. However, spermatia and/or urediniospores may be absent from the lifecycle of some macrocyclic rusts. In contrast, microcyclic or short-cycled rust species produce only teliospores and basidiospores.

P. violaceum is a macrocyclic autoecious rust fungus capable of producing all five spore states in Australia (Washington, 1985). Figure 2.3 summarises the sequence of spore states in macrocyclic rust fungi and their nuclear condition.

Table 2.1 Specialised fruiting bodies associated with the development of various spore states in the Uredinales rust fungi.

Spore state	Spore type	Fruiting body
0	Spermatium	Spermagonium
I	Aeciospore	Aecium
II	Urediniospore	Uredinium
III	Teliospore	Telium
IV	Basidiospore	Basidium

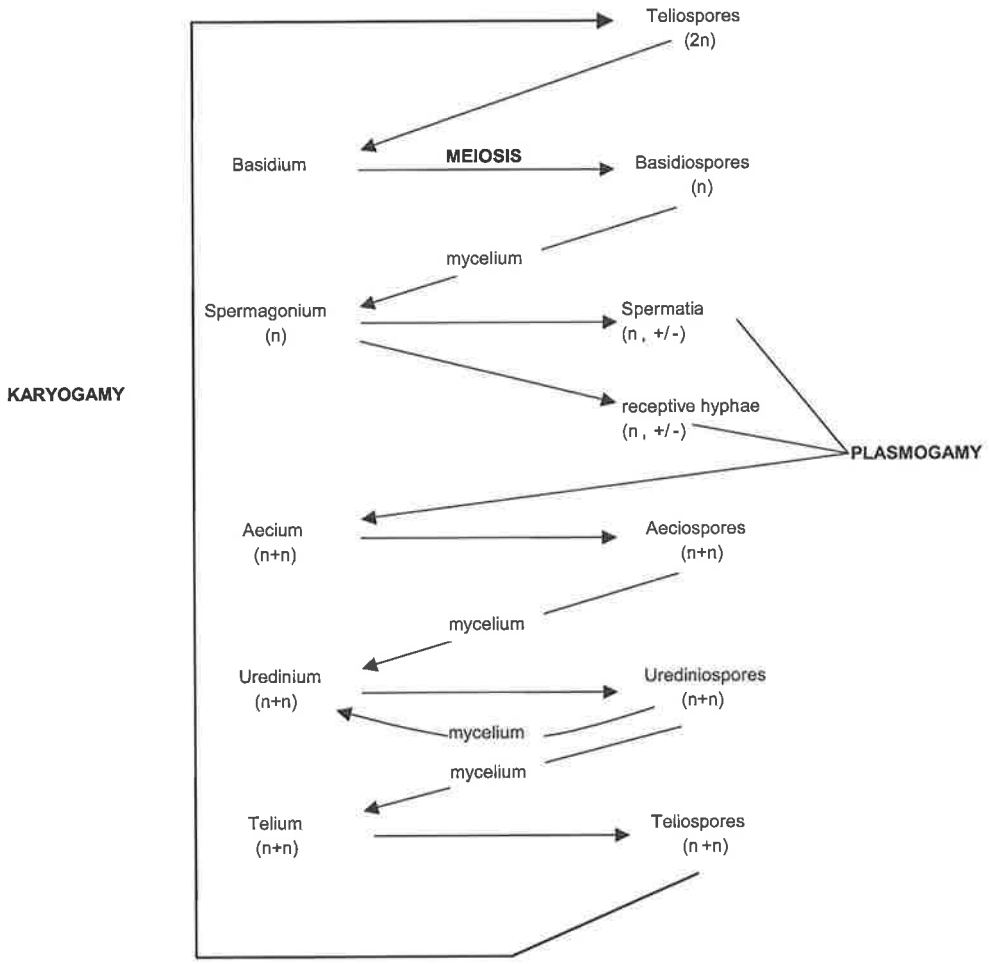

Figure 2.3 Sequence of spore states in macrocyclic Uredinales (adapted from Agrios, 1997). Nuclear condition of each state is given: haploid, n; diploid, n+n; 2n denotes a diploid state after karyogamy (nuclear fusion) and + and – denotes the two different mating types.

2.3.3.2 Life history of *P. violaceum* in relation to host phenology

The lifecycle of *P. violaceum* (Figure 2.3) is now described in relation to the phenology of European blackberry (Washington, 1985; Evans & Bruzzese, 2003).

Dikaryotic (n+n) teliospores (III) represent the over-wintering sexual spore state, which survive during the semi-dormant period of the blackberry's lifecycle, but are produced in increasing number between mid summer and winter as uredinia differentiate to telia. Teliospores mature on leaves that have not senesced in winter (Evans & Burzzese, 2003) and as blackberry enters active growth in spring, karyogamy occurs in the teliospores (n+n → 2n) which germinate to produce the heterobasidium. Meiosis takes place in the basidium giving rise to haploid (n) basidiospores (IV) which are released onto new leaves of developing floricanes and possibly onto leaves of primocanes (Evans & Burzzese, 2003). On infection, basidiospores produce haploid mycelia that form spermagonia containing spermatia (0) and receptive hyphae.

Spermatia are produced in a sweet sticky mass, which likely attract insects that disseminate the spermatia to other spermagonia. Spermatia act as male gametes, fertilising receptive hyphae of the opposite mating type. Fertilisation results in the production of dikaryotic mycelium to produce aecia containing aeciospores (I; n+n). Aeciospores are then disseminated by wind to susceptible host tissue from mid to late spring coinciding with the emergence of primocanes. Uredinia result from the infection of host tissue with aeciospores.

The formation of uredinia and production of urediniospores (II) coincide with the active growth of the host. Urediniospores are dispersed over potentially large areas by wind (Brown & Hovmøller, 2002) and represent the asexual phase and the only repeating spore state of the

macrocyclic life cycle. That is, multiple generations of uredinia are produced during a growing season. During warmer periods of summer and cooler periods of autumn/winter, when weather conditions become suboptimal for fungal growth, a proportion of uredinia may differentiate to form telia.

2.3.4 Disease signs and symptoms

A general term used for uredinia and telia is rust 'pustule' or 'sorus'. An easily recognised sign of active blackberry-rust disease is the presence of yellow-orange pustules, or uredinia, typically on the abaxial surface of blackberry leaves (Figure 2.4). Symptoms of uredinium production may include a corresponding discoloration on the adaxial surface of leaves (Figure 2.4). Although pustules are observed primarily on leaves, pustules may also develop on floral parts.

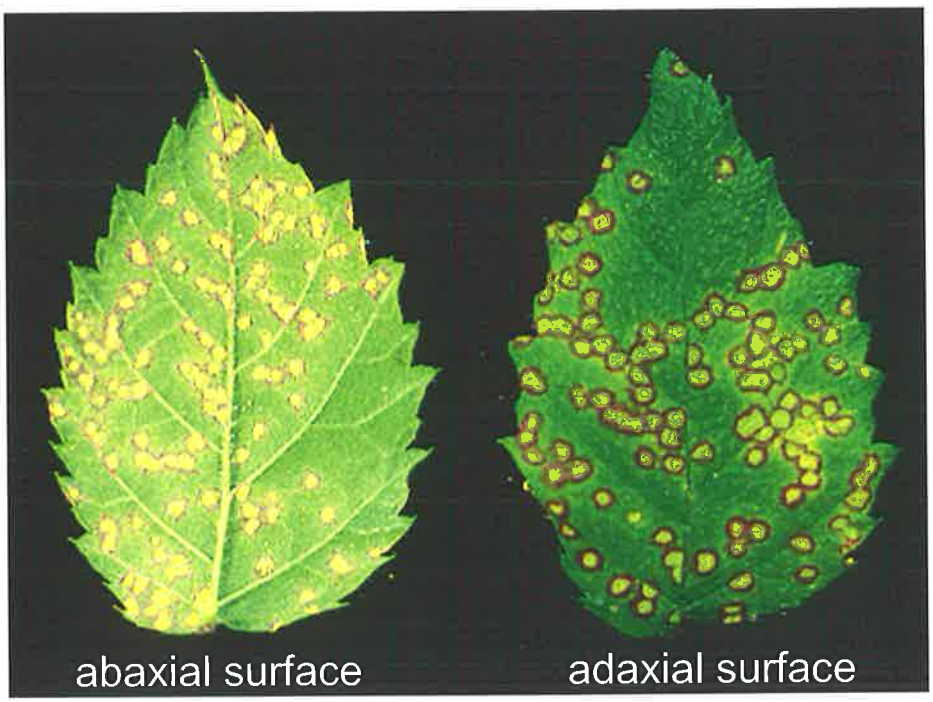

Figure 2.4 Uredinia on the abaxial surface and the corresponding discolouration on the adaxial surface of leaves of *R. vestitus* inoculated with *P. violaceum* strain SA1. Photograph supplied by K. J. Evans.

2.3.5 Disease epidemiology

Epidemiology is the study of temporal and spatial changes in disease caused by populations of pathogens on populations of hosts (Campbell & Madden, 1990). Given that plant disease develops when there is a favourable environment, a susceptible host and a virulent pathogen (Agrios, 1997), plant disease epidemics are the intersection of these factors as a function of time and space (Burdon, 1993; Thrall & Burdon, 1997). Knowing which environmental parameters and host growth factors are correlated to disease increase allows the use of these variables to predict the impact of rust disease.

2.3.5.1 Weather and climate

Environmental mapping using 'geographic information systems' (GIS) can be used to define the climate that is likely to favour significant levels of rust on susceptible blackberry taxa. Results of GIS mapping in Victoria indicate that locations where disease impact is high also have an annual rainfall of greater than 800 mm and an average maximum daily temperature in January of approximately 20°C (Pigott *et al.*, 2003). However, weather rather than climate is used to predict disease progression in a particular locality. For many plant diseases, temperature determines the rate of disease development for susceptible plant tissue whereas moisture (free water, relative humidity) determines the amount of disease that develops at a given temperature (Campbell & Madden, 1990). Weather variables most limiting epidemics of blackberry rust in the field have not been defined, although an indication of optimum values of temperature and moisture can be gleaned from results of controlled environment studies. Evans & Bruzzese (2003) showed that high infection efficiency for urediniospores was achieved following inoculation and incubation of young, detached leaves if infection occurred at 18-20°C with high relative humidity for 12 h. Temperatures that were above or below the apparent optimum for infection led to longer latent periods for uredinia and

uredinia that differentiated more rapidly to telia (Evans & Bruzzese, 2003). Under field conditions, Evans *et al.* (2005) showed latent period for uredinia for susceptible plants inoculated with strain SA1 of *P. violaceum* was approximately 150 degree days above a base temperature of 6°C. More data are needed to determine the per annum level of disease incidence and severity, especially the timing of disease onset and number of generations of uredinia, in relation to active shoot growth, that impact on the biomass of blackberry. One can envisage a blackberry-taxon and region specific decision rule that states that if the level of disease has not reached a certain percentage incidence or severity by a particular date, then weed management measures other than biological control should be implemented where possible (E. Bruzzese, personal communication).

While weather conditions may favour the development of rust disease in a particular locality, adjacent patches of blackberry, possibly different genotypes, have been observed to express marked differences in disease expression (Figure 2.5; Evans *et al.*, 2005). Explanations for this observed difference will be explored in Section 2.4.

2.3.5.2 Disease impact in Australia

Since the first record of *P. violaceum* in Australia in 1984 (Marks *et al.*, 1984), Mahr and Bruzzese (1998) have demonstrated a significant reduction in biomass of *R. polyanthemus* and *R. ulmifolius* as a result of parasitism by *P. violaceum*. Other reports of biomass reduction are anecdotal, although it is clear that adequate biological control has been mainly restricted to higher rainfall areas south of the Great Dividing Range in south-eastern Victoria (Mahr & Bruzzese, 1998; Pigott *et al.*, 2003). A large proportion of the blackberry infestation in Australia escapes severe disease. Where disease is severe, it is not known if biological control

is through parasitism by the descendants of the unauthorised strains, strain F15 or hybrids of the various strains.

Figure 2.5 Example of two blackberry infestations in Victoria where the plants in the background are more severely diseased than plants in the foreground. Photograph courtesy of D. McLaren.

2.4 Host-pathogen interactions

Before disease can develop in a blackberry infestation, there must be a compatible weed biotype and pathogen strain interaction. The recent taxonomic update of exotic *Rubus* in Australia revealed that European blackberry comprises at least 15 closely related taxa (Evans *et al.* 2004b) that can vary in their response to a particular strain of *P. violaceum*, even among DNA phenotypes of the same taxon (Evan *et al.* 2005). Therefore, the success of biological control depends on the presence of an appropriate range of virulence genes among naturalised populations of the rust that can overcome an unknown range of resistance genes in the *R. fruticosus* agg., in Australia.

Genetic variation among European and Australian strains of *P. violaceum*, using virulence markers, has been identified (Evans *et al.*, 2000; Morin & Evans, 2003; Evans & Gomez, 2004). A preliminary report of host specialisation in *P. violaceum* can be found in Evans & Gomez (2004) and Evans *et al.* (2004a). Pathotypes of *P. violaceum* present in Australia were identified as part of the current biological program for blackberry, along with at least one additional pathotype that was identified among additional strains selected in Europe for release in Australia in 2004 (Morin & Evans, 2003). Isolate V1 of *P. violaceum*, isolated from western Victoria, was able to incite disease in a broad sample of taxa and DNA phenotypes of European blackberry from Australia (Evans & Gomez, 2004).

Despite the increased knowledge of climatic and genetic considerations that limit or promote disease, many questions remain as to what limits disease on certain blackberry biotypes in particular locations. The existence of qualitative and quantitative resistance to rust disease might explain the difference in disease severity illustrated in Figure 2.5. In some cases, perhaps the rust strain with corresponding virulence does arrive on a host biotype, but it

arrives so late in the growing season that initiation of the epidemic is delayed, resulting in ineffective disease levels at critical times in the season (Burdon *et al.*, 1996). Another explanation may be leaf age-related resistance, which for *P. violaceum* corresponds to an exponential decline in densities of uredinia as leaves age beyond full expansion (Figure 2.6; Evans & Bruzzese, 2003). Leaf age-related resistance has been observed in numerous interactions between obligate pathogens of perennial hosts including the Uredinales pathosystems, *Phragmidium rubi-idaei* on red raspberry (Anthony *et al.*, 1985) and *Hemileia vastatrix* on coffee (Coutinho *et al.*, 1994). If two neighbouring plants grow at different rates due to differences in microclimatic conditions or inherent genetic factors, then this might result in two blackberry canopies having different proportions of old and young leaves and therefore differences in the proportion of the canopy available for infection (Evans & Gomez, 2004). Whether or not different strains of *P. violaceum* vary in infection efficiency over different aged leaves is unknown. If certain strains of *P. violaceum* can infect a greater proportion of older leaves, then this may provide criteria for selecting more effective strains for improved biological control (K. Evans & E. Bruzzese, personal communication). Evans *et al.* (2005) also discuss the possibility of disease severity varying among co-located taxa that have significantly different shoot densities.

The role of pathogen-host genetics in disease development goes well beyond finding a compatible plant-pathogen interaction. Section 2.5 introduces the population genetics of rust fungi as the basis for exploring how advantageous genes or genotypes of the rust, such as those which confer virulence to a target blackberry taxon, either move and persist among populations or become restricted in distribution.

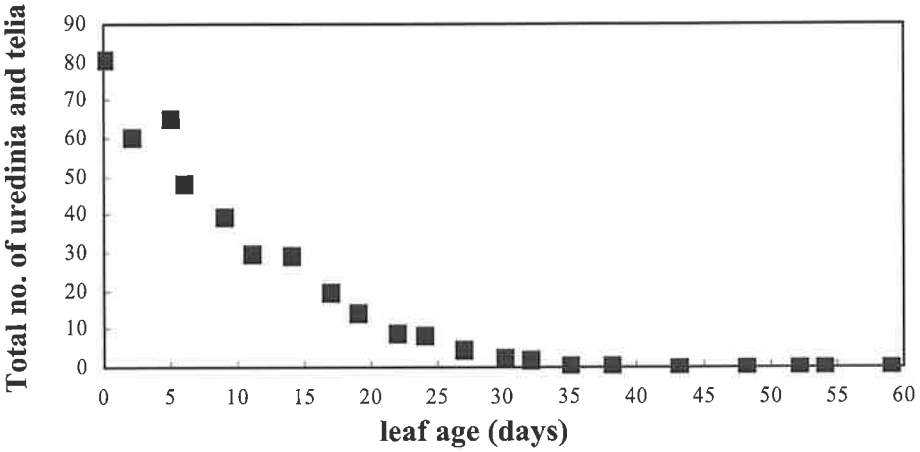

Figure 2.6 The relationship between numbers of uredinia (and telia) per square centimetre of leaf and leaf age, reproduced from Evans & Bruzzese (2003).

2.5 Population genetics of rust fungi in relation to biological control

Any successful plant pathogen requires the opportunity to reproduce and proliferate advantageous genotypes if it is to retain an exclusive niche or food source (Kohn, 1994). Thus if a pathogen is to be successful in an environment it must evolve in response to changing conditions. Mechanisms for generating new pathogenic variants in fungi include mutation, sexual and/or mitotic recombination (Burdon & Silk, 1997). There is evidence that somatic hybridization in rust fungi occurs under controlled conditions, although evidence for mitotic recombination in a field situation has been reported infrequently (Park *et al.*, 1999).

The genetic structure of a population refers to the amount and distribution of genetic variation within and among populations (McDonald, 1997). Population genetic structure reflects a population's evolutionary history and its potential to evolve. McDonald & Linde (2002) list

high mutation rate, large population sizes, high gene flow, mixed reproductive system and efficient directional selection as factors likely to create high evolutionary potential in plant pathogens. Rust fungi potentially satisfy all these criteria in agricultural systems. However, much less is known about rusts in natural ecosystems. Furthermore, the relative importance of each evolutionary force may vary considerably depending on the pathogen-host association and the stage of the pathogen's life cycle (Burdon & Silk, 1997).

2.5.1 Evolution in natural *versus* agricultural ecosystems

The potential for pathogen evolution has been most evident in agricultural ecosystems. In agriculture, plant pathogens undergo strong directional change in response to defined selection pressures in a field, whether it is overcoming new resistance genes in field crops, or evolving resistance to frequently used pesticides (Brown, 1996; McDonald, 1997). One can then assume that in agricultural systems, the proliferation of advantageous pathogen genes or genotypes can lead to severe epidemics, placing constant strain on plant productivity (Burdon, 1993; Brown, 1996; McDonald, 1997). However, the population biology of agricultural *versus* natural pathosystems is immensely different. Although the same general principles of population biology apply to plant pathogens of agricultural and natural ecosystems, Burdon (1993) argues that different constraints and opportunities imposed by natural ecosystems, compared to agricultural production systems, will change the relative importance of evolutionary factors that drive population change in these two environments. Understanding the evolutionary forces that drive change in populations of *P. violaceum* may provide clues for selection of strains and development of release strategies for effective biological control in the long term. An understanding of what constitutes a pathogen population in natural ecosystems is needed in order to make valid assumptions of likely evolutionary processes which drive pathogen population change in natural ecosystems.

2.5.2 Metapopulation theory: populations within a population

A population refers to a group of individuals of a species which occupies a defined geographic region. The metapopulation view (Levins, 1971) of the diversity and evolution of plant pathogens in natural ecosystems differs greatly from models based on plant pathogen evolution in uniform agricultural ecosystems, where mutation rate and frequency-dependent selection are the most important processes driving pathogen change (Burdon & Silk, 1997). In natural ecosystems, plants may exist as individuals or small aggregates of individuals, unevenly distributed across time and space. Although small pockets of individuals may appear to exist independently of each other, they may be linked through migration events. Populations that evolve semi-independently are referred to as "local populations" or demes, which belong to a larger metapopulation. Metapopulation theory recognises the fragmented distribution of demes and typically demonstrates a turnover of local populations through population crashes and migration events resulting in distributions of demes that change over time.

There is strong evidence that host-pathogen interactions in natural ecosystems reflect metapopulation structure. The evidence provided by Thrall *et al.* (2001) for metapopulation structure in the *Linium marginale-Melampsora lini* pathosystem, may be analogous to that occurring in the *R. fruticosus-P. violaceum* complex. European blackberry is predominantly apomictic, producing a comparatively small number of reproductive propagules relative to the extremely large numbers of urediniospores produced by *P. violaceum*. The propagules of the host are dispersed on a much smaller spatial scale when compared with urediniospores of *P. violaceum* which are windborne and are able to disperse over long distances (Figure 2.7). The further local populations of the host are from each other, the more likely variation in frequencies of host resistance genes will be observed due to geographic isolation (Thrall &

Burdon, 1997). In contrast, the rust pathogen has potential for high rates of gene flow. Variation in pathogenicity of the *P. violaceum* population is likely to be less obvious across the spatial scale of the interacting metapopulation. However, since variation in host resistance genes is likely to occur within the spatial scale of the metapopulation, local populations of the host might be acting as a "selective sieve" (Thrall & Burdon, 1997). That is, variation in virulence genes is maintained among pathogen populations associated with local populations of the *R. fruticosus* agg., that have evolved semi-independently.

Although the potential for pathogen dispersal is great, disease is not always guaranteed on a spatial and temporal scale across the metapopulation. This is largely due to the uneven distributions of host resistance and habitat across the metapopulation. Therefore, stochastic events, such as season to season fluctuations in microclimate, the probability of survival of pathogen propagules, or the probability that a virulent strain finds a susceptible host and climate, will be deciding factors for disease development and subsequent incidence (Thrall & Burdon, 1997). Thus, processes such as selection, migration and random genetic drift will play greater roles in pathogen population structure in situations where pathogens have wide dispersal and frequent population crashes followed by random immigration and re-establishment (Burdon, 1993; Thrall & Burdon, 1997). In rust systems where urediniospore dispersal is potentially great, and boom and bust cycles are inherent to the pathogen lifecycle, disease incidence will likely vary in time and space across the metapopulation due the stochastic nature of pathogen strain recruitment. Metapopulation theory is likely to explain why disease incidence and severity across the range of the European blackberry infestation in Australia can vary in spatial scale, from season to season and from small to large degrees.

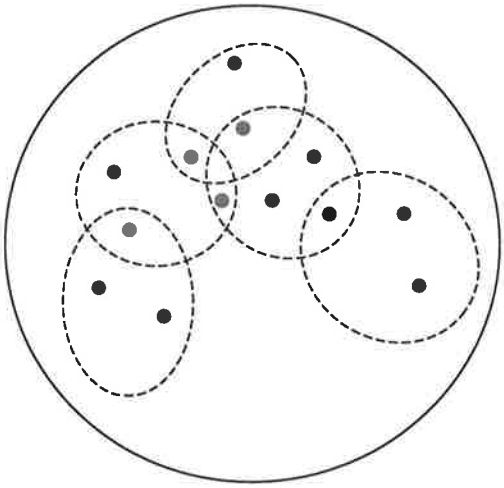

Figure 2.7 'Interaction metapopulation' hypothesised for the *R. fruticosus-P. violaceum* interaction, where the spatial scale of host dispersal (dashed line) is less than the dispersal of urediniospores (outer circle). Host dispersal might extend beyond the dashed line following dispersal of seed by birds, foxes or native animals. Solid circles (black and red) indicate local populations with red circles showing linkage among local populations of the host. Adapted from a typical metapopulation model of wind dispersed rusts and their hosts first illustrated by Thrall & Burdon (1997).

2.5.3 Gene flow

Having highlighted the importance of matching weed and pathogen diversity, the question remains about how population biology of an agent affects the establishment and persistence of introduced genes and genotypes following the release of new strains of *P. violaceum* into existing populations of the fungus in Australia. Gene flow is concerned with the movement of genes or genotypes among populations separated geographically and can cause evolution directly through the loss (emigration of individuals) or gain (immigration) of genes or genotypes among populations. Pathogens that have high degrees of gene flow are expected to have greater genetic diversity, since high gene flow increases the effective population size by increasing the size of the genetic neighbourhood (McDonald & Linde, 2002). The rust fungi produce asexual spores that are dispersed over large areas (Brown & Hovmøller, 2002). Rust fungi, therefore, may have expansive genetic neighbourhoods in which genetic information can be exchanged. For effective biological control, a high rate of gene flow among populations of *P. violaceum* is desirable to ensure the movement and integration of useful genes and genotypes, such as those that confer virulence.

McDonald & Linde (2002) hypothesised that long distance dispersal and frequent gene flow of asexual propagules poses a greater risk for development of disease epidemics than movement of sexual spores because asexual propagules represent a linked set of alleles which have already been selected for a particular environment. However, this assumes that the host and environment are uniform over the distribution area which may not be the case in plant-pathogen interactions of natural ecosystems (Burdon, 1993). Little is known about the size of the genetic neighbourhood of *P. violaceum* in Australia. The ability to detect and measure gene flow among geographically isolated populations of *P. violaceum* will provide

information that will allow for the improvement of successful strain establishment in the field through the development of appropriate selection and release strategies for new strains.

2.5.4 Reproductive mode

Phragmidium violaceum alternates between cycles of sexual and asexual reproduction. However, the relative contribution of clonal and sexual reproduction to the genetic structure of populations of rust fungi is poorly understood. DNA markers are increasingly used to elucidate reproductive mode within and among populations of plant pathogens (Milgroom, 1996).

Like gene flow, recombination plays an important role in the augmentation of genetic variation within populations. Whereas gene flow directly affects the evolution of populations through the loss or gain of alleles and hence, changed allele frequencies, sexual recombination does not alter allele frequencies within populations that are strictly sexual and which undergo random mating. However, recombination results in variation through the formation of new combinations of genes following outcrossing, ultimately driving changes in genotype frequency. Therefore the frequency of sexual recombination plays an important role in the genotypic diversity of a population. Recombination will drive population change through the creation of new allele combinations, however, persistence of beneficial allele combinations, such as those conferring virulence and fitness, is not necessarily, guaranteed. On the other hand, asexual reproduction, combined with natural selection can maintain allele combinations best adapted to the immediate environment (McDonald & Linde, 2002).

Reproductive mode has implications for the selection of rust strains for weed biocontrol. If the agent has a strictly clonal mode of reproduction, then virulent strains of the agent can be

matched to weedy biotypes. Ideally, a virulence gene tightly linked to a fitness gene would create a high potential for severe disease. Conversely, if the mode of pathogen reproduction is sexual, then genotype integrity of pure strains introduced for biological control is not guaranteed if the agent undergoes sexual recombination with co-introduced or existing rust strains. However in systems that have mixed reproductive modes, the organism potentially benefits from advantages of recombination and clonality. In such cases, recombination allows the formation of new genotypes which can be tested in the environment, while clonality along with selection can maintain allele frequencies best suited to the immediate environment (McDonald & Linde, 2002).

An example of the effect of pathogen reproductive mode in weed biocontrol can be found in the skeleton weed-rust pathosystem. Skeleton weed, *Chondrilla juncea*, exists as three clonal forms in Australia. The introduction of *Puccinia chondrillina*, the biological control agent of *C. juncea*, into Australia resulted in the effective control of the narrow leaf form of the weed enabling the other two forms of *C. juncea* to colonize wheat crops in regions where the narrow leaf form existed previously. *P. chondrillina* cannot reproduce sexually in the Australian climate, unlike populations in its native range (Chaboudez & Sheppard, 1995). Since Australian populations of *P. chondrillina* were clonal, additional strains of *P. chondrillina* from Turkey were matched for virulence to the other two forms of *C. juncea* in Australia. Since further research on *C. juncea* ceased in 1996, the impact of additional strains of *P. chondrillina* on *C. juncea* in Australia has not been assessed (Evans & Gomez, 2004). An introduced pathogen population that is strictly clonal may still have a high evolutionary potential as demonstrated by *P. striiformis* f. sp. *tritici* in Australian cereal crops, where new pathotypes are generated by high mutation rates combined with strong selection for new or

altered virulence (Steele *et al.*, 2001). The evolutionary potential of *P. chondrillina* in Australia remains unknown.

2.5.5 Selection of molecular markers for population genetic studies

The advent of polymorphic molecular markers, based on electrophoretic separation of DNA and proteins, has provided researchers with powerful and precise tools by which gene flow and recombination can be detected and measured (McDermott & McDonald, 1993; Brown, 1996; McDonald, 1997). Comprehensive reviews of the basic properties of a range of genetic markers and their selection for a given research question in population biology are presented by McDonald & McDermott (1993), Brown (1996), McDonald (1997), Parker *et al.* (1998) and Sunnucks (2000). The value of common molecular markers used in population genetics studies of rust fungi will now be reviewed.

2.5.5.1 The issue of dominance

Molecular markers are either dominant or co-dominant. Dominant marker systems, such as 'randomly amplified polymorphic DNA' (RAPD) and 'amplified fragment length polymorphisms' (AFLPs), cannot distinguish heterozygous and homozygous individuals, as markers are scored as being present or absent (null state). Application of these types of markers, especially in dikaryotic (n+n) and diploid (2n) organisms, can result in the underestimation of genetic diversity within and among populations, as different alleles at some loci may not be identified when they belong to the null class. Analytical problems associated with dominance can be overcome or lessened by increasing the number of individuals or loci assayed (McDonald & McDermott, 1993; McDonald, 1997). In comparison, co-dominant markers, such as single-locus microsatellites, isozymes and most

RFLPs, identify homologous alleles and thus distinguish between heterozygous and homozygous states.

The issue of dominant *versus* co-dominant markers can assist the decision of whether the data set is generated using (a) multiple, single-locus markers for DNA haplotyping or (b) multilocus DNA patterns or fingerprints. Both approaches are suitable where the research objective is to separate all genetically different individuals for the purpose of genotyping. DNA fingerprinting is often used for genotyping because a single marker system can produce unique patterns for each genotype. Genotypes identified by DNA fingerprinting are referred to as DNA phenotypes, because the DNA pattern generated represents only a subset of the entire genome.

2.5.5.2 Isozymes and RFLPs

Isozymes are simple and reliable genetic markers that require minimal development. When compared with other marker systems, very little variation exists in isozymes at the intraspecific level in rust fungi (Burdon *et al.*, 1982; Newton *et al.*, 1985). Burdon & Roelfs (1985), for example, screened 65 races of *P. graminis* f.sp. *tritici* from 13 countries and detected 13 loci with 10 enzyme systems. Five loci revealed no variation, whereas two to four alleles were detected at the remaining loci. In another study, isozyme markers were combined with RFLPs and virulence phenotypes to characterise the genetic structure of Australian populations of the rust fungus *Melampsora lini* occurring on *Linum marginale*, an indigenous species of flax in Australia (Burdon & Roberts, 1995). Of 107 isolates of *M. lini* collected from a region of the Kosciusko National Park, New South Wales, Australia, six isolates were distinguished from the rest by one enzyme system. This information when combined with pathotyping and RFLP data suggests that the six isolates represented two

closely related races of *M. lini* and were recent immigrants to the Kosciusko area at the time of the study.

Analysis of RFLPs involving hybridization of anonymous or generic DNA probes, while technically demanding, remains a robust and useful technique. In rust fungi, it may be applied for preliminary analyses of intraspecific genetic variation (Evans *et al.*, 2000) and for discriminating races (Anderson & Pryor, 1992). The generic M13 probe (Rogstad *et al.*, 1988; Zimmerman *et al.*, 1989), for example, can be applied to any organism for preliminary analysis of intraspecific genetic variation. Using an M13 probe, DNA banding patterns were found to be somatically stable and the marker could discriminate closely-related strains of *P. violaceum* and clones of its blackberry host (Evans *et al.*, 1998; Evans *et al.*, 2000). No preliminary sequence information is required and, unlike generic polymerase chain reaction (PCR)-based techniques like RAPDs, contamination by small quantities of non-target DNA will not confound the result.

2.5.5.3 PCR-based markers

PCR has found widespread application in many fields of biology and has become established as a basic tool of biological research. A key component of the reaction is the oligonucleotide primers that are used to amplify targeted regions in DNA. The type of primer divides PCR markers into two general groups; those based on arbitrary primers and those known as sequence tagged sites (STSs) using primers designed from a known sequence.

Arbitrarily-primed PCR

RAPDs were adopted enthusiastically by many laboratories in the early 1990s, but the ease of contamination with non-target DNA led to extreme measures of laboratory hygiene. In rust fungi, RAPDs have been applied to studies of gene flow (Braithwaite *et al.*, 1994) and of linkage of molecular markers to virulence phenotypes (Lui & Kolmer, 1998). A major disadvantage of RAPDs is poor reproducibility in different laboratories (Devos & Gale, 1992; Perez *et al.*, 1998). The availability of more informative and reproducible techniques, such as AFLP, means that RAPDs are now utilised for a limited number of applications, including preliminary identification of genetic variation, 'within laboratory' strain identification or to aid development of sequence tagged sites.

Subsequent PCR-based fingerprinting techniques were designed to improve the reproducibility of amplification by using longer primers and more stringent reaction conditions. An example is 'sequence characterised amplified regions', SCAR, in which RAPD fragments are end-sequenced so that longer PCR primers can be designed for use in PCR (McDermott *et al.*, 1994). Another improvement was designed to increase the efficiency of identifying polymorphic loci. Examples include (a) microsatellite-primed PCR (Gupta *et al.*, 1994), where specific subsets of the genome are randomly amplified and (b) AFLP markers, where the genomic DNA is enriched for particular sequences prior to random amplification (Vos *et al.*, 1995). AFLPs are widely adopted at present because they are highly polymorphic and reproducible (Majer *et al.*, 1996). The process of generating AFLP is illustrated in Figure 2.8. No prior knowledge of DNA sequences is required in developing AFLP markers, ensuring minimal development time. For example, Steele *et al.* (2001) investigated variation in AFLP among five geographically distinct isolates of *Puccinia striiformis* f. sp. *tritici* collected from the United Kingdom, Denmark and Columbia. With

each primer set tested, an average of 6.5 polymorphic bands was detected. All five isolates tested could be uniquely identified from amplicons generated using six primer combinations.

Although AFLPs are classed as dominant markers, pedigree information from sexual crosses from several organisms has revealed co-dominant AFLP markers at frequencies of 4-15% among all polymorphic loci (Waugh *et al.*, 1997; Boivin *et al.*, 1999). Furthermore, a novel extension of the AFLP technique, called 'selective amplification of microsatellite polymorphic DNA' (SAMPL), also referred to as 'microsatellite AFLP' (Morgante & Vogel, 1994), appears to generate co-dominant markers at even higher frequencies then those reported for AFLPs (Morgante & Vogel, 1994; Paglia & Morgante, 1998). Thus the use of high ratio multiplex markers, which generate many markers for a few primer sets and which cover a wide spread of the genome, has the potential to remove some bias in linkage analyses associated with studies of diploid organisms using traditional dominant markers.

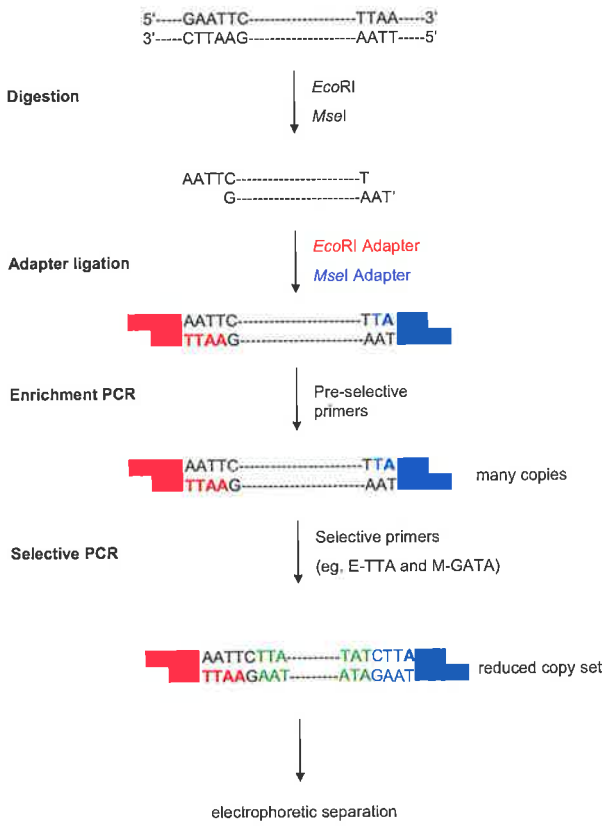

Figure 2.8 The generation and detection of AFLPs from the digestion of genomic DNA with the restriction enzymes *Eco*RI and *Mse*I. Variation among individuals will arise from the presence or absence of restriction enzyme sites among individuals due to mutation, dclction and insertion events. Adapted from Zabeau & Vos (1993).

Sequence-tagged sites

Microsatellites are simple, tandemly-repeated motifs that are dispersed thoughout the genome in transcribed as well as non-transcribed sequences. They exhibit site-specific length variation and often represent polyallelic genetic loci with a wide range of evolutionary rates (Olsen *et al.*, 1989). Although considered the ideal species-specific, single-locus marker for population studies (Blouin *et al.*, 1996; Jarne & Lagoda, 1996), their development can be laborious and expensive, in that it requires sequence information obtained from cloned DNA, and the resulting primers can rarely be applied to other species. However, methods for identifying clones with microsatellite sequences and sequencing strategies are rapidly becoming more efficient (Hoelzel, 1998; Scribner & Pearce, 2000; Burgess *et al.*, 2001).

2.5.6 Application of molecular markers in population studies of *P. violaceum*

2.5.6.1 Estimating gene flow

There are several indirect methods for estimating gene flow, based on the analysis of allele frequencies in populations. Some of these methods have been reviewed by McDermott & McDonald (1993). In particular, measuring the degree of population differentiation through the calculation of Wright's F-statistic (Wright, 1951) or the partitioning of genotypic variation within and among populations in an 'analysis of molecular variance' (AMOVA; Excoffier *et al.*, 1992) allows inferences to be made on the likelihood of gene flow among populations. High rates of gene flow reduce the likelihood of population differentiation caused by natural selection or random genetic drift. For instance, from the determination of Φ-statistics, from AMOVA, Samils *et al.* (2001a) found differentiation to be low among three populations of *M. larici-epitea* from Sweden. The high dispersal capacity of urediniospores and aeciospores among these populations (gene flow) was the likely reason why population differentiation was low.

Other approaches of detecting gene flow include observation of frequencies of rare alleles (Slatkin, 1985), or via correlation of phylogenetic distance and geographic distance through nested-clade analyses (Templeton, 1998).

2.5.6.2 Obtaining evidence for clonality and recombination

Evidence for clonal reproduction can be obtained by simple observations of genotype over-representation (Pei & Ruiz, 2000). Clonal lineages within and among populations can also be detected (Anderson & Kohn, 1995). However, the presence of clonal lineages does not necessarily mean there is an absence of sex. Likewise, the presence of variation does not necessarily mean recombination is occurring, as other forces, such as mutation and high rates of gene flow, may be contributing to the genetic diversity of a population.

There are several approaches to test marker data for the occurrence of recombination. One such method is to test for association among amplified loci by calculating the "Index of Association", I_A (Maynard Smith *et al.*, 1993). This statistic is a measure of multilocus linkage disequilibrium and the analysis compares the variance of all pairwise distances to an expected variance where there is no linkage disequilibrium (random mating). Random mating is simulated by re-sampling alleles at each locus, without replacement, over many repetitions (>500) with the null hypothesis that the population is randomly mating (I_A=0). If the observed I_A is significantly greater than zero, the null hypothesis is rejected in favour for gametic disequilibrium.

Another method for ascertaining reproductive mode relies on phylogenetic inferences of clonal and recombining populations. The ancestry of individuals in clonal populations can be well correlated to a phylogenetic tree of good fit since each individual has one ancestor and

because there is no horizontal transfer of genes. However, in recombining populations, there will be little or no phylogenetic consistency because each locus may have a different phylogeny. The test, also called the "parsimony tree length permutation test" (PTLPT; Burt *et al.*, 1996), involves comparing the observed length of the most parsimonious tree to the lengths expected in a recombining population. As with I_A, recombination is simulated by reassembling alleles at each locus over many permutations, with the null hypothesis being panmixis. If the observed length of the most parsimonious tree is significantly shorter than the expected, then the null hypothesis of random mating is rejected.

Both the I_A test and the PTLPT have been used to test for recombination in numerous microorganisms, where multilocus variation has been observed, but for which the basis of variation was not well understood. However, many of these studies have concerned haploid organisms. Milgroom (1996) discusses the difficulty of estimating gametic disequilibrium in diploid and dikaryotic fungi, especially when the marker system is dominant. However, with the advent of high ratio multiplex markers, such as AFLP and SAMPL, the I_A test may be used in studies of diploid populations with increased confidence. For example, the I_A test was applied to AFLP data to show that two Swedish populations of the diploid rust fungus *Melampsora larici-epitea* were panmictic whereas a significant level multilocus disequilibrium was observed in a population from Northern Ireland (Samils *et al.*, 2001b).

Similarly, the PTLPT has been used to test for random mating in *P. striiformis* f. sp. *tritici* (Hovmøller *et al.*, 2002). This diploid rust fungus is thought to be strictly clonal and its alternate host has not been found. Although little multilocus variation exists in the fungus, high rates of mutation coupled with strong directional selection at virulence loci appears to be the main evolutionary forces generating new pathotypes of the rust (Steele *et al.*, 2001). From

AFLP typing of *P. striiformis* f. sp. *tritici* populations from France, Germany and the United Kingdom, Hovmøller *et al.* (2002) revealed 20 electrophoretic types among 42 isolates. Testing for recombination using the PTLPT on clone corrected data (see below) revealed the observed tree length to be 37 steps which was significantly shorter than the expected mean length of trees if random mating were occurring (82 steps). This allowed for the null hypothesis that populations of *P. striiformis* f. sp. *tritici* in north-western Europe were undergoing random mating to be rejected and that they were likely to be clonal.

At best, the I_A and PTLPT provide estimates of the deviation of population structure from panmixis (Milgroom & Peever, 2003), and should not be used as a relative measure of recombination. Although evidence for random mating can be significant, it cannot be assumed to be absent if both tests reject the null hypothesis of panmixis. In populations that undergo alternate cycles of clonal and sexual reproduction, recombination may occur at very high rates. However, there may be a strong directional selection in a population so that certain genotypes proliferate through clonal reproduction. If high rates of recombination are followed by clonal selection leading to over-representation of genotypes, then analyses based on a null hypothesis of panmixis would be confounded. Likewise, the tests may be confounded due to the migration and fixation of rare alleles into a population or even through loss of certain genotypes from strong directional selection. The problem of genotype over-representation can be minimized by an appropriate sampling procedure. Variables that can be addressed during sampling include sampling time, sample area and appropriate sample sizes (Samils *et al.*, 2001b), plus clone correction of the data set, where putative clones are removed, such that there is one representative of each electrophoretic phenotype present in the population sample (Chen & McDonald, 1996).

2.6 Summary

The use of DNA markers throughout the biological control process has merit, including identification of host diversity, cataloguing and identifying strains of biological control agents, and studying the fate of a strain once it has been released into the environment. The recent taxonomic update of exotic *Rubus* in Australia highlights the diverse nature of the *R. fruticosus* taxa naturalised in this country, which makes research on biological control complicated. To further compound the biocontrol issue, the success of establishment of the first sanctioned biocontrol strain of *P. violaceum*, F15, is questionable, based on results of recent RFLP studies (Evans *et al.*, 2000). However, recombination events may have occurred in the field, in which case the persistence of the F15 genotype would have been diminished. Since there is no information about the prevalence of recombination in the field, the fate of F15 since its release in 1991 remains speculative.

The goal of the blackberry rust biological control program is not simply matching virulent pathogen strains to susceptible weed biotypes but also ensuring that each naturalised blackberry infestation in Australia has access to appropriate rust strains. Knowledge of the population structure of *P. violaceum* in the field and how it changes is the basis for understanding what constitutes an ideal founding population for effective biological control. Molecular markers offer a means by which to study evolutionary processes within and among plant pathogen populations. In particular, the high multiplex ratio markers, such as AFLP, offer an efficient means to assess many polymorphic loci. SAMPL, a modification of the AFLP technique, allows for the analysis of potentially greater levels of polymorphisms with a higher proportion of co-dominance than AFLP.

Understanding the relative importance of various evolutionary forces in *P. violaceum*, such as recombination and gene flow, will contribute to the theory of strain selection. It will also contribute to the development of release strategies that maximise the opportunity for appropriate strains of *P. violaceum* and/or their virulence genes to establish and persist where needed for effective, long-term, biological control of European blackberry.

2.7 Project aims

Having defined knowledge gaps and current priorities for research, the objectives of the work presented in this thesis are to:

1 Develop, compare and validate the use of AFLP and SAMPL for genotyping isolates of *P. violaceum* from Europe, Australia and New Zealand, and for application in population genetic studies.

2 Apply DNA markers to determine the structure of genetic variation within and among five populations of *P. violaceum* in Victoria for estimating gene flow and the prevalence of recombination.

3 Determine if three genetically distinct rust strains, representing three virulence phenotypes, vary in infection efficiency on leaves of *R. anglocandicans* as a function of leaf age.

3 General material and methods

3.1 Propagation of plant material and fungal isolates

3.1.1 Propagation of plant material

Young leaves from potted plants of *R. anglocandicans* A. Newton were used for the propagation of single uredinium-derived isolates of *P. violaceum*. Crowns of *R. anglocandicans* collected from field infestations were propagated clonally either by taking semi-hardwood to hardwood cuttings or by daughter plant production following tip-rooting of vegetative cane apices in potting soil. Cuttings with three nodes were selected from the vegetative shoot. The base of the cutting was treated with 3 $g.kg^{-1}$ indol-3-butyric acid (Yates, Milperra, NSW) and planted into trays containing sand at a depth of 15 cm or a 1:1 sand:peat mixture. After 6 to 8 weeks, cuttings with roots were planted into 20 cm diameter pots containing commercial garden potting mix (Nu-Earth Premium Potting Mix). Plants were kept in a controlled environment room (CER) at 20°C with a photoperiod of 16 h (halogen white light, 100 $\mu mol.m^{-2}s^{-1}$). Plants were watered every second day and fertilized every 6 weeks with a liquid fertilizer (Aquasol, Hortico, N:P:K 23:4:18). Two spotted mites (*Tetranychus urticae*) were managed by applying label rates of the ovicide, Apollo® (AgrEvo), and the miticide, Omite® (Crompton Uniroyal Chemical), every 6 weeks.

3.1.2 Propagation of *P. violaceum*

The method by which urediniospores of *P. violaceum* were multiplied was based on methods described by Evans *et al.* (2000), as described below:

3.1.2.1 Leaf harvest and inoculation

Young leaves were harvested from potted plants and individual leaflets were excised from the detached leaves and placed, abaxial surface uppermost, on 25 ml of 1% water agar (Bitek™ Agar, Difco, Becton Dickinson) contained in 100 mm x 20 mm Falcon® (Becton Dickinson) tissue culture dishes. Detached leaflets were inoculated in one of two ways, depending on the source of inoculum, as follows:

A. Inoculation of leaflets using frozen spores of a known mass:

Frozen spores stored at -80°C in 1.5 ml Eppendorf tubes were heat-shocked for 3 min in a 40°C water bath. Sterile distilled water (SDW) was added to spores to form a suspension of 0.5 mg spores.ml^{-1}.

Leaflets were inoculated with the spore suspension using a Preval® power sprayer unit (Precision Valve Corporation). The spore suspension was sprayed on the detached leaflets to provide a fine and even coverage of droplets on the epidermis. After inoculation, leaflets were incubated in a growth cabinet at 20°C with a 16 h photoperiod (fluorescent white light, 40 µmol.m^{-2}.s^{-1}).

B. Inoculation of leaflets from diseased material:

Single uredinium-derived individuals were isolated from infected leaves by transferring spores from a single pustule to detached leaflets on water agar. This was achieved by touching individual pustules with a sterilised artists' paint brush (synthetic, size 0 or 1) and spores lightly brushed onto 'clean' detached leaflets. Leaflets were incubated as described above.

3.1.2.2 Leaflet sterilization and collection of spores

Following 6 days of incubation and before pustules were erumpent, inoculated leaflets were surface sterilised in a 0.15% solution of mercuric chloride. Under aseptic conditions, leaflets were soaked in the mercuric chloride solution for 1.5 min, and then rinsed three times in SDW. Leaflets were placed in fresh 1% water agar plates, abaxial surface uppermost and the surface allowed to dry in the laminar flow hood.

Plates were incubated, as above, for another 7 days, at which time pustules had developed and were ready for harvesting. Spores to be used in subsequent inoculations were harvested using a cyclonic spore harvester, as illustrated in Evans *et al.* (1996), and collected in pre-weighed Eppendorf tubes. Tubes were then weighed and the final mass of harvested spores determined.

For those leaflets which were inoculated from diseased leaf material from the field, a second round of single pustule isolation was performed by transferring spores from individual pustules to newly harvested leaflets. Incubation and sterilization of leaflets was repeated and the spores harvested as described above.

Harvested spores were then dried at room temperature (*ca* 20°C) for 24 h on racks in sealed plastic containers containing a saturated solution of $CaCl_2$. Tubes were then sealed with Parafilm® (American National Can), snap frozen in liquid nitrogen and stored at -80°C until needed.

3.2 Extraction of DNA from urediniospores of *P. violaceum*

DNA was extracted from urediniospores of each isolate using a modified CTAB extraction protocol. A 500 µl volume of CTAB buffer, pre-heated to 65°C, was added to 5 mg of urediniospores and 20 sterile 2 mm diameter glass beads in a 2 ml microcentrifuge tube. The CTAB buffer contained 20 $g.L^{-1}$ hexadecyltrimethylammonium bromide (CTAB), 1.4 M NaCl, 100 mM Tris.HCl pH 8.0, 20 mM EDTA pH 8.0, 2 $ml.L^{-1}$ ß-mercaptoethanol and 10 $g.L^{-1}$ polyvinylpyrrolidone-360 (PVP-360). Tubes were placed on a horizontal flat-bed vortex adapter (Mo Bio) and the samples vortexed at maximum velocity (setting 8) using a Vortex Genie 2 (Scientific Industries) for 2 min.

Tubes were then incubated at 65°C for 20 min and nucleic acids extracted with 500 µl chloroform-isoamyl alcohol (24:1 v/v) for 10 min. After centrifugation at 20 800 x ***g*** (r_{av} 9.5 cm) for 10 min, the upper aqueous phase (*ca* 400 µl) was removed to a new tube and 2/3 volume (*ca* 270 µl) of ice-cold isopropanol added. DNA was allowed to precipitate for 1 h on ice before centrifugation at 20 800 x ***g*** for 10 min. The supernatant was discarded and the DNA pellet was then washed twice in ice-cold 70% ethanol for 10 min. Samples were centrifuged at 20 800 x ***g*** for 10 min and the ethanol discarded. Pellets were allowed to air-dry before being dissolved in 20 – 40 µl of SDW. Nucleic acid preparations (2 µl) were separated by electrophoresis on a 1% TAE agarose gel at 70 V for 1.5 h alongside 250 ng of a λ-*Hin*dIII DNA quantitative standard. After electrophoresis, the gel was stained for 15 min with

ethidium bromide (1 $\mu g.ml^{-1}$ in TAE buffer). Nucleic acids were visualised under UV light, and the concentration of the DNA estimated by relating the intensity of bands of the genomic DNA to the intensity of bands provided by the quantitative standard. The remaining DNA preparations were stored at -20°C until needed.

3.3 Preparation of size ladder for AFLP and SAMPL

A 30 – 330 bp AFLP DNA ladder (Life Technologies) was used to provide a relative size reference for AFLP and SAMPL products. The ladder consisted of 31 fragments at 10 bp increments between 30 and 330 bp with one fragment at 1668 bp and two diffuse bands at 10 and 20 bp.

The AFLP ladder was labelled according to the manufacturer's protocol which is summarised here. The DNA ladder was end-labelled with [γ-^{32}P] ATP by combining 2 µl of 30 – 330 bp ladder with 10 U T4 polynucleotide kinase, 1X Exchange Reaction Buffer (250 mM imidazole pH 6.4, 60 mM $MgCl_2$, 5 mM 2-mercaptoethanol, 350 µM ADP) and 10 µCi [γ-^{32}P] ATP in a final reaction volume of 5 µl. Contents were collected at the bottom of the tube by brief centrifugation and incubated at 37°C for 10 min. The reaction was then terminated by heating to 65°C for 15 min. TE buffer, 5 µl, was added to the labelled ladder followed by 25 µl of denaturing loading buffer (98% v/v de-ionized formamide, 10 mM EDTA pH 8.0, 0.025% w/v bromophenol blue and 0.025% w/v xylene cyanol). The mixture was then denatured at 70°C for 5 min and placed on ice immediately. Two 3 µl aliquots of the mixture were separated by electrophoresis (section 3.4.5) on each gel along with AFLP and SAMPL products.

3.4 Detection of SAMPL

3.4.1 Preparation of adapters

Double-stranded adapters (Table 4.2, p. 57) were prepared by combining the following components in two separate microcentrifuge tubes: (1) *Hae*III adapter solution: 64 µl of H-adap1 oligonucleotide (500 $ng.\mu l^{-1}$) and 56 µl of H-adap2 oligonucleotide (500 $ng.\mu l^{-1}$). (2) *Pst*1 adapter solution: 3.4 µl P-adap1 oligonucleotide (1 $\mu g.\mu l^{-1}$), 3.0 µl P-adap2 oligonucleotide (1 $\mu g.\mu l^{-1}$) and 113.6 µl of sterile water. Tubes were incubated at 94°C for 5 min, 65 °C for 10 min, 25°C for 10 min and at 4°C for 10 min. Adapter solutions were stored at -20°C until further use.

3.4.2 Digestion and ligation of DNA preparations

DNA, 250 ng, from isolates of *P. violaceum* was digested with 5 units each of *Hae*III (Promega) and *Pst*I (Promega) in the presence of 1X Restriction Buffer C (Promega) and 2.5 µg BSA, in a final volume of 25 µl. Mixtures were incubated for 4 h at 37°C followed by termination of the reaction at 70°C for 15 min.

A ligation mixture, consisting of 1 µl each of *Hae*III and *Pst*I adapter solutions, 1X T4 ligase buffer, 1 unit T4 DNA ligase, adjusted to 10 µl with SDW, was added to the digested DNA. Ligation reactions were incubated at 20 ± 2°C for 8 h and diluted 1 in 10, in SDW. Ligation preparations were stored at -20°C until needed.

3.4.3 Pre-selective amplification of digested DNA preparations

Pre-amplification reactions were performed in 200 µl thin-walled PCR tubes in a total volume of 51 µl containing 5 µl of diluted ligation preparation, 1 unit Taq DNA polymerase (Promega; storage buffer A), 1X magnesium-free PCR buffer (Promega), 1.5 mM $MgCl_2$ (Promega), 200 µM each dNTP, 75 ng primer P-0 (Table 4.2) and 75 ng of primer H-0 (5'–GATGAGTCCTGAGCC–3'). Pre-amplifications were carried out in an Eppendorf Mastercycler Gradient PCR machine with a thermocycle consisting of 20 cycles of 94°C for 30 s, 56°C for 60 s and 72°C for 60 s. Each pre-amplification product was then diluted 1 in 50 with SDW and diluted pre-amplification products were stored at -20°C if not used immediately.

3.4.4 Selective PCR amplification

Variable number tandem repeat (VNTR) primers were end-labelled with [γ-^{32}P]ATP by combining 5**x** ng of VNTR primer, 2**x** µCi [γ-^{32}P]ATP, 0.2**x** U T4 polynucleotide kinase (Promega), and 1X kinase buffer (Promega) in a final volume of 0.5**x** µl, where **x** is the number of AFLP reactions. Labelling was performed at 37°C for 1 h and the reactions were terminated at 70°C for 10 min.

Selective SAMPL reactions were performed in 200 µl thin-walled PCR tubes in a final volume of 20 µl consisting of 5 ng of [γ-^{32}P]ATP end-labelled VNTR primer, 30 ng of primer H-G (5'–GATGAGTCC TGAGCCG–3'), 200 µM each dNTP, 1X magnesium-free PCR buffer (Promega), 2.5 mM $MgCl_2$, 200 µM each dNTP, 0.5 units Taq DNA polymerase (Promega) and 5 µl of diluted pre-amplification product. Selective PCRs were performed in an Eppendorf Mastercycler Gradient PCR machine using a touchdown thermocycle where the annealing temperature was lowered by 0.7°C after each cycle consisting of 13 cycles of 30 s

at 94°C, 30 s at 65°C, 60 s at 72°C. This was followed by 23 cycles of 30 s at 94°C, 30 s at 56°C and 60 s at 72°C. After the selective PCR, 20 µl of a denaturing formamide loading buffer was added to each PCR tube. The samples were denatured at 95°C for 5 min and immediately placed on ice.

3.4.5 Electrophoresis conditions and visualisation of SAMPL

3.4.5.1 Gel preparation

Denaturing polyacrylamide gel electrophoresis was used to separate SAMPL. SequaGel® 6 (National Diagnostics) was prepared and used according to the manufacturer's protocol, and is briefly described below:

A 6% polyacrylamide sequencing gel with 6 M urea was prepared by mixing SequaGel Monomer Solution with SequaGel Complete Buffer at a ratio of 4:1 in a final volume of 60 ml in a clean beaker. Freshly prepared 10% (w/v) ammonium persulfate, 400 µl, was added to the SequaGel mix and the solution gently swirled and drawn into a clean 60 ml syringe. Using the syringe, the solution was poured horizontally between two horizontal glass plates separated by 0.4 mm plastic spacers. The glass plates had previously been washed thoroughly with Extran® (MERCK Pty Ltd) detergent and rinsed with reverse osmosis (RO) water, followed by a final rinse with 100% ethanol and dried by wiping with extra low lint paper wipers (Kimwipe®, Kimberly-Clark® Professional).

Once the gel solution had been poured, the flat edge of a 96 well shark's-tooth comb was inserted at the top of the gel, forming a flat gel front. The gel was allowed to polymerise for at

least one hour before use. Prior to use, the sharks-tooth comb was removed from the polymerised gel and the front washed with RO water followed by 1X TBE buffer.

3.4.5.2 Electrophoresis conditions

Using a Hoefer SQ3 Sequencer electrophoresis unit, the gel was pre-run in 1X TBE buffer at 55 W constant power until the gel temperature reached between 45 and 50°C. After the pre-run, the gel front was rewashed with 1X TBE buffer and the shark's-tooth comb re-inserted to form wells ready for loading samples. SAMPL were then separated by loading 3 µl of each denatured selective PCR product per well and the samples electrophoresed at 55 W until the xylene cyanol dye front migrated two-thirds of the length of the gel (*ca* 1.5–2 h).

3.4.5.3 Gel drying and autoradiography

The assembly was removed from the electrophoresis unit and the plates cooled at room temperature for 20 min. The glass plates were then carefully disassembled and the gel transferred to 3 mm chromatography paper (Whatman® International). The exposed gel was covered with cling wrap and the gel dried at 80°C in a Model 583 Gel Dryer (Bio-Rad) for 1 h. Once dry, gels were exposed to Kodak X-OMAT AR Film for 16 h, after which, the film was developed using a CP1000 (Agfa) automated developer.

4 Development of molecular markers for *P. violaceum*

4.1 Introduction

DNA markers are useful tools for elucidating processes driving pathogen change and pathogen population dynamics. The determination of factors contributing to pathogen evolution can be used to gain insight on the dynamics of plant disease epidemics (Milgroom & Peever, 2003). The development of efficient DNA markers will allow the study of the likely evolutionary processes contributing to pathogen evolution of *P. violaceum* in Australia.

Prior to authorised releases of additional strains of *P. violaceum* in Australia in 2004, Evans *et al.* (2000) identified restriction fragment length polymorphisms (RFLPs) using M13 DNA to probe genomic DNA of Australian isolates of *P. violaceum* digested with the restriction enzyme *Hae*III. The robustness of this technique was demonstrated when 13 DNA phenotypes were identified among 18 single uredinium-derived isolates of *P. violaceum* from various locations in mainland Australia between 1997 and 1999 (Evans *et al.*, 2000). This DNA marker also identified strain F15 of *P. violaceum* as being genetically distinct from the 18 isolates screened in the same study. The disadvantage of the RFLP protocol is that it requires relatively large quantities of DNA when compared to a PCR-based marker system. Furthermore, the M13 RFLP technique of Evans *et al.* (2000) was limited to analysis of restriction fragments in the range 4 to 9 kbp and additional markers were deemed necessary to expand the proportion of the genome analysed for elucidating genetic diversity.

AFLPs are used frequently in studies of genetic diversity in plants and fungi due to their ease of development, efficiency, high multiplex ratio and reproducibility (Zabeau & Vos, 1993; Majer *et al.*, 1996). AFLPs have been demonstrated in several species of Uredinales (Pei &

Ruiz, 2000; Samils *et al.*, 2001a; Samils *et al.*, 2001b; Hovmøller *et al.*, 2002; Keiper *et al.*, 2003; Yourman & Luster, 2004). SAMPL, a modification of the AFLP procedure (Morgante & Vogel, 1994), combines the advantages of AFLP with the analysis of highly variable microsatellite regions of eukaryotic genomes. SAMPL has been reported to be more powerful than AFLPs in discriminating between closely related individuals in several plant complexes (Paglia & Morgante, 1998; Singh *et al.*, 2002; Tosti & Negri, 2002; Roy *et al.*, 2002) and in the wheat stripe rust pathogen, *Puccinia striiformis* f. sp. *tritici*, which is strictly clonal (Keiper *et al.*, 2003).

This chapter details the development of a modified SAMPL technique using adapters linked to the blunt end restriction enzyme, *Hae*III, following on from the useful resolution identified by RFLPs using this same enzyme (Evans *et al.*, 2000). The superiority of SAMPL, relative to AFLP, in describing genetic variation among isolates of *P. violaceum* from Europe and Australasia, is demonstrated.

4.2 Materials and methods

4.2.1 Origin of isolates and extraction of DNA

A total of 44 single uredinium-derived isolates of *P. violaceum* were analysed through the course of developing markers for the fungus (Table 4.1). Eighteen single uredinium-derived isolates of *P. violaceum* from Australia originated from collections made by Evans *et al.* (2000). For the purpose of genetic analysis, isolates of *P. violaceum* from Australia and the seven single uredinium-derived isolates collected in New Zealand are referred to collectively as the Australasian isolates. Eleven of the 19 European isolates were collected by Bruzzese & Hasan (1986) and eight isolates were recovered from Australian genotypes of European

blackberry growing in an experimental *Rubus* trap garden at the CSIRO European laboratories, Montpellier, France in 2000 (Scott *et al.*, 2002). Single uredinium-derived isolates of *P. violaceum* from Australia and isolates collected by Bruzzese & Hasan (1986) in Europe were propagated on detached leaflets of *Rubus anglocandicans* A. Newton. Strains collected from the trap garden in France were propagated on detached leaves of the *Rubus* clone from which the isolate was collected originally (Scott *et al.*, 2002). Apart from strain F15, all European isolates were propagated and maintained by CSIRO in France. Isolates of *P. violaceum* collected in New Zealand were propagated on detached leaflets of *R. ulmifolius* Schott and urediniospores were stored using procedures described by Bruzzese & Hasan (1986) or Evans *et al.* (2000). Propagation of these isolates was performed by Landcare Research, New Zealand. Voucher specimens of uredinia of Australian isolate SA1 of *P. violaceum* are stored at the Plant Pathology Herbarium of the New South Wales Department of Primary Industries as DAR 75508, DAR 75509 and DAR 75561, respectively.

DNA from the Australian and European isolates was extracted from urediniospores of each isolate using a modified CTAB extraction protocol (as detailed in section 3.2). Nucleic acids from urediniospores of New Zealand isolates of *P. violaceum* were extracted by staff from Landcare Research, New Zealand, using the method reported by Evans *et al.* (2000).

4.2.2 Marker development: AFLP

The following 20 primer pair combinations were used to generate fingerprints for isolates SA1, V1 and V2: E-AA + M-0, E-AA + M-A, E-AA + M-C, E-AA + M-G, E-AA + M-T, E-AC + M-0, E-AC + M-A, E-AC + M-C, E-AC + M-G, E-AC + M-T, E-0 + M-0, E-0 + M-A, E-0 + M-C, E-0 + M-G, E-0 + M-T, E-A + M-0, E-A + M-A, E-A + M-C, E-A + M-G and E-A + M-T. Primer pairs E-AC + M-C, E-0 + M-0 and E-0 + M-T were also used to generate

Table 4.1 Origin of isolates collected in Australia (reproduced from Evans *et al.* 2000), New Zealand and Europe.

Isolate	Source		Year collected
	Location[a]	Host[b]	
Australia			
V1	Cobden to Port Campbell Road, Vic.	*R. leucostachys*	1997
V2	Foster, Vic.	*R. leucostachys*	1997
SA1	Cleland Conservation Park, SA	*R. anglocandicans*	1998
WA10	Nanarup, WA	*R. anglocandicans*	1998
WA11	Yakamia drain, WA	*R. anglocandicans*	1998
WA12	West of Hall, WA	*R. anglocandicans*	1998
WA9	Albany, WA	*R. anglocandicans*	1998
WP21	Sassafras, NSW	*R. anglocandicans*	1998
WP69	Princetown, Vic.	*R. anglocandicans*	1998
WP43	Strzelecki Ranges, Vic.	*R. cissburiensis*	1998
WP59	Creswick, Vic.	*R. laciniatus*	1998
WP52	Foster, Vic.	*R. leucostachys*	1998
WP70	Gellibrand, Vic.	*R. vestitus*	1998
WP42	Jeeralong, Vic.	*Rubus sp.*	1998
WP90	Hiawatha, Vic.	*R. anglocandicans*	1999
9901	Belair Nation Park, SA	*R. anglocandicans*	1999
9902	Mount Lofty, SA	*R. anglocandicans*	1999
R3WP3	Red Hill, Vic.	*R. leucostachys*	1999
New Zealand			
PV17B	Kaikoura, Canterbury, SI	*R. cissburiesis*	2002
PV13F	Rainbow Mountain, Taupo, NI	*R. nemoralis*	2002
PV15E	Eltham, Taranaki, NI	*R. procerus*	2002
PV23C	Conical Hill, Southland, SI	*R. procerus*	2002
PV11B	Parau, Auckland, NI	*R. ulmifolius*	2002
PV12A	Waioeka, Bay of Plenty, NI	*R. ulmifolius*	2002
PV8B	Pahiatua, Wellington, NI	*R. vestitus*	2001
Europe[c]			
BG25	Rila, Bulgaria	*R. procerus*	1979
BG26	Kalojanovo, Bulgaria	*R. procerus*	1979
E3	Navaleno, Spain	*R. procerus*	1978-1980
E6	San Jorge, Spain	*R. ulmifolius*	1978-1980
F12	La Mejanelle, France	*R. fruticosus* subg. *Vestiti*	1978-1980
F14	St. Victor, France	*R. ulmifolius*	1978-1980
F15[d]	Chalon-sur-Saône, France	*R. procerus*	1978-1980
I19	Tovo Santa Agata, Italy	*R. procerus*	1978-1980
YU20	Prozor, Yugoslavia	*R. procerus*	1979
YU21	Mostar, Yugoslavia	*R. ulmifolius*	1979
YU22	Skopje, Yugoslavia	*R. procerus*	1979
G32-TG-00-1-1[e]	Montpellier trap garden[f]	*R. anglocandicans*	2000
G32-TG-00-2-2[e]	Montpellier trap garden	*R. anglocandicans*	2000
G32-TG-00-4-1	Montpellier trap garden	*R. anglocandicans*	2000
G21-TG-00-1-1[e]	Montpellier trap garden	*R. leucostachys*	2000
G6-TG-00-4-1[e]	Montpellier trap garden	*R. leucostachys*	2000
G7-TG-00-2-1	Montpellier trap garden	*R. leucostachys*	2000
G28-TG-00-2-1[e]	Montpellier trap garden	*R. vestitus*	2000
G14-TG-00-4-1[e]	Montpellier trap garden	*R.* sp. clone SR43	2000

[a]Vic. = Victoria, WA = Western Australia, SA = South Australia, NSW = New South Wales, Australia. NI = North Island and SI = South Island, New Zealand.
[b]The taxon described as *R.* affin. *armeniacus* by Evans *et al.* (2000) has been renamed here *R. anglocandicans,* according to Evans & Weber (2003).
[c]Refer to Bruzzese & Hasan (1986) for a map or European collection sites during 1978-1980.
[d]Strain F15 was recovered from frozen urediniospores, originally multiplied and stored at the Keith Turnbull Research Institute (Department of Primary Industries, Victoria) in 1991.
[e]One of eight strains of *P. violaceum* released in Australia in 2004.
[f]All plants in the CSIRO Montpellier trap garden originated from naturalised infestations of *R. fruticosus* agg., in Australia.

fingerprints for seven reference isolates of *P. violaceum*: F15, SA1, V1, V2, WA9, WA12 and WP69. AFLPs were detected using AFLP™ Analysis System for Microorganisms and the AFLP Microorganism Primer Kit (Life Technologies), according to the manufacturer's protocol, as summarised below.

4.2.2.1 Digestion and ligation of DNA preparations

DNA (250 ng) from the seven reference isolates of *P. violaceum* were digested with 2.5 units each of restriction enzymes *Eco*RI and *Mse*I in 25 µl of reaction buffer. Reactions were incubated at 37°C for 2 h followed by termination of the reaction at 70°C for 15 min.

To each digested sample, 24 µl of adapter ligation solution (Life Technologies, *Eco*RI/*Mse*I adapters, 0.4 mM ATP, 10 mM Tris-HCl pH 7.5, 10 mM Mg-acetate, 50 mM K-acetate), and 1 unit T4 DNA Ligase (Life Technologies) were added. Ligation preparations were incubated at 20°C ± 2°C for 2 h. Following ligation, preparations were diluted 1 in 10 by mixing 10 µl of ligation preparation with 90 µl of SDW. The remaining ligation preparation was stored at -20°C.

4.2.2.2 Pre-amplification of digested DNA preparations

Pre-amplification reactions were performed as described in section 3.4.3 with the exception of 75 ng primer E-0 (life technologies) and 80 ng primer M-0 (life technologies) used instead of primers P-0 and H-0.

4.2.2.3 Selective PCR amplification and detection of AFLPs

A labelling master mix consisting of 5**x** ng of *Eco*RI primer, 2**x** μCi [γ-^{32}P]ATP, 0.2**x** units T4 polynucleotide kinase (Promega), and 1X kinase buffer (Promega) in a final volume of 0.5**x** μl was prepared, where **x** was the number of AFLP reactions. Labelling reactions were incubated at 37°C for 1 h and the reactions were terminated at 70°C for 10 min.

Selective AFLP reactions were performed in a final volume of 20 μl consisting of 5 ng of [γ-^{32}P]ATP end labelled *Eco*RI (E-n) primer, 30 ng *Mse*I (M-n) primer, 200 μM each dNTP, 1X magnesium-free PCR buffer (Promega), 1.5 mM $MgCl_2$, 200 μM each dNTP, 0.5 units Taq DNA polymerase (Promega) and 5 μl of diluted pre-amplification product. Selective PCRs were performed in an Eppendorf Mastercylcer Gradient PCR machine using the touchdown thermocycle as described in section 3.4.4.

4.2.2.4 Electrophoresis of AFLP products and autoradiography

Following selective PCR, 20 μl of formamide loading buffer was added to each reaction and mixed well. Samples were denatured at 95°C for 3 min and immediately placed on ice.

For each sample, 3 μl of amplified fragments were separated electrophoretically at constant power (40W) using a 40 x 30 cm, 0.4 mm thick, 8% denaturing polyacrylamide gel (19:1 acrylamide:bis-acrylamide; 6 M urea; 1X TBE buffer) pre-heated to 50°C on a Hoefer SQ3 Sequencer electrophoresis apparatus. After electrophoresis, gels were dried at 80°C for 1 h and exposed to Kodak X-OMAT AR film for 16 h.

4.2.3 Modification of AFLP procedure

The AFLP procedure was modified for the use of the restriction enzymes *Hae*III and *Pst*I. *Pst*I adapters and their associated primers were obtained from those reported by Zabeau & Vos (1993) and Vuylsteke *et al.*, (1999). Adapters linked to the blunt end restriction enzyme, *Hae*III, were developed for this project and the sequences for this and the respective primers are listed in Table 4.2. Blunt ended adapters for use with the restriction enzyme *Hae*III were developed based on *Mse*I adapter sequences developed by Vos *et al.* (1995). *Pst*I and *Hae*III adapters were then prepared as described in section 3.4.1.

4.2.3.1 Detection of AFLPs using DNA digested with *Hae*III and *Pst*I

DNA, 250 ng, extracted from the seven reference isolates of *P. violaceum* was digested with *Hae*III and *Pst*I and the associated linkers ligated as detailed in section 3.4.2.

Pre-amplification and selective AFLP reactions were performed as described in sections 4.2.2.2 and 4.2.2.3, with the following changes:

1) Pre-amplifcation PCR (section 4.2.2.2):

   *EcoR*I and *Mse*I primers were replaced with 75 ng each of *Pst*I (P-0) and *Hae*III (H-0) primers in the pre-amplification.

2) End-labelling of primers (section 4.2.2.3):

   Selective *Pst*I primers (5 ng per reaction) were end labelled instead of end labelling *Eco*RI primers.

3) Selective AFLP reactions (section 4.2.2.3):

Labelled *Eco*RI primer was replaced with 0.5 ng of labelled selective *Pst*I primer and *Mse*I primer was replaced with selective *Hae*III primer (Table 4.2).

AFLP products were then denatured and separated by electrophoresis in 8% denaturing polyacrylamide gels as described in section 4.2.2.4.

Table 4.2 Sequences of adapters and primers used in modified AFLP and SAMPL analysis

Oligonucleotide		
*Hae*III adaptors	H-adap1	5' – GAC GAT GAG TCC TGA G – 3'
	H-adap2	5' – CTC AGG ACT CAT – 3'
*Pst*I adaptors	P-adap1	5' – CTC GTA GAC TGC GTA CAT GCA – 3'
	P-adap2	5' – TGT ACG CAG TCT AC – 3'
AFLP primer sequences		
*Hae*III pre-selective primer	H-0	5' – GAT GAG TCC TGA GCC – 3'
*Hae*III selective primer	H-A	5' – GAT GAG TCC TGA GCC A – 3'
*Hae*III selective primer	H-T	5' – GAT GAG TCC TGA GCC T – 3'
*Hae*III selective primer	H-C	5' – GAT GAG TCC TGA GCC C – 3'
*Hae*III selective primer	H-G	5' – GAT GAG TCC TGA GCC G – 3'
*Pst*I pre-selective primer	P-0	5' – GAC TGC GTA CAT GCA G – 3'
*Pst*I selective primer	P-AC	5' – GAC TGC GTA CAT GCA GAC – 3'
*Pst*I selective primer	P-AA	5' – GAC TGC GTA CAT GCA GAA – 3'
VNTR primer sequences used for SAMPL analysis		
[A]Intron Splice Junction Primer	R1	5' – GTC CAT TCA GTC GGT GCT – 3'
$(CAC)_5$	CAC_5	5' – CAC CAC CAC CAC CAC – 3'
$(GACA)_4$	$GACA_4$	5' – GAC AGA CAG ACA GAC A – 3'
$(GGAT)_4$	$GGAT_4$	5' – GGA TGG ATG GAT GGA T – 3'
$(TCT)_5$	TCT5	5' – TCT TCT TCT TCT TC T – 3'
$(GATA)_4$	$GGAT_4$	5' – GGA TGG ATG GAT GGA T – 3'

[A] Weining and Langridge (1991)

4.2.4 Marker development: SAMPL

The detection of SAMPL required small changes in the AFLP procedure. These changes are detailed below and the SAMPL protocol described in full in section 3.4 with the exception that amplicons were visualised on 8% denaturing polyacrylamide gels.

DNA, 250 ng, extracted from the seven reference isolates of *P. violaceum* were digested with *Pst*I and *Hae*III and their respective adapters (Table 4.2) ligated and pre-amplification performed as described in section 3.4.

Changes from the AFLP procedure, described in section 4.2.2.3, were made in primer labelling, with 5 ng of a VNTR primer (Table 4.2) labelled instead of selective *Eco*RI (E-n) primers. This was followed by the use of 5 ng of the end-labelled VNTR primer per selective PCR reaction. Table 4.2 lists the VNTR primers used for SAMPL analysis.

The reproducibility and stability of amplified loci generated by primer pairs $(GACA)_4$ + H-G and R1 + H-G were tested across three clonal generations of strain F15. Spores representing three generations of strain F15 were obtained as follows: strain F15 was propagated from frozen spores as indicated in section 3.1. A subset of spores arising from the first generation was transferred to clean leaflets of *R. anglocandicans* for propagation of the second generation of spores. Remaining spores from the first generation were stored at -80°C. The third generation of spores was obtained from inoculations made with a subset of spores from the second generation.

4.2.5 Characterisation of European and Australasian isolates of *P. violaceum*

Fingerprints of the 44 isolates of *P. violaceum* listed in Table 4.1 were generated from SAMPL amplified using primer pairs, $GACA_4$ + H-G and R1 + H-G. SAMPL were generated and visualised on 6% denaturing polyacrylamide gels, as described in section 3.4.

4.2.6 Statistical analysis

The presence (1) or absence (0) of SAMPL or AFLP loci was scored for each of the seven reference isolates of *P. violaceum* used in method development. The resulting binary matrices produced for each primer combination were used to estimate Shannon's information index and Nei's gene diversity for each primer pair (Shannon & Weaver, 1949; Nei, 1973). Percentage polymorphic loci were also calculated. These indices and percentage polymorphic loci were calculated using POPGENE version 1.32 software for Windows (Yeh & Boyle, 1997).

Similarly, binary matrices were produced and diversity indices were estimated for European and Australasian isolates across 51 scoreable loci generated from primer pairs $GACA_4$ + H-G and R1 + H-G. Genetic similarity and grouping divergence among European and Australasian isolates of *P. violaceum* were examined using the phylogenetic software package TREECON for Windows (Van de Peer & De Wachter, 1994). The algorithm for genetic distance described by Nei & Li (1979) was used and a dendrogram based on the unweighted pair group method with arithmetic mean (UPGMA) was generated from 1,000 bootstrap replicates.

4.3 Results

4.3.1 AFLP

No genetic variation was observed among isolates SA1, V1 and V2 using the 20 AFLP primer pairs listed in section 4.2.2 (autoradiograph not shown). Likewise, no genetic variation was detected among the seven representative isolates of *P. violaceum* (F15, SA1, V1, V2, WA9, WA12, WP69) using primer pairs E-AC + M-C, E-0 + M-0 and E-0 + M-T (autoradiograph not shown).

4.3.2 Modified AFLP procedure

Fingerprints of isolates SA1, V1, V2, WA9, WA12, WP69 and F15 generated using primer pairs P-AC + H-A, P-AC + H-T, P-AC + H-C, P-AC + H-G, P-AA + H-A, P-AA + H-T, P-AA + H-C and P-AA + H-G revealed polymorphic loci, in contrast to AFLPs generated using adapters linked to *Eco*RI and *Mse*I. All primer pairs generated in excess of 30 scoreable loci. Primer pairs P-AC + H-T and P-AC + H-C revealed no variation, whereas primer pairs P-AC + H-A and P-AC + H-G revealed one and four polymorphic loci, respectively (Figure 4.1). Primer pairs P-AA + H-A, P-AA + H-T, P-AA + H-C and P-AA + H-G revealed one, four, three and ten discernable polymorphic loci, respectively (Figure 4.2).

4.3.3 SAMPL

SAMPL fingerprints (Figure 4.3) revealed higher levels of polymorphisms among isolates SA1, V1, V2, WP69, WA9, WA12 and strain F15 when compared with AFLP fingerprints generated using primer combinations linked to *Pst*I and *Hae*III. Using 8% denaturing polyacrylamide gels, primer pairs H-G + $GGAT_4$, H-G + R1, H-G + $GACA_4$, H-G + CAC_5, revealed 23, 12, 19 and 13 polymorphic loci, respectively. Polymorphic loci generated by

primer pair H-G + $GGAT_4$ were generally small (80 – 100 bp), whereas other SAMPL primer pairs produced polymorphic loci that were distributed evenly in the size range for SAMPL. Primer pairs TCT_5 + H-G and $GATA_4$ + H-G consistently failed to amplify the DNA. Amplicons generated using primer pairs $(GACA)_4$ + H-G and R1 + H-G were reproducible when DNA extracted from urediniospores representing three clonal generations of strain F15 was used (autoradiograph not shown).

4.3.4 Statistical comparison of modified AFLP and SAMPL

SAMPL, generated using any one of the six primer pairs tested, and modified AFLP markers, generated using all six primer pairs, resolved seven genotypes among the seven reference isolates of *P. violaceum* used during method development (Table 4.3). SAMPL revealed higher levels of diversity than AFLPs despite fewer primer pairs being used to generate SAMPL markers compared to AFLP markers.

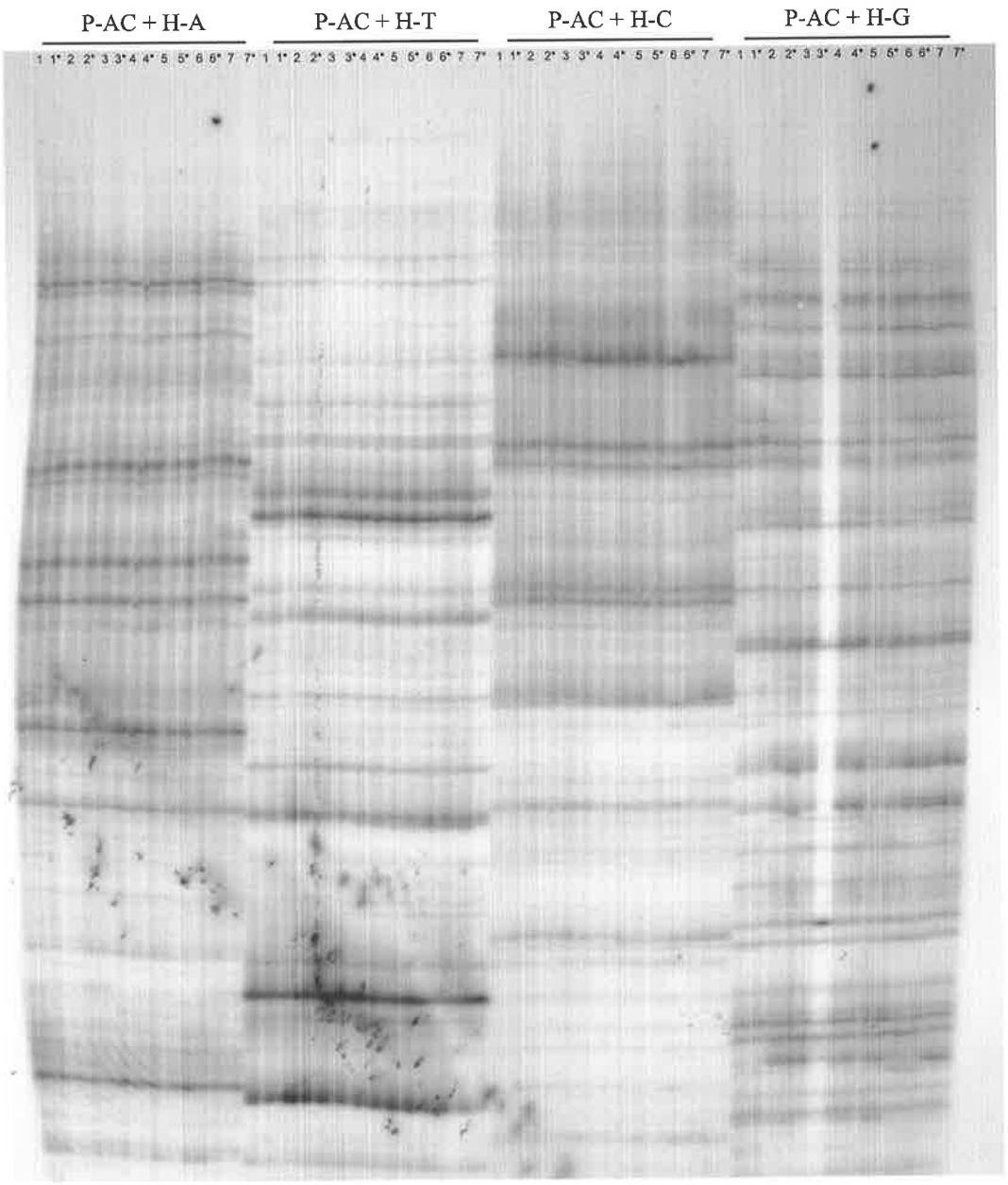

Figure 4.1 Autoradiograph displaying patterns of modified AFLP loci obtained for seven isolates of *P. violaceum*: F15 (1 or 1*), SA1 (2 or 2*), V1 (3 or 3*), V2 (4 or 4*), WP69 (5 or 5*), WA9 (6 or 6*), WA12 (7 or 7*). Primer pairs are indicated above the respective lanes of the autoradiograph. Labelled amplicons were separated electrophoretically using 8% denaturing polyacrylamide gels. Loci separated in the size range of 1000 to 50 bp.

* indicates fingerprint for the same isolate using a different DNA preparation from a different batch of spores.

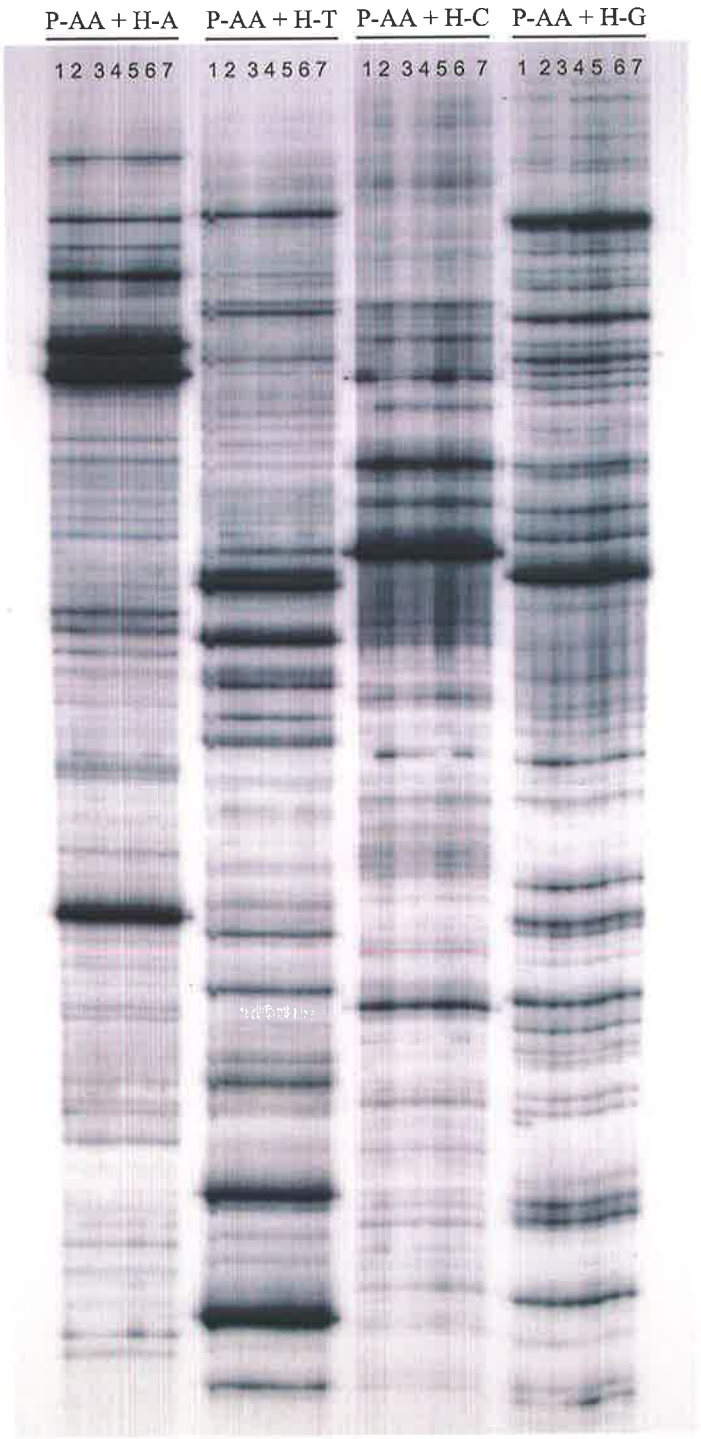

Figure 4.2 Autoradiograph displaying patterns of modified AFLP loci obtained for seven isolates of *P. violaceum*: F15 (1), SA1 (2), V1 (3), V2 (4), WP69 (5), WA9 (6), WA12 (7). Fingerprints generated using four primer pairs: P-AA + H-A, P-AA + H-T, P-AA + H-C, P-AA + H-G and labelled amplicons separated by electrophoresis on 8% denaturing polyacrylamide gels.

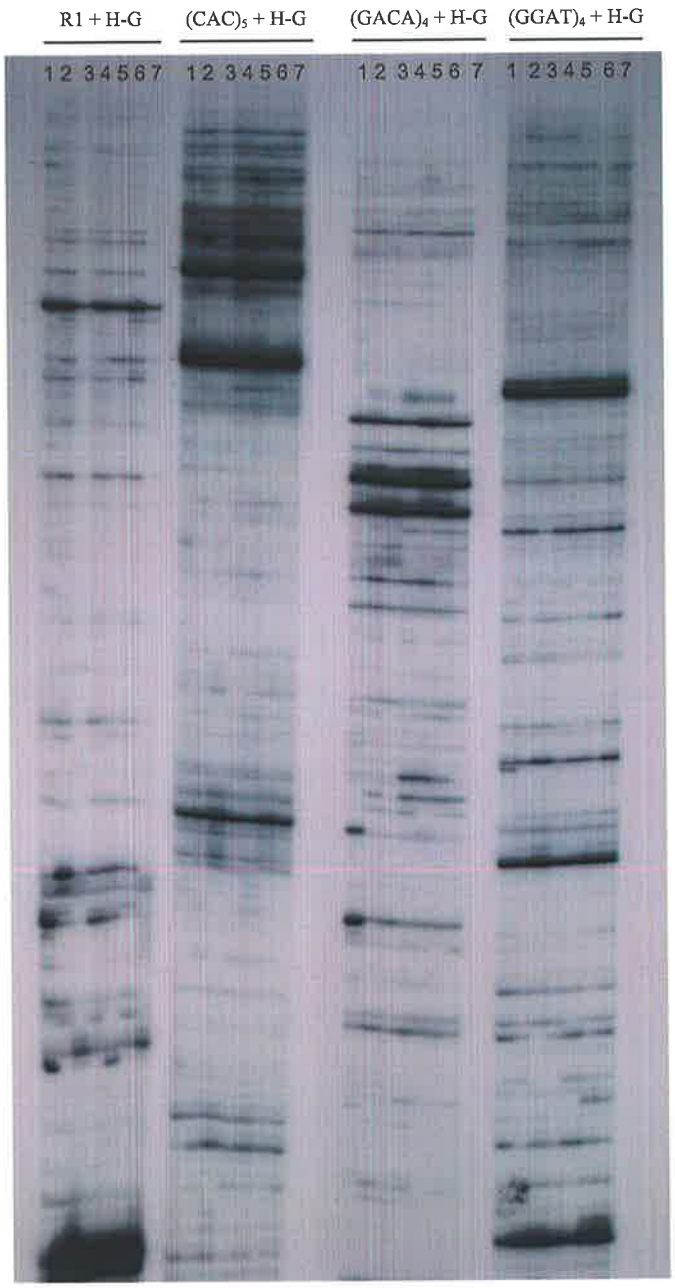

Figure 4.3 Autoradiograph displaying patterns of SAMPL obtained for seven isolates of *P. violaceum*: F15 (1), SA1 (2), V1 (3), V2 (4), WP69 (5), WA9 (6), WA12 (7). Fingerprints were generated using four primer pairs: R1 + H-G, CAC_5 + H-G, $GACA_4$ + H-G, $GGAT_4$ + H-G and labelled amplicons separated electrophoretically on 8% denaturing polyacrylamide gels.

Table 4.3 Total number of polymorphic loci and estimates of diversity indices for *Hae*III/*Pst*I AFLP and SAMPL markers for seven reference isolates: F15, SA1, V1, V2, WP69, WA9 and WA12. Amplicons separated on 8% denaturing polyacrylamide gels.

Marker system	No. of polymorphic loci	Polymorphic loci (%)	No. of genotypes identified	Shannon's information index	Nei's gene diversity
AFLP (total)	29	13	7	0.08	0.06
P-AA + H-A	1	2	2	0.02	0.01
P-AA + H-C	7	18	4	0.08	0.12
P-AA + H-G	10	21	4	0.08	0.12
P-AA + H-T	3	8	3	0.03	0.05
P-AC + H-G	5	16	5	0.07	0.10
P-AC + H-A	3	16	4	0.07	0.10
SAMPL (total)	67	42	7	0.15	0.23
$R1_4$ + H-G	12	44	7	0.15	0.23
$GACA_4$ + H-G	19	43	7	0.16	0.23
CAC_5 + H-G	13	35	7	0.15	0.21
$GGAT_4$ + H-G	23	44	7	0.16	0.23

4.3.5 Comparison of SAMPL among European and Australasian isolates of *P. violaceum*

When SAMPL fingerprints were compared visually on 6% polyacrylamide gels, Australasian isolates could be distinguished from the group of European isolates (Figure 4.4). A total of 20 genotypes were identified among 25 Australasian isolates of *P. violaceum* and 18 genotypes were identified among 19 European isolates of the rust fungus using primer pairs $(GACA)_4$ + H-G and R1 + H-G. Diversity indices and the proportion of polymorphic loci were higher for the European isolates than for the Australasian isolates (Table 4.4).

The UPGMA dendrogram revealed that Australian and New Zealand isolates formed one distinct cluster that was separated from two clusters identified for European isolates (Figure 4.5). Apart from isolate PV11B of *P. violaceum*, all other isolates from New Zealand were interspersed among the Australian isolates. Isolate F15 of *P. violaceum*, the original biological control strain from France, and the trap garden isolates, from Montpellier, clustered with the European isolates. Although clustering appeared to be correlated to geographic location, the dendrogram exhibited low bootstrap support at the majority of clades. There was no obvious association between isolates and hosts from which they were obtained

Table 4.4 Diversity indices calculated for Australasian (Australia and New Zealand), Australian and European isolates across 51 scored loci generated using SAMPL primer pairs $(GACA)_4$ + H-G and R1 + H-G.

Population	Shannon's information index	Nei's gene diversity index	Percentage polymorphic loci
Australasian (n=25)	0.09	0.12	22
Australian (n=18)	0.11	0.09	19
European (n=19)	0.12	0.18	37

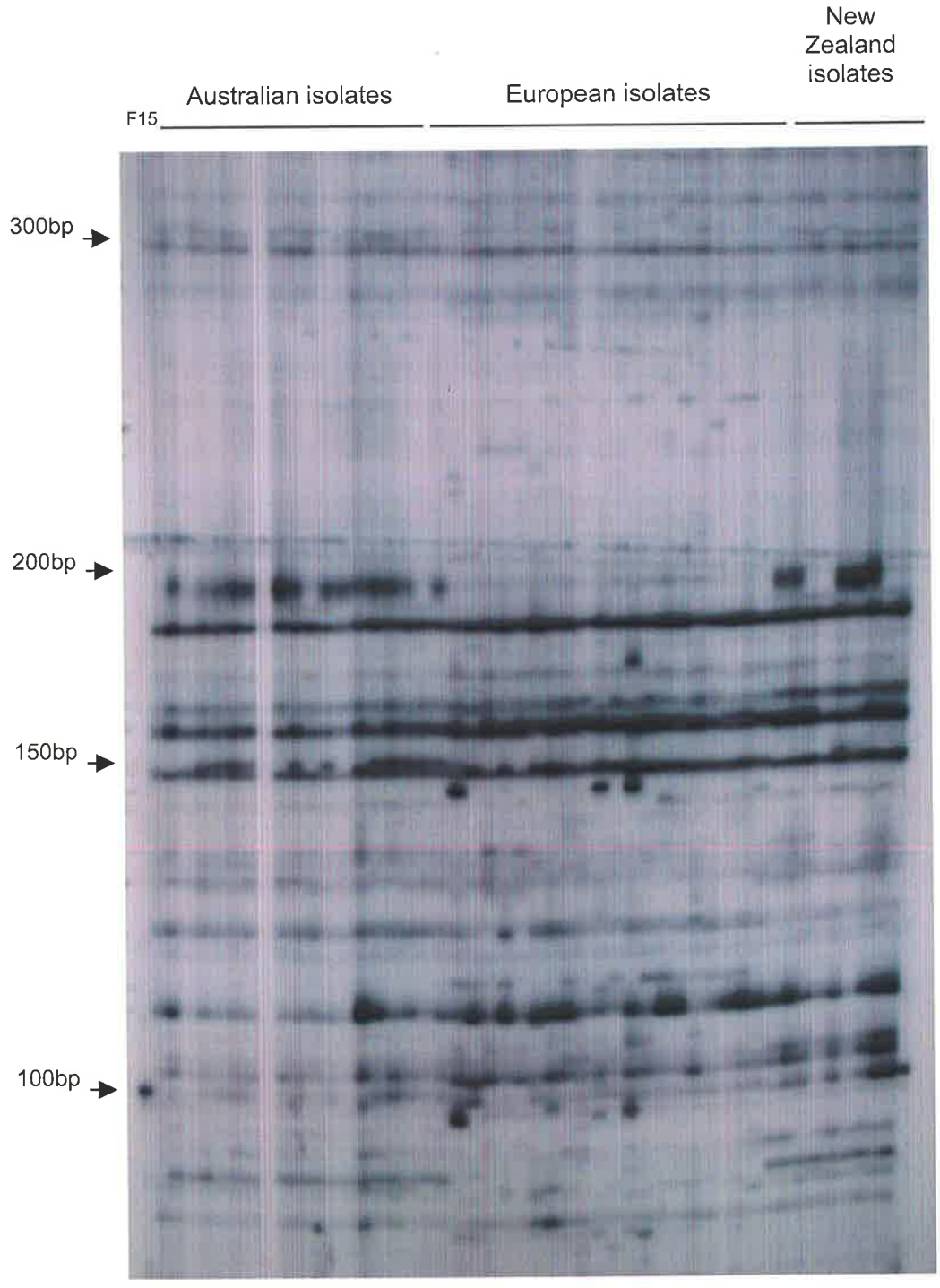

Figure 4.4 SAMPL fingerprints generated using primer pair $GACA_4$ + H-G reveal a distinct visual contrast between Australasian and European isolates of *P. violaceum*. Radiolabelled amplicons were separated electrophoretically using 6% denaturing polyacrylamide gels.

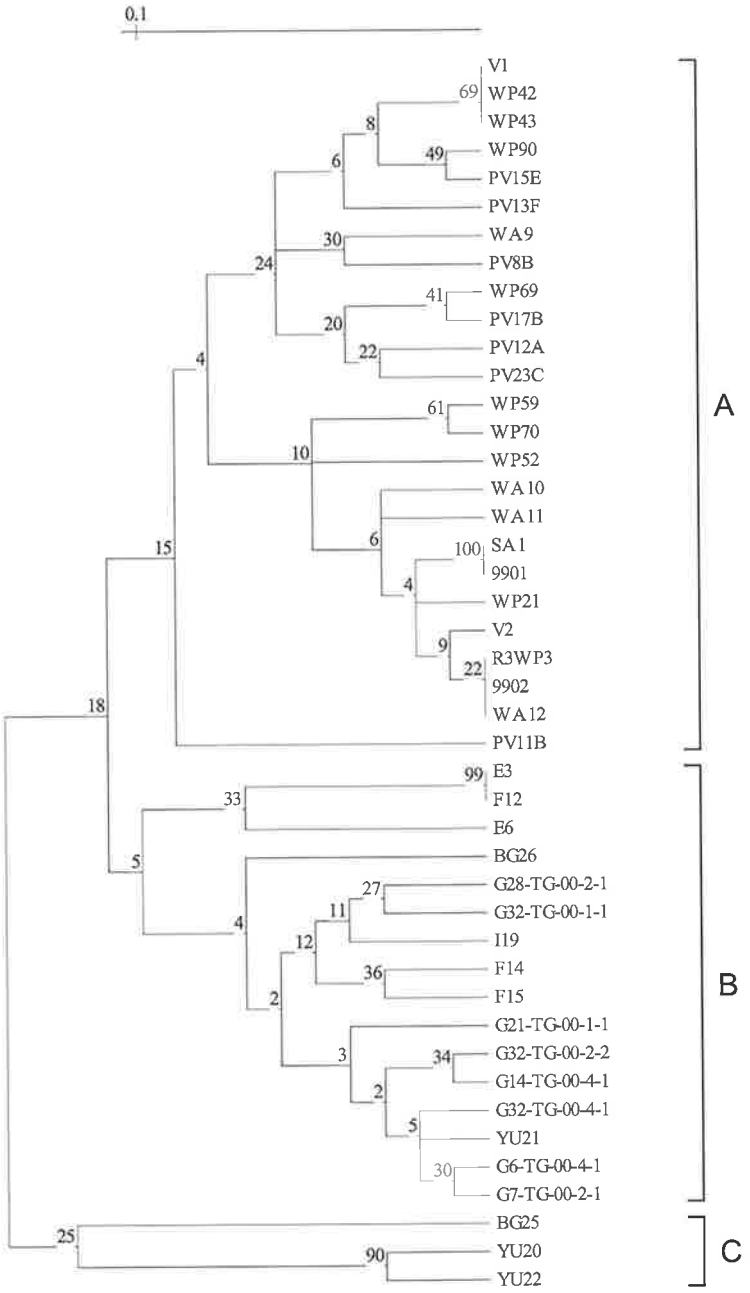

Figure 4.5 Dendrogram of genetic distance among Australasian and European isolates generated using UPGMA with bootstrap of 1,000 replicates of 51 SAMPL. Although low bootstrap support (values at nodes) of less than 50% indicate weak phylogenetic groups among isolates, Australasian isolates form a cluster (A) that is separated from two clusters (B and C) comprising the European isolates. Genetic distance calculated based on the algorithm reported by Nei and Lei (1979). No obvious relationship was observed between isolates and hosts from which they were obtained.

4.4 Discussion

This study highlights the importance not only of screening AFLP primers for their ability to generate polymorphisms but also choosing restriction enzymes that generate polymorphisms for the species being studied, as practised during development of RFLP markers. In contrast to results obtained by application of AFLP for genetic analysis of other Uredinales by means of commonly-used linkers and primers (Pei & Ruiz, 2000; Samils *et al.*, 2001a; Hovmøller *et al.*, 2002; Keiper *et al.*, 2003; Yourman & Luster, 2004), genetic diversity was not detected in *P. violaceum* using AFLP. It was expected that genetic variation in the Australian population of *P. violaceum* would be low. This expectation was based on the fact that the first introduction of *P. violaceum* was unauthorised, limited to a small area (Marks *et al.*, 1984) and subsequently thought to have consisted of few genotypes. However, the result using standard AFLP was inconsistent with our earlier finding, using RFLP analysis, that isolate F15 did not share any *Hae*III restriction fragments with 14 of 18 isolates of *P. violaceum* sampled in mainland Australia between 1997 and 1999 (Evans *et al.*, 2000). The result for standard AFLP analysis was a function of the technique, because the screening process included a broad range of isolates from Europe, which is presumed to be the centre of diversity for this rust fungus. The fact that extremely low diversity among isolates was observed even when using the M-0 and end-labelled E-0 primers in the selective PCR, which should allow for visualisation of all amplicons containing the *Eco*R1 linker sequence, also supported the deficiency of this technique.

The results for SAMPL were consistent with findings of previous studies in which SAMPL were found to have greater power in discriminating closely related genotypes than did AFLP markers. In a study comparing SAMPL, AFLP and sequence-specific amplification polymorphisms, Keiper *et al.* (2003) found SAMPL to be the most informative marker system

in assessing genetic variation among isolates of five cereal rust pathogens. Similarly, the relative utility of SAMPL, RAPDs and AFLPs was compared during characterisation of genetic variation among 11 cowpea (*Vigna unguiculata* subsp. *unguiculate*) landraces and two commercial varieties (Tosti & Negri, 2002). Although all three marker systems were able to differentiate the individuals being analysed, diversity indices were marginally greater for SAMPL than for AFLPs and RAPDs. Additionally, fewer SAMPL primer combinations were needed to obtain similar discrimination when compared with AFLP and RAPD analyses. In another study, SAMPL was found to be more efficient than AFLP in differentiating closely related accessions of neem, *Azadirachta indica* (Singh *et al.*, 2002).

The high levels of diversity observed among the European isolates when compared with Australasian isolates provided qualitative evidence that *P. violaceum* shares its centre of diversity with its coevolved host (*Rubus fruticosus* agg.) in Europe (Gustafsson, 1943). The comparatively low level of variation observed among Australian isolates is typical of a founder effect where a small number of individuals have migrated from a diverse extant population to new geographic locations, forming independent populations that are typically less diverse than the extant population. The findings of this study are consistent with observations that few and distinct disease foci were observed in a restricted area following the first detection of *P. violaceum* in Australia in 1984 (Marks *et al.*, 1984).

Cluster analysis supported the hypothesis that *P. violaceum* in Australia represents a genetic subset of *P. violaceum* found in Europe. Although *P. violaceum* isolates clustered based on geography, low bootstrap support indicated that substructuring among the majority of *P. violaceum* isolates was weak. One interpretation of this finding is that there has been insufficient time for significant genetic differentiation of the Australian population from the

European population, assuming *P. violaceum* was first introduced to Australia about 20 years ago. Isolate F15 of *P. violaceum* from France, the biological control strain sanctioned for release in Australia in 1991, did not cluster with the Australian isolates following analysis by UPGMA. Given that Australian isolates used in this study were collected between 1997 and 1999, it appears that the strain F15 genotype did not persist in the Australian environment 6–8 years after its release. Either strain establishment did not occur or if it did occur, recombination events between isolate F15 and Australian wild type strains may have taken place. In the latter event, the F15 genotype might no longer exist intact but some of its genes would remain in the Australian population of the fungus. The results presented here support questions raised by Evans *et al.* (2000) regarding the success of establishment of strain F15.

The aerial migration of rust spores from Australia to New Zealand has been documented (Close & Moar, 1978) and, prior to this study, it was postulated that wind currents transported spores of *P. violaceum* from Australia across the Tasman Sea, resulting in the establishment of disease in New Zealand blackberry *circa* 1990 (Pennycook, 1998). Although the mechanics of migration cannot be established with certainty, UPGMA cluster analysis appears to provide compelling evidence that the New Zealand population of *P. violaceum* is very likely to have originated from Australia.

Clustered within one of two genetic groups representing the European isolates, the trap garden isolates appeared to be a subset of the potential genetic diversity in Europe. There are at least two explanations for this finding, assuming the European population of *P. violaceum* in 2000 was just as diverse as it was in 1978-1980. Firstly, the genotypes of *P. violaceum* available for selection were restricted to spores present in air currents over southern France in a single growing season. Secondly, a selection pressure was imposed, whereby pathotypes of *P.*

violaceum were selected based on their compatibility with Australian biotypes of the *R. fruticosus* agg. Indeed, the use of Australian plant material as trap plants ensured selection of biocontrol strains that were virulent under field conditions.

The separation of the Australasian cluster from the European population, plus evidence supporting an Australian origin for *P. violaceum* in New Zealand, illustrates the merit of using SAMPL with adapters that recognise polymorphic restriction sites, such as *Hae*III, in *P. violaceum*. This technique can now be used to study evolution of *P. violaceum* in its native and introduced range, especially when it is important to estimate genotype frequencies. As part of biological control programs, SAMPL can be used to identify pathogen strains with certainty when selecting and maintaining isolates by clonal propagation. Otherwise, pathogen isolates must be identified by their virulence pathotype following lengthy bioassay using a differential set of host genotypes, if available. SAMPL can also be used to track the fate of pathogen strains released for biological control, although the fate of specific genotypes may be confounded in situations where the agent undergoes frequent sexual recombination.

In the next chapter, the application of SAMPL is extended to elucidate the contribution of gene flow and reproductive mode to evolution of populations of *P. violaceum* in Australia.

5 Population genetics and metapopulation structure of *P. violaceum* in Australia

5.1 Introduction

There are at least 15 species of European blackberry naturalised in Australia, which display varying susceptibility to different strains of *P. violaceum* (Evans & Gomez, 2004). Selecting strains of a pathogen that are virulent to target weed biotypes is an important prerequisite for any successful biological control program. The ultimate success of the agent, however, depends on its ability to establish, persist, spread and suppress the weed in its introduced range.

The persistence of a biological control agent is determined by the fitness of pathogen strains released in a new environment. If the agent reproduces clonally then persistence will depend on the release of a fit clone that can maintain beneficial gene linkages through successive clonal generations. If the agent reproduces sexually and undergoes high rates of recombination, then linkages of genes, beneficial or not, may be disrupted. Hence, the persistence of introduced virulence genes or genotypes of the pathogen is not guaranteed in sexually recombining populations (McDonald & Linde, 2002). However, gene diversity in the founding population of the agent may enable the pathogen to evolve and adapt to its new environment through natural selection.

Assuming introduced virulence genes persist following introduction of one or more pathogen strains, then the role of space and time is also central in the understanding of how individuals move and establish across the landscape (gene flow). In the case of an obligate pathogen which enters a new geographic range, its spatial distribution relies on the distribution of its

host, and the host, along with environmental considerations, becomes the underlying component of the pathogen's habitat. Furthermore, because of the considerable genetic variation in the weed, the pathogen's habitat is fractured both spatially and genetically. In natural ecosystems, the distribution of host demes reflects a metapopulation structure, where populations may be evolving semi-independently (Burdon, 1993; Thrall & Burdon, 1997). Because plants are generally restricted in their movement, neighbourhood structures over short distances may result, such that neighbouring demes are likely to share a similar disease resistance structure when compared with demes that are geographically isolated. As a result, populations become genetically isolated by distance.

Aerial plant pathogens, especially those with an extensive dispersal range compared to their host, appear to have a metapopulation structure in natural ecosystems that is often characterised by major episodes of extinction and recolonisation that occur at irregular intervals in time (asynchrony). Thus stochastic events such as random genetic drift and migration are likely to play important roles in maintaining genetic differentiation among pathogen demes (Burdon, 1993), leading to weak associations between genetic and geographic distance in the pathogen metapopulation when compared with its host.

Multiple demes of the pathogen, structured ephemerally across time and space, influence disease incidence across the host-pathogen metapopulation. This phenomenon is in contrast to a single epidemiologically homogenous population or a series of isolated and mutually exclusive host-pathogen interactions (Ericson *et al.*, 1999). If weed-pathogen interactions exhibit metapopulation structure, then the likely consequences for selection, release and post-release evolution of pathogen strains towards an effective biological program should be explored.

In the experiments reported in this chapter, SAMPL was utilised to determine the population genetic structure of *P. violaceum* in Victoria and test the hypothesis that demes of *P. violaceum,* collected from geographically isolated infestations of European blackberry, reflect a metapopulation structure. SAMPL was also applied to test the hypothesis that *P. violaceum* undergoes random mating early in the growing season of European blackberry in Victoria.

5.2 Materials and methods

5.2.1 Description of populations

Individuals (isolates) from four Victorian populations of *P. violaceum* were sampled from naturalised infestations of European blackberry in spring 2003 (Table 5.1, Figure 5.1). One population from Whitfield was sampled in the spring of 2002 and 2003. Pairwise distance between each location is displayed in Table 5.2.

The Whitfield infestation was located on private property in the King Valley region of north-eastern Victoria. The Tarwin East infestation was located on private property in the Strzelecki ranges, *ca* 189 km south of the Whitfield infestation. Situated approximately 90 and 218 km west of Tarwin East were the roadside blackberry infestations in Korumburra and Wangerrip, respectively. *R. anglocandicans* was the underlying blackberry taxon in the Whitfield, Tarwin East and Wangerrip infestations, while *R. leucostachys* represented the underlying taxon in the Korumburra infestation (F. Mahr, personal communication).

Table 5.1 Origin of populations sampled in Victoria in November 2002 and 2003.

Population	Source			Year of collection
	Location[a]	Location coordinates (decimal degrees)	Host	
KV1	Whitfield	S36.78461 E146.39137	*R. anglocandicans*	2002
KV2	Whitfield	S36.78461 E146.39137	*R. anglocandicans*	2003
WR	Wangerrip	S38.73053 E143.31193	*R. anglocandicans*	2003
TE	Tarwin East	S38.51753 E146.24407	*R. anglocandicans*	2003
KB	Korumburra	S38.42533 E145.79568	*R. leucostachys*	2003

[a]Refer to Fig. 5.1

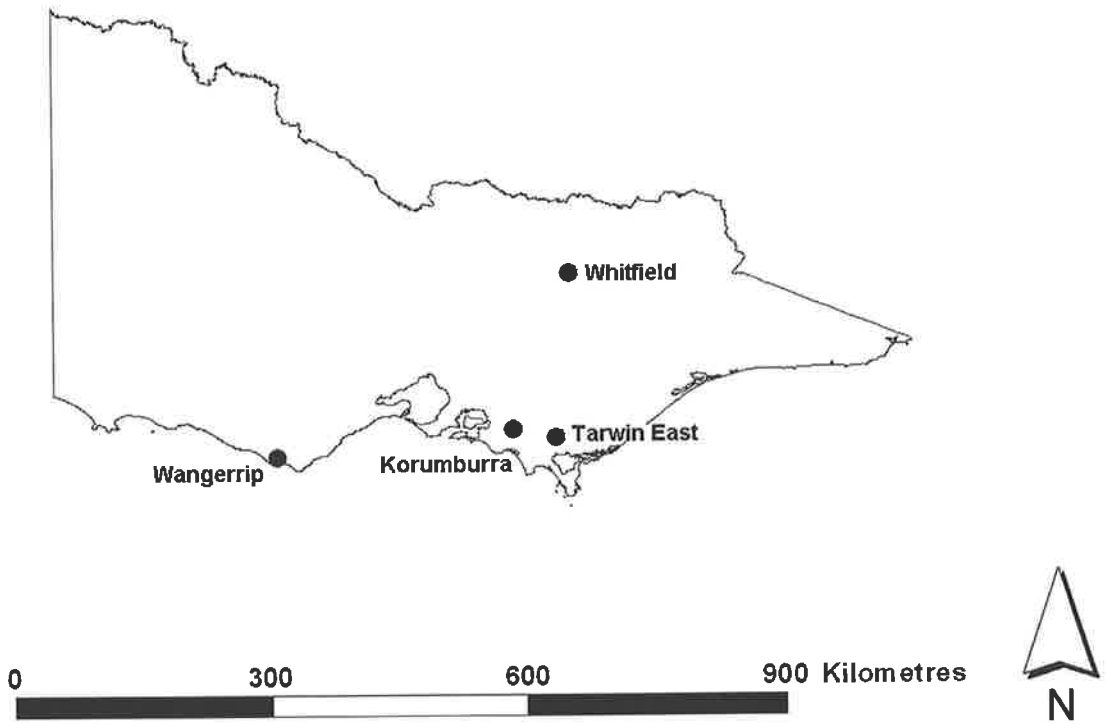

Figure 5.1 Location of populations sampled in Victoria in 2002 and 2003.

Table 5.2 Pairwise distances (kilometres) among locations sampled for population genetic analyses

	Whitfield	Wangerrip	Tarwin East	Korumburra
Whitfield	0			
Wangerrip	246	0		
Tarwin East	189	218	0	
Korumburra	193	255	40	0

5.2.2 Population sampling

In November 2002, population KV1 at Whitfield was sampled using a hierarchical sampling strategy (Figure 5.2). Due to the difficulty of distinguishing single plants within the field, leaves were sampled on a 'per location' basis. Seven locations in the field were sampled along random transects designated in the field. From each location, four leaves arising from different primocanes were detached and leaflets placed individually in 10 cm-diameter and 2 cm-deep Petri plates containing 20 ml of sterile 1% water agar (Bitek™ Agar, Difco, Becton Dickson) and transported by courier mail to Adelaide, South Australia. Within 4 days of arrival, urediniospores from each of three uredinia per leaflet were transferred aseptically to individual detached leaflets of *R. anglocandicans*, using a sterile artist's paint brush, as described in section 3.1. Urediniospores from uredinia that developed on these 'clean' leaflets were transferred to healthy leaflets for multiplication as described in section 3.1.

In 2003, sampling was performed by randomly selecting 30 individuals from each site along multiple transects in the field. The number of transects ranged from 2 to 5 depending on the size of the infestation. Four populations in Victoria (Figure 5.1) were sampled in this fashion. One compound leaf was collected at each of 30 randomly selected locations along transects, with the aim of recovering at least 20 individuals (single uredinium-derived isolates) from these 30 leaves. Detached leaves were placed in Petri plates containing 1% water agar and

transported to Adelaide as described previously. To maximise recovery of individuals, urediniospores from three randomly selected uredinia per leaf were transferred as mixed inoculum to individual clean leaflets. Single uredinia arising from the mixed inoculation were isolated by transferring their urediniospores to clean leaves for multiplication as described in section 3.1.

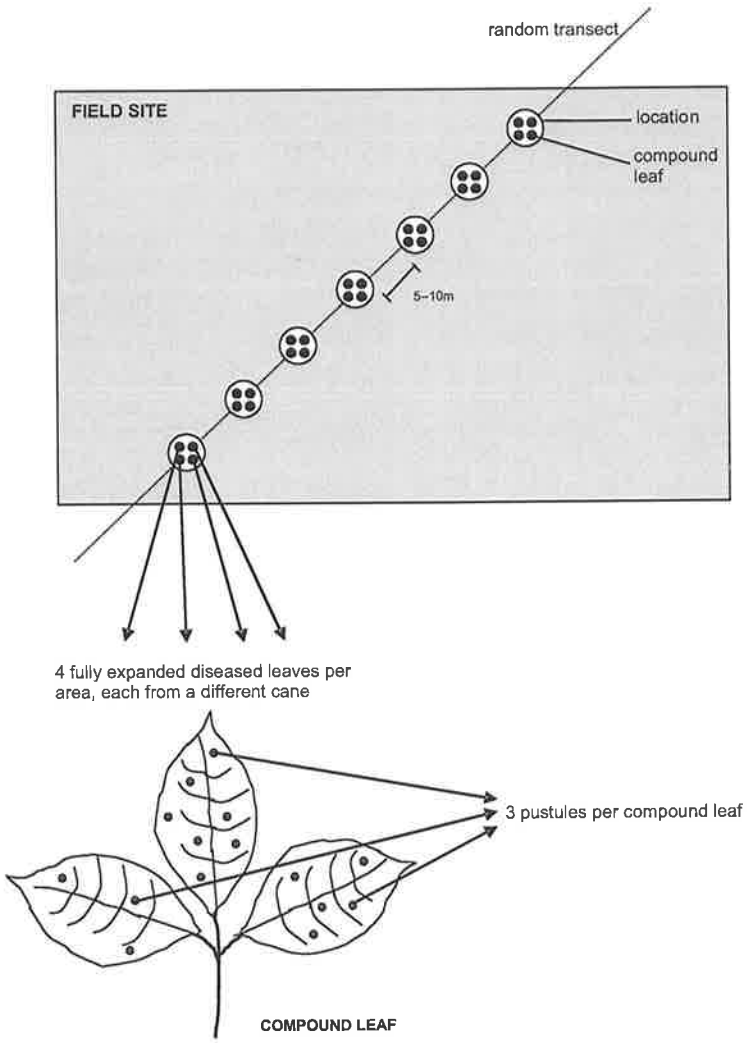

Figure 5.2 Schematic illustration of hierarchical sampling strategy used to sample the Whitfield infestation in November 2002. The seven large (white) circles represent different locations sampled within the field site. Each location was separated by 5–10 m. Solid black circles represent sampled leaves arising from different primocanes.

5.2.3 Genetic analyses

DNA was extracted from urediniospores of each individual as described in section 3.2. SAMPL fingerprints were generated for each individual and visualised as described in section 3.4 using primer pairs $(GACA)_4$ + H-G and R1 + H-G. The presence or absence of 51 loci was scored for each individual and these data used for subsequent population-genetic analyses.

Genetic variation for each population was assessed by calculating Nei's gene diversity index (Nei, 1973) and Shannon's information index (Shannon & Weaver, 1949). Diversity indices were calculated using POPGENE software version 1.32 for Windows. Analysis of molecular variance (AMOVA; Excoffier *et al.*, 1992) was performed using Arlequin Version 2.001 for Windows (Schneider *et al.*, 2000) to assess the spatial distribution of genetic variation and the likely contribution of gene flow within and among the populations. AMOVA estimates variance components for SAMPL phenotypes and partitions genotypic (multilocus) variation within and among populations based on the proportion of difference in the number of variable loci between all pairs of SAMPL fingerprints. The parameters estimated by AMOVA, termed Φ statistics (Excoffier *et al.*, 1992), are analogous to Wright's *F*-statistics (Fst; Wright, 1969) and they describe the level of population differentiation. The degree of differentiation between each population was also measured by calculating pairwise Φ statistics and Wright's Fst values using Arlequin and Tools for Population Genetic Analysis (TFPGA) version 1.3 for Windows (Miller, 1998), respectively. The calculation of Wright's Fst was based on the algorithm adjusted for dominant data (Lynch & Milligan, 1994). Genetic isolation by distance was investigated by plotting pairwise Fst and Φ values against their respective geographic distance.

To test for random mating, multilocus disequilibrium was measured by calculating the Index of Association (I_A; Maynard Smith *et al.*, 1993) using the Multilocus software (Agapow & Burt, 2001). The observed I_A for each population was compared to an expected I_A under random mating simulated through the reshuffling of clone-corrected data over 1,000 repetitions.

5.3 Results

5.3.1 Genetic diversity

Table 5.3 summarises the genetic diversity observed in each population in 2002 and 2003. In 2002, 29 SAMPL phenotypes were identified among 38 isolates recovered from population KV1 at Whitfield (Table 5.3).

In 2003, 20-23 isolates were recovered from a potential maximum of 30 from each field site, giving a total of 87 isolates for genetic analysis (Table 5.3). SAMPL (Figures 5.3 and 5.4) revealed considerable genotypic variation in populations KV2, TE and WR, with *ca* 91% of individuals in these three populations representing unique electrophoretic phenotypes (Table 5.3). The population from Korumburra, KB, was characterised by little variation, with 19 of the 20 isolates represented by a single electrophoretic phenotype. Therefore, this population was determined to have a clonal structure from genotype overrepresentation. In total, 61 SAMPL phenotypes were identified among the 87 isolates collected in 2003. Populations KV2 and WR shared one electrophoretic phenotype, as did populations KB and TE. Incidentally, the SAMPL phenotype shared by populations KB and TE represented the rare phenotype in the KB population. Population KV1 (2002) did not share any SAMPL phenotypes with population KV2 sampled in the following growing season.

Table 5.3 Number of unique individuals and diversity indices observed for populations of *P. violaceum* sampled in Victoria in 2002 and 2003, calculated across 51 loci generated using SAMPL primer pairs $(GACA)_4$ + H-G and R1 + H-G.

Populations	Number of individuals recovered (n) per number sampled	Number of electrophoretic genotypes identified	Nei's gene diversity index	Shannon's information index
KV1	38/84	29	0.0969	0.1380
KV2	21/30	19	0.1486	0.2123
TE	23/30	21	0.1152	0.1661
WR	23/30	21	0.0908	0.1313
KB	20/30	2	0.0350	0.0544

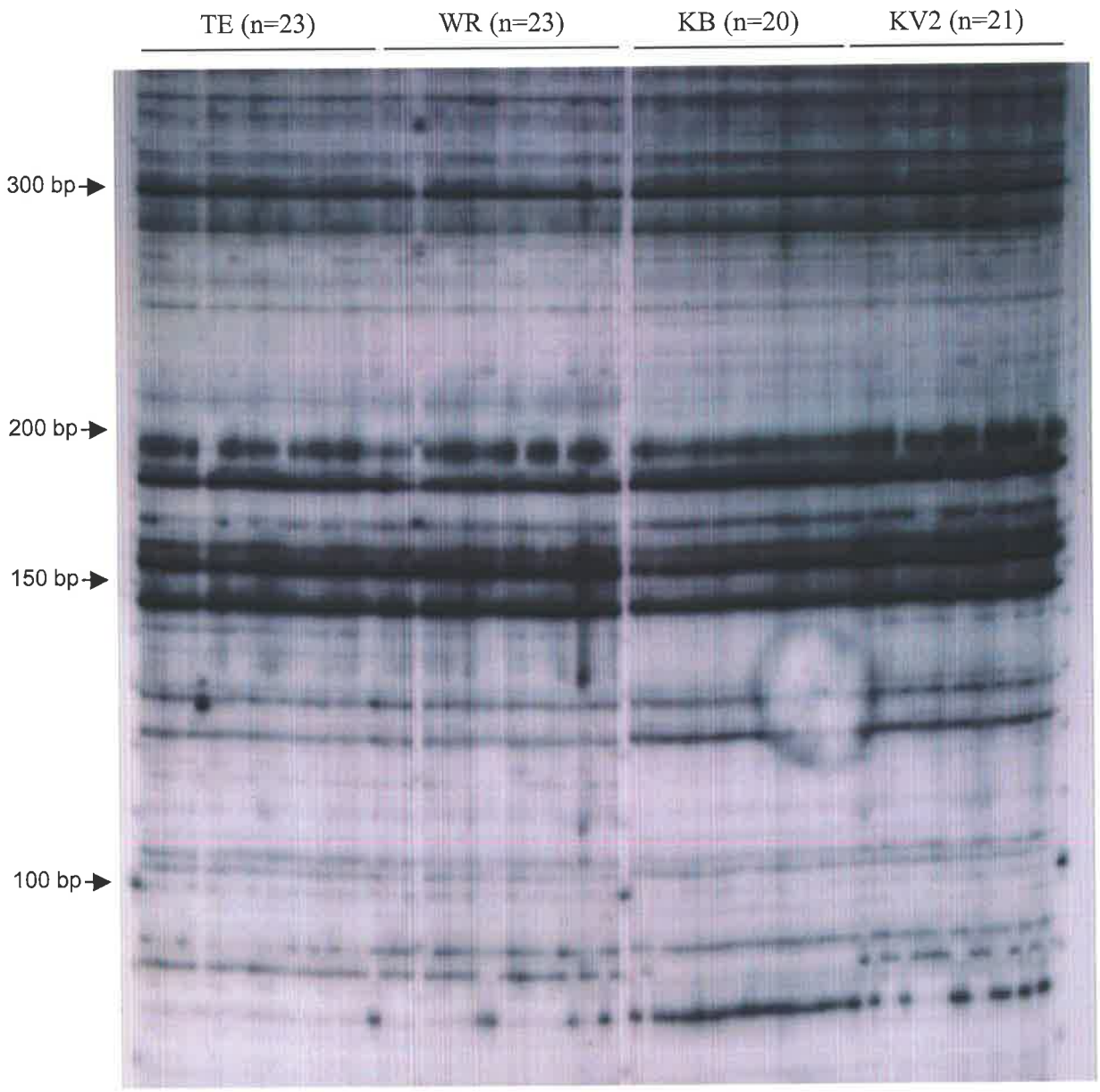

Figure 5.3 SAMPL profiles generated using primer pair $(GACA)_4$ + H-G for isolates recovered from Tarwin East (TE), Wangerrip (WR) Korumburra (KB) and Whitfield (KV2) in 2003.

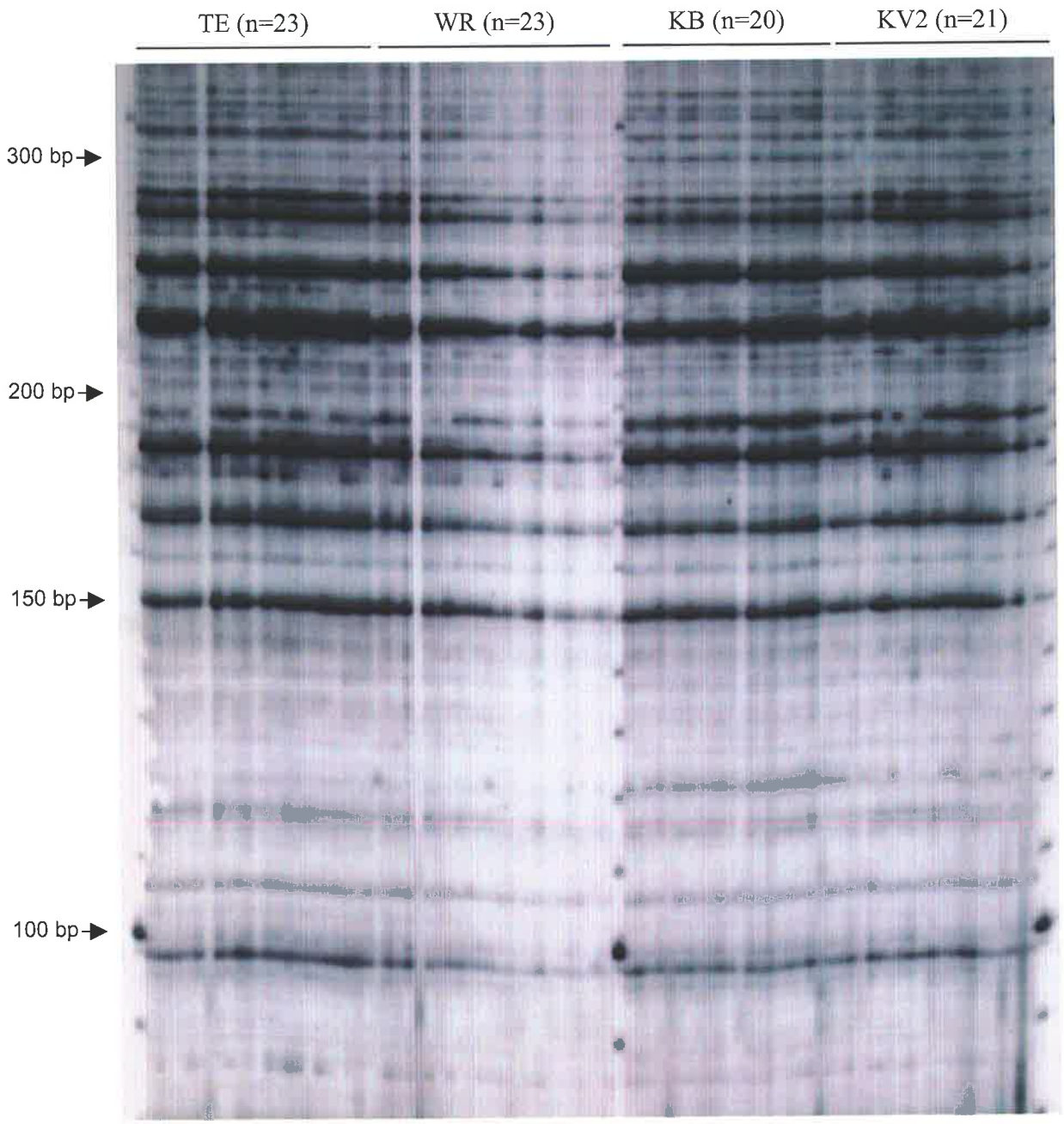

Figure 5.4 SAMPL profiles generated using primer pair R1 + H-G for isolates recovered from Tarwin East (TE), Wangerrip (WR) Korumburra (KB) and Whitfield (KV2) in 2003.

5.3.2 Validation of sampling strategy in 2003

The low recovery rate of isolates from the KV1 population in 2002, 38 out of a potential maximum of 84 isolates, meant that the sample size was insufficient to analyse the genetic structure of isolates collected within and among leaves. However, AMOVA revealed that there was no significant genetic differentiation among the seven locations in the single field (Φ = 0.0417, P=0.140; Table 5.4). This result validated the sampling strategy for populations in 2003, whereby single leaves, from which an individual was isolated, were selected at 30 random locations along transects.

Table 5.4 AMOVA of 38 isolates of *P. violaceum* collected across seven locations within a single field site in Whitfield, Victoria, in November, 2002.

Source of variation	Degrees of freedom	Sums of squares	Variance components	% Variation	Φ	P
Among locations within the field	6	12.463	0.07139	4.17	0.0417	0.140
Within locations	31	52.169	1.68287	95.83		

5.3.3 Genetic differentiation among populations in 2003

AMOVA revealed that 59.9% of genotypic variation was present within populations and 40.1% of the variation was observed among populations (Table 5.5). Overall, AMOVA revealed significant and high levels of differentiation among the four populations (Φ=0.4019, P<0.0001), indicating that gene flow may be infrequent among these populations. Pairwise comparison of Φ between any two populations supported this observation, revealing significant differentiation between any two populations (P<0.0001; Table 5.6). Furthermore population KV1, sampled in 2002, was significantly different from all populations sampled in 2003. There did not appear to be a correlation between pairwise Φ or Fst and geographic distance for the populations sampled in 2003 (Figure 5.5).

Table 5.5 AMOVA of 87 individuals of *P. violaceum* collected across four populations in Victoria in 2003

Source of Variation	Degrees of freedom	Sums of squares	Variance components	% Variation	Φ	P
Among populations	3	99.43	1.427	40.1	0.4019	<0.0001
Within populations	83	176.92	2.131	59.9		

Table 5.6 Number of SAMPL phenotypes shared between populations (upper diagonal) and pairwise comparison of Φ (lower diagonal; P<0.0001 for each pairwise comparison)

	Wangerrip	Tarwin East	Korumburra	Whitfield 2003	Whitfield 2002
Wangerrip	-	0	0	1	0
Tarwin East	0.17407	-	1	0	0
Korumburra	0.73732	0.58181	-	0	0
Whitfield2003	0.28023	0.12705	0.35267	-	0
Whitfield2002	0.49284	0.39886	0.56989	0.28376	-

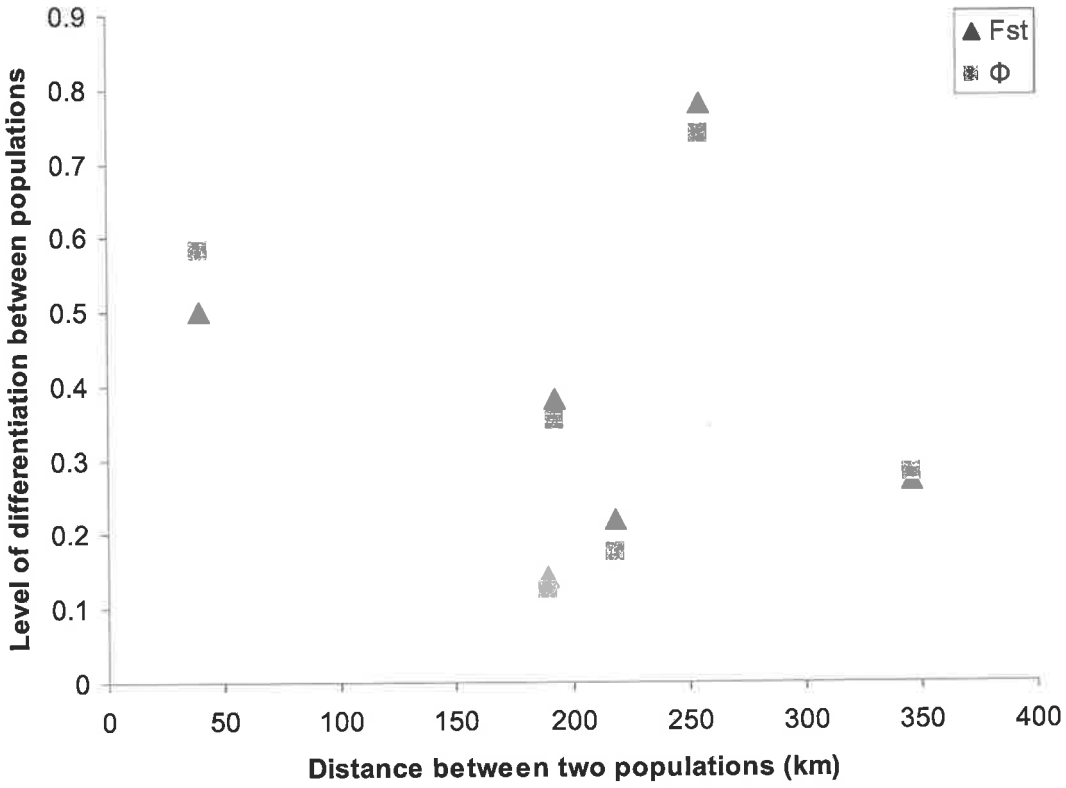

Figure 5.5 The relationship between genetic differentiation (expressed as Φ or Fst) and geographic distance.

5.3.4 Testing for random mating

The KB population was omitted from the calculations of the I_A since only two electrophoretic phenotypes were identified among the 20 isolates collected. When the probability of panmixia was calculated, populations KV1 and WR showed evidence of panmixia (random association of loci), with I_A=-0.101/P=0.83 (Figure 5.6) and I_A=-0.088/P=0.715 (Figure 5.7), respectively. Populations TE and KV2 revealed significant levels of non-random associations among their multilocus phenotypes, with I_A=0.413/P<0.05 and I_A=1.117/P<0.05, respectively (Figure 5.7). Therefore, the hypothesis for random mating within each of these populations was rejected.

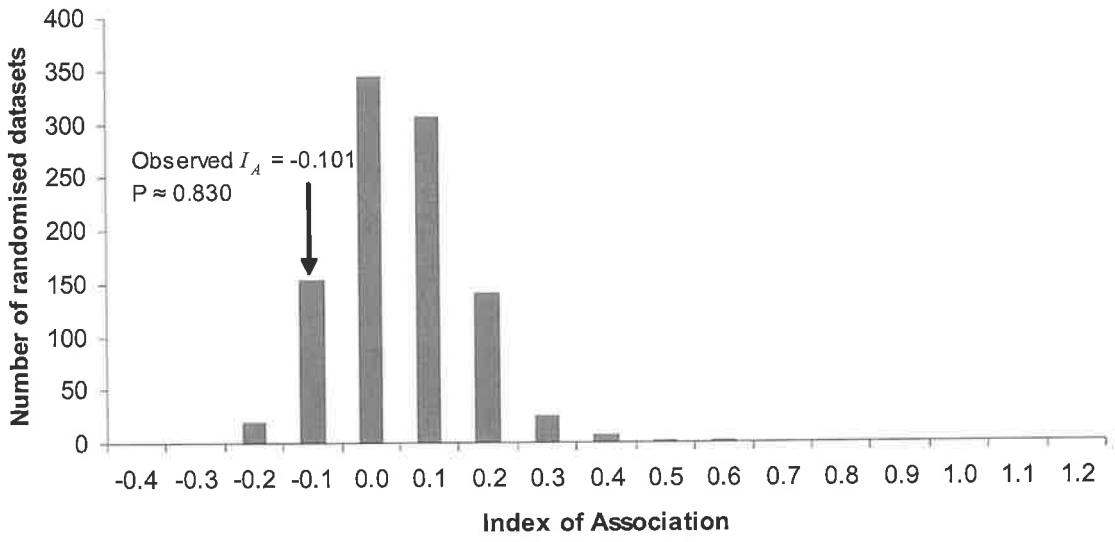

Figure 5.6 Observed index of association (I_A; arrow) compared to indices computed from 1,000 artificially recombined datasets for the Whitfield population (KV1) sampled in 2002.

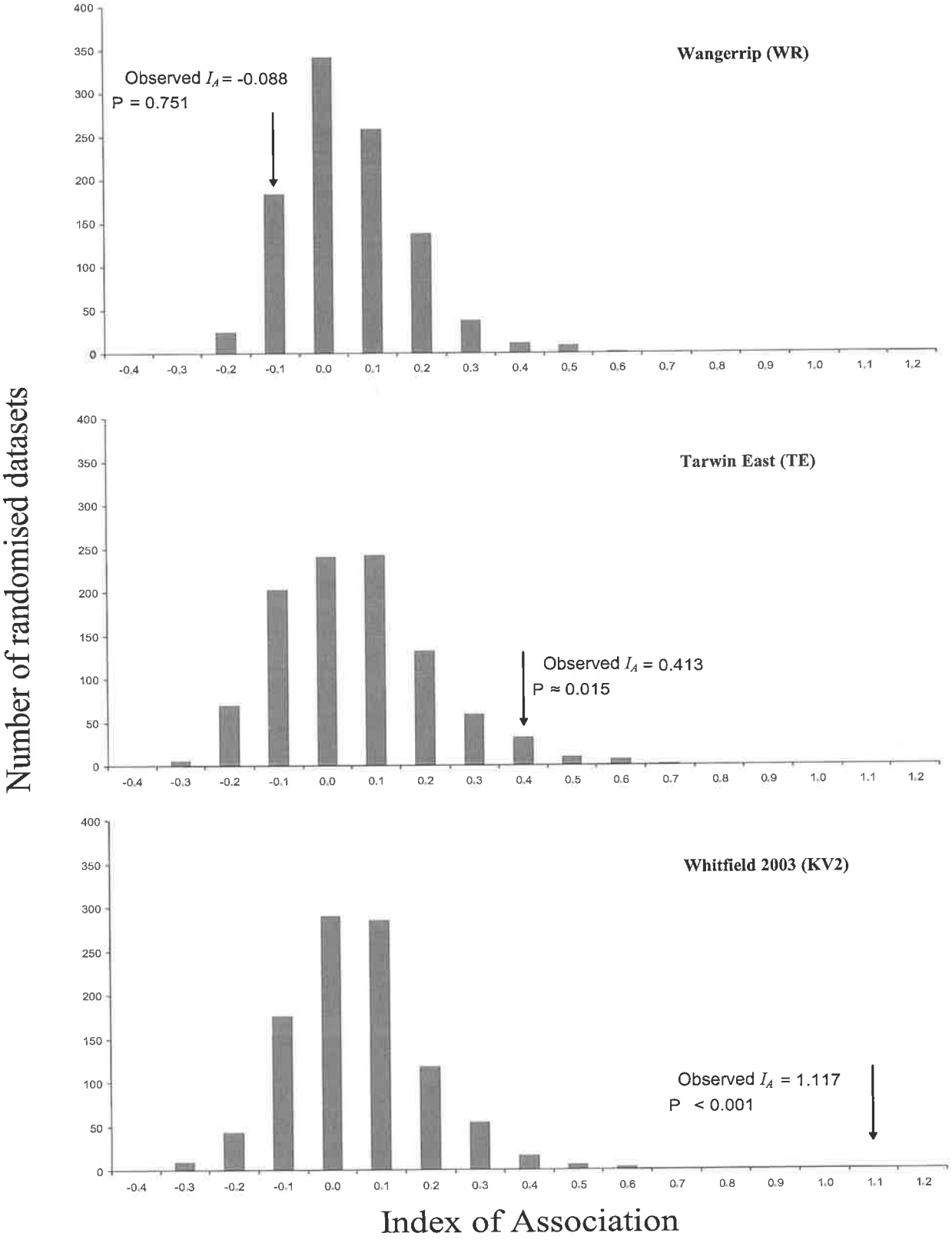

Figure 5.7 Observed index of association (arrow) compared to indices computed from 1,000 artificially recombined data sets for populations WR, TE and KV2 sampled in 2003.

5.4 Discussion

In the seven years between the first report of *P. violaceum* in Australia, as localised disease foci in 1984, and the release of strain F15 in 1991, it was recognised that this rust had become widespread across the Australian blackberry infestation (Watson, 1991). Long distance and frequent dispersal of large numbers of urediniospores represents a successful reproductive strategy of the Uredinales fungi for finding suitable hosts across expansive areas (Brown & Hovmøller, 2002). Evidence for long distance transport of *P. violaceum* was presented in the previous chapter in relation to wind-mediated movement of urediniospores between Australia and New Zealand. While results from AMOVA indicated that genotype flow was infrequent among populations of *P. violaceum* in Victoria, the lack of genetic isolation by distance among these populations supports the occurrence of varying scales of spore dispersal and the chance establishment of these spores on susceptible hosts. Milgroom & Lapari (1995) quantified a similar phenomenon among highly differentiated populations of the ascomycete *Cryphonectria parasitica* (chestnut blight fungus), where differentiation was not correlated to geographic distance.

An explanation for significant differentiation among populations of *P. violaceum* in Victoria comes from understanding that gene flow is not only a function of long-distance movement, but also subsequent establishment and persistence of genes or individuals among demes. The vastly different ecology of agricultural *versus* natural plant communities needs to be recognised (Burdon, 1993). Due to the strong selection pressures imposed in agricultural systems, processes such as frequency dependent selection are of great importance to pathogen survival. In contrast, the genetic structure of pathogen populations of natural plant communities is thought to be driven by stochastic events with habitat, as a function of host and climate, unevenly distributed across the environment (Burdon, 1993).

Populations of rust fungi experience population crashes and recolonisation in response to changes in climate and habitat. After a population crash, recolonisation occurs by the chance arrival of a virulent pathotype. Therefore, migration and random genetic drift (stochastic chance effects) are likely to influence all pathogen demes and will be the most likely cause of differentiation among these demes (Burdon, 1993). As a consequence, the stochastic nature of pathogen strain recruitment and the heterogeneity of climate may also lead to the asynchrony of timing at which epidemics occur across local populations of the host. In effect, the scale of gene flow can vary significantly from season to season due to the asynchrony of disease development among local populations of the pathogen. The stochastic nature of migration and genetic drift, coupled with the asynchrony of disease development and incidence among populations of the rust, may explain why significant differentiation among populations of *P. violaceum* in Victoria was observed across time and space.

The hypothesis that *P. violaceum* undergoes random mating early in the growing season of European blackberry in Victoria was supported for two different sites sampled in different years (KV1, 2002 and WR, 2003). Although determination of I_A rejected the hypothesis of panmixia at TE and KV2, recombination may be occurring within these populations, possibly at very high rates. Factors such as random genetic drift can lead to non-random associations among loci and may skew the I_A towards linkage disequilibrium (Samils *et al.*, 2001b). However, KV2 and TE had higher diversity indices than the other populations studied; this is atypical of random genetic drift which causes reduced heterozygosity and ultimately lower levels of genetic variation. Thus, factors other than drift may have biased the calculation of I_A. In particular, genetic variation in these populations may have been underestimated if the sample size was too small or sampling was not conducted on the appropriate temporal or

geographic scale. The question of sampling is discussed further in relation to metapopulation theory below.

Metapopulation theory is an appropriate approach to describe pathogen populations of natural plant communities as it recognises the fragmented habitat and uneven distribution of host genotypes and climate. The concept of what constitutes a rust population and the nature in which demes interact in the metapopulation need to be considered. In this study, the presence of the rust within a weed infestation was considered a population. However, in reality, pathogen demes may represent part of an extensive genetic neighbourhood, where they are linked by frequent migration events such as wind-dispersal of urediniospores. Additionally, because the source and sinks of migrants (genetic neighbourhood) will change due to the asynchrony of disease development across local populations of the pathogen from season to season, what was deemed an appropriate sample size and geographic scale at which sampling was performed may not hold true between sampling periods. Consequently, this can lead to the possibility of underestimating variation especially where extensive genetic neighbourhoods exist.

Another factor that may lead to the underestimation of genetic variation is the host genotype used to isolate field collections of *P. violaceum*. In this study, a clone of *R. anglocandicans* was used to propagate and maintain isolates recovered from the field. Although three of the four sites sampled were infestations of *R. anglocandicans*, the rust resistance structure of these populations and of the plant material used to propagate isolates may vary. If so, then genes for disease resistance might be present in plant material used to propagate fungal isolates, and hence only a proportion of individuals that are virulent to the propagation material will survive. The presence of incompatible host-pathogen interactions would

undoubtedly underestimate the potential levels of diversity of sampled populations. Evans *et al.* (2005) did not find any difference in susceptibility to *P. violaceum* isolate SA1 between two clones of *R. anglocandicans* assayed under field conditions. However, it is unknown whether incompatible host-pathogen interactions may have biased analyses used in this study. Ideally, clones of host plants from which individuals were originally recovered from in the field should be used for propagation to maximise recovery of individuals.

The varying scales of gene flow, resulting in shifting spatial scales of the local demes across time may explain why the determination of the I_A revealed a significant shift from panmixa to significant non-random associations among loci between 2002 and 2003 at Whitfield. The sampling strategy employed in this study aimed to target populations early in the disease cycle so as to minimise the effect of collecting too many clones from the field which would have underestimated initial levels of diversity and skewed certain analyses such as the I_A. In 2002, it is possible that gene flow was infrequent in the Whitfield population thus the sampling strategy employed was suitable to obtain a representative collection of individuals which had arisen from random mating in the immediate infestation. However, in 2003 gene flow may have been more frequent, extending the geographic and genetic range of the Whitfield population. While the presence of aecia and the subsequent development of urediniospores were not monitored in this study, it is unlikely that gene flow occurred from the movement of urediniospores, given that sampling occurred early in the growing season. However, gene flow might have occurred from the dissemination of spermatia from neighbouring demes of the rust, via insect vectors which aid in outcrossing during the sexual phase of the rust lifecycle (Roy, 1993). If this was the case, then evidence for random mating in two populations (KV1 and WR) plus observations of high genotypic variation and the presence of sexual spores in the field (Washington, 1985) suggests that sexual recombination

plays an important role for maintaining genotypic diversity within populations of *P. violaceum*. However, mutation cannot be ruled out as a possible source of variation in *P. violaceum* because nothing is known about its frequency in the field. Knowing that demes vary in the spatial and temporal course of disease, the results of analyses of I_A when combined with those for population differentiation support the hypothesis that stochastic processes such as random genetic drift, founder effects and migration play important roles in evolution of *P. violaceum*.

The population from Korumburra (KB) had an obvious clonal structure in 2003, which may have resulted from genetic drift, likely from a founder event. Although *R. leucostachys* is a genetically variable taxon (Evans *et al.*, 2004b), it is not known whether the host was genetically variable at this field site, nor whether it had a complex genetic structure for rust resistance. If the Korumburra infestation had a complex resistance structure, then a fit clone of *P. violaceum* virulent to this taxon at this site may have been selected for and persisted through multiple generations each growing season. Disease severity at this field site is generally high because weather conditions are often conducive for multiple cycles of infection by *P. violaceum*. Hence, rapid selection of a fit clone of *P. violaceum* is plausible. Alternatively, the lack of genotypic diversity in *P. violaceum* may have been a result of a genetic bottleneck arising from a founder effect or extinction event. Following such events, the resulting clone may have proliferated over several generations before sampling, and during this time, little or no gene flow of unique genotypes had occurred with other populations. The rare genotype observed at this site was also found at the Tarwin East infestation, indicating that there had been possible migration of urediniospores between the populations of the pathogen at these two infestations. Weather systems generally move west to east in Victoria, and so migration from KB to TE seems likely.

Further research is required to elucidate the geographic range of *P. violaceum* metapopulation/s in Australia to provide a better indication for the spatial scale of gene flow. Additional sampling within the blackberry growing season, after the occurrence of multiple clonal generations, would allow detection of genotype flow through the direct observation of migrating clonal genotypes (urediniospores) among populations. Experimental design using appropriate blackberry clones for isolation of *P. violaceum* also needs to be refined to maximise recovery of genetically different individuals.

The results of this study highlight the asynchrony of life histories among pathogen populations within and among seasons and support the concept of metapopulations as the most appropriate scale by which to study *P. violaceum* in Australia. In conclusion, these results support a model in which sexual recombination and stochastic processes such as random genetic drift, migration and founder effects play important roles in the genetic structure of *P. violaceum* in Victoria.

6 Variation in infection efficiency among strains of *P. violaceum* in relation to leaf age

6.1 Introduction

In previous chapters, diversity in DNA phenotype among isolates of *P. violaceum* in Australia was demonstrated. Isolates of *P. violaceum* may vary in other phenotypic characters, such as the range of weed biotypes in which they can incite severe rust disease (virulence) or the amount of disease incited in a particular weed biotype (aggressiveness). One phenotypic character that has not been studied to date in this or any other pathosystem is variation among rust strains for infection efficiency as a function of the age of the leaf they are infecting.

Species of Uredinales infect young, green plant tissue and obtain nutrients from living plant cells. As plant tissue ages, infection efficiency diminishes, as has been observed for *Phragmidium rubi-idaei* on red raspberry (Anthony *et al.*, 1985), *Hemileia vastatrix* on coffee (Coutinho *et al.*, 1994) and *Melampsora larici-populina* on *Populus* species (Sharma *et al.*, 1980). Similar observations have been made for *Rubus anglocandicans*, a species of European blackberry that, when inoculated with urediniospores *of P. violaceum*, exhibited a quantifiable and exponential decrease in the density of uredinia as leaf age increased (Evans & Bruzzese 2003).

The proportion of young and newly developed leaves in a plant canopy can vary among plants depending on local and regional growing conditions and genetically determined characters such as cane density or inherent growth rate of shoots. For instance, canes of *R. anglocandicans* can elongate up to 8 cm per day under ideal environmental conditions (Bruzzese, 1998), resulting in the continual production of young leaf tissue susceptible to

infection by *P. violaceum*. However, if canes stop growing due to environmental stress, such as drought, then the proportion of young, susceptible leaves decreases, which may result in poor disease development. Whether different strains of *P. violaceum* vary in infection efficiency in relation to leaf age is unknown. If significant differences among strains of *P. violaceum* exist, then strain selection using additional selection criteria may improve the effectiveness of blackberry biological control. For example, if one strain has a high infection efficiency on older leaves when compared to another strain, then biological control might be improved when environmental conditions become sub-optimal for blackberry shoot growth. Alternatively, combining strains of *P. violaceum* that infect efficiently over a wide range of leaf ages will expand the amount of susceptible host tissue (ecological niche) available for growth and reproduction of the biological control agent.

The aim of this experiment was to test the hypothesis that strains of *P. violaceum* that vary in virulence and DNA phenotype also vary in their infection efficiency as a function of leaf age. A strict definition of infection efficiency is the number of spores that germinate and establish infection in relation to the amount of spores present on the host surface. We use the term 'infection efficiency' broadly as the number of uredinia that develop for a given concentration of inoculum.

6.2 Materials and methods

6.2.1 Isolates and strains of *P. violaceum*

Isolates V1 and V2, from Victoria, and strain F15 of *P. violaceum* (Table 4.1, Chapter 4), were selected for this experiment based on the fact that they represent three virulence phenotypes (Evans & Gomez, 2004) and three DNA phenotypes following analysis of SAMPL (Chapter 4) or M13/RFLP analysis (Evans *et al.*, 2000). Strain F15 of *P. violaceum* also appears to be genetically distant from isolates V1 and V2 (Chapter 4).

6.2.2 Plant material, inoculation and incubation conditions

A single crown of *R. anglocandicans* collected from a naturalised infestation near Bradford in the Adelaide Hills was established as a potted plant (see section 3.1.1). A specimen from the same blackberry infestation was confirmed to be *R. anglocandicans* by R. M. Barker and D. E. Symon of the State Herbarium of South Australia. When primocanes from the potted crown had elongated sufficiently, daughter plants were generated from them by tip-rooting single primocane apices in separate 40 cm-diameter pots containing a commercial grade potting mix (Nu-Earth Premium Potting Mix). Two daughter plants were propagated in a controlled environment room at 20°C with a 16-h photoperiod (halogen white light, 100 $\mu mol.m^{-2}s^{-1}$). Plants were fertilized with a liquid fertilizer (Aquasol, Hortico, N:P:K 23:4:18) at the beginning of the experiment when the daughter shoots were beginning to emerge from the soil surface.

One week after cane emergence, the three most vigorous shoots in each pot were selected and the remaining shoots pruned and discarded. Each day after shoot emergence, newly formed leaves were tagged and dated when the length of the terminal leaflet blade was at least 3 cm.

The age of each leaf was defined as the number of days from the date of tagging to the date leaves were inoculated. Fifteen days after shoot emergence, each of the three shoots per pot was assigned at random for inoculation with strain F15, V1 or V2. Leaves ranged in age from 0 to 17 days at the time of inoculation.

6.2.3 Inoculation, incubation and disease assessment

Urediniospores were tested for viability by spraying a fine mist of each suspension on 1% water agar in Petri plates. Petri plates were incubated for 16 h in the dark and the proportion of viable spores determined by counting the number of spores with germ tubes greater than the width of the spore for 200 randomly selected spores.

Urediniospores of each strain or isolate of *P. violaceum* were multiplied on detached leaves as described in Chapter 3 and stored at 5°C for 2 days in the dark prior to inoculation. A suspension of 0.25 $mg.ml^{-1}$ of urediniospores in water was applied as a fine mist with a hand-held Preval Spray Power unit (see Chapter 3) to the abaxial surface of all leaves on the shoot selected. Droplets were allowed to dry before each plant was incubated in a separate plastic tent for 24 h at 100% relative humidity provided by sonicating humidifiers (Figure 6.1) in a controlled environment room (20°C, 16 h photoperiod, halogen white light, 100 $\mu mol.m^{-2}s^{-1}$). A concurrent spore viability test indicated that the urediniospores were mostly nonviable (germination percentage $\leq$ 5%), and so a second inoculation was attempted 24 h after the first inoculation using urediniospores recovered from material cultured and stored frozen since 2000. Frozen spores were prepared for inoculation as described in section 3.1.3B and the inoculum prepared by suspending spores in water and adjusting the concentration to 5,000 $spores.ml^{-1}$ with the aid of a haemocytometer. Germination of urediniospores was greater than 60% in this spore viability test and the experiment was continued as planned. Given that new

leaves emerged between the first and second inoculations on some canes, these new leaves were inoculated once only.

Following inoculation and incubation in the plastic tent, plants were removed from the tents and remained in the controlled environment room where they were watered and monitored daily for the appearance of uredinia. The latent period for uredinia was defined as the number of days for uredinia to appear after inoculation, and was recorded for each leaf. Fourteen days after inoculation, leaves were harvested, the total number of pustules per compound leaf was counted and leaflet area determined using an electronic planimeter (Paton and CSIRO, Australia). Infection efficiency was expressed as the number of uredinia per square cm of leaf area.

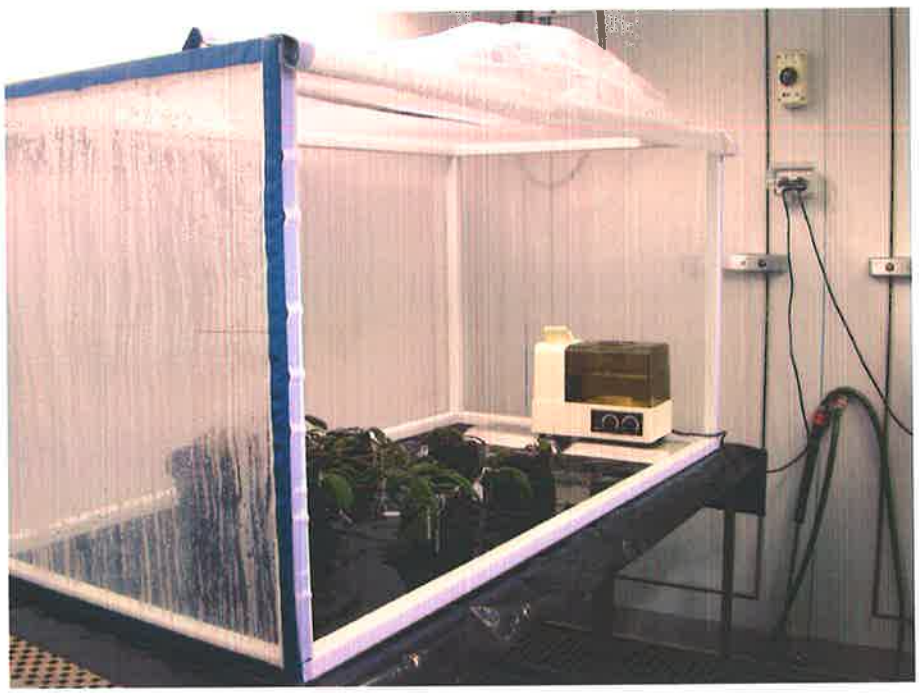

Figure 6.1 Plastic tent in which a blackberry plant was incubated at 100% humidity for 24 h following inoculation of leaves with urediniospores of *P. violaceum*.

6.2.4 Statistical analysis and model development

Genstat version 6.0 for Windows was used to perform non-linear regression analysis, based on logistic models, of leaf age as the explanatory variable of uredinium density for each strain and isolate of *P. violaceum*. Accumulated analysis of variance (A-ANOVA) was performed to determine whether or not non-linear parameter estimates in the logistic models of isolates V1 and V2, and strain F15 of *P. violaceum* were significantly different at P=0.05. If they were not significantly different, then regression analysis was performed again using constant non-linear estimates for each strain of *P. violaceum*. A-ANOVA was then performed again to determine whether or not linear parameter estimates in the logistic models of the two Victorian isolates and strain F15 of *P. violaceum* were significantly different. If they were significantly different, then regression analyses of pairs of *P. violaceum* strains/isolates were performed as described above to test if any two strains/isolates shared parameter estimates for their regression lines. If so, then data sets for each isolate and strain of *P. violaceum* were combined if regression models were statistically similar. Logistic models were then developed for single or multiple isolates/strains of *P. violaceum* as indicated by regression analysis.

Once appropriate models were developed, fitted values of the response variable and the 'rate parameter' of each model were presented graphically as a function of leaf age. The 'rate parameter' is the rate of change in uredinium density with change in leaf age, calculated as the first derivative of uredinium density with respect to leaf age.

The area under each curve between $2 \leq x \leq 25$ days was also estimated as the sum of 20 equally divided segments (rectangles) under each curve as follows:

$$\text{Area} \approx f(x_0).\Delta x + f(x_1).\Delta x + f(x_2).\Delta x + f(x_3).\Delta x + \ldots + f(x_{20}).\Delta x$$

$$\text{Area} \approx \sum_{i=0}^{19} f(x_i).\Delta x$$

where,

$$\Delta x = \frac{25 - 2}{20} = 1.15$$

6.3 Results

6.3.1 Observations of latent period and pustule densities

The latent period of uredinia was 8 days for each leaf regardless of age. This result includes leaves of age 0, which were inoculated once only. No telia formed during the course of the experiment. Younger leaves generally supported greater densities of uredinia than older leaves (Figure 6.2). There appeared to be an inverse logistic relationship between uredinium density and leaf age, for leaves greater than 2 days old (Figure 6.3). Isolates V1 and V2 appeared to produce greater densities of uredinia than strain F15 for leaves aged between 2 and 10 days. For leaves older than 10-12 days, pustule densities appeared to decline more rapidly for isolates V1 and V2 when compared with pustule densities that were apparently higher for strain F15 on these older leaves. Strain F15 continued to produce pustules on leaves that were 17 days old, whereas it appeared that pustule production was ceasing on 17 day-old leaves inoculated with isolates V1 or V2.

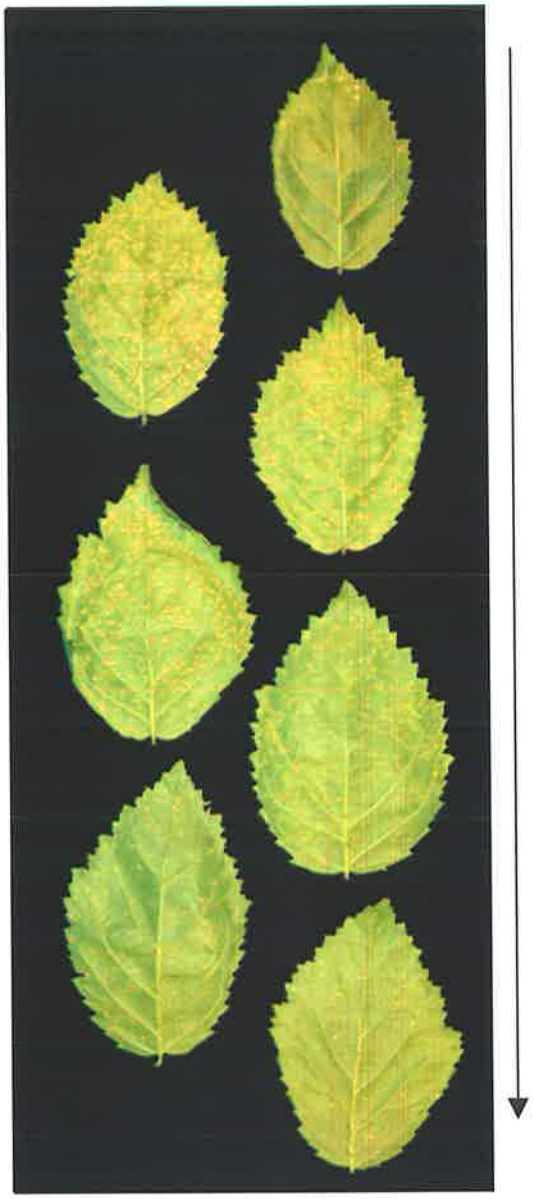

Figure 6.2 The effect of increasing leaf age at the time of inoculation on pustule density. Arrow indicates increasing leaf age.

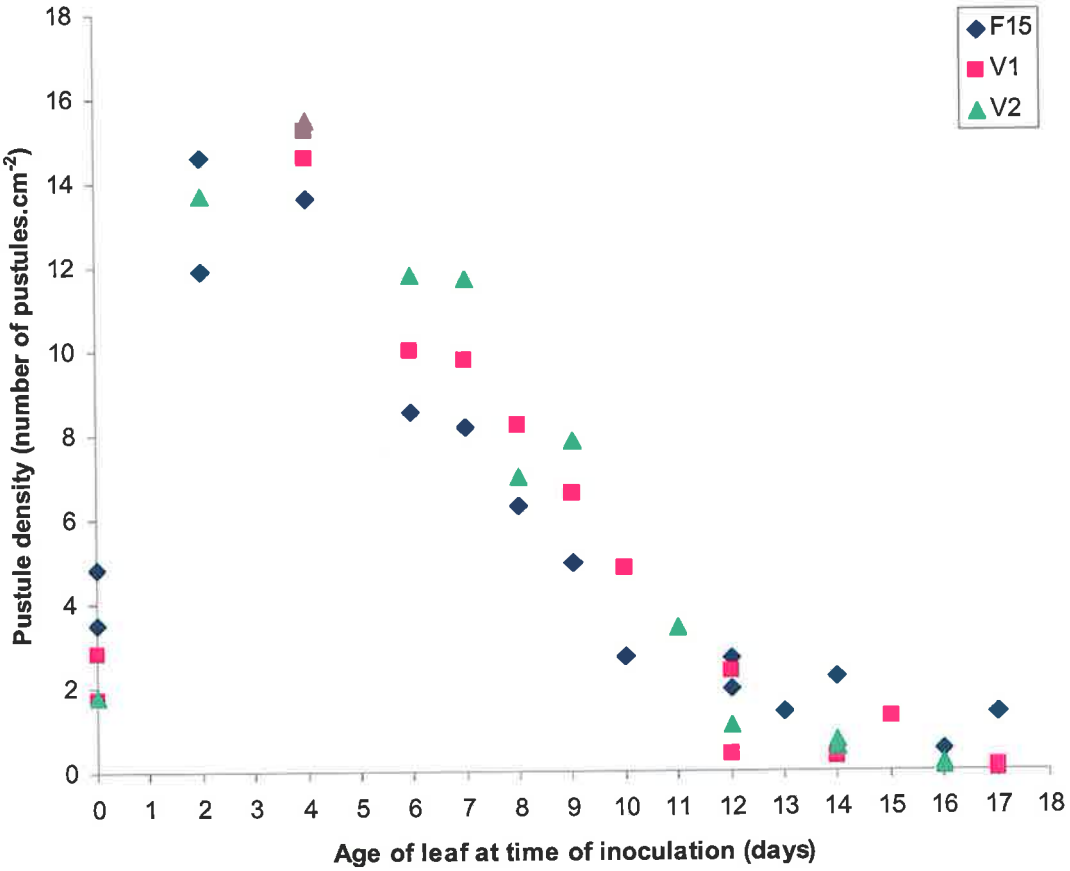

Figure 6.3 Inverse logistic relationship between uredinium density and leaf age at the time of inoculation.

6.3.2 Regression analyses and model development

For analytical purposes, data for leaves less than 2 days old at the time of inoculation were omitted during development of logistic models. Non-linear regression analysis indicated that leaf age had a significant effect on pustule density ($P<0.001$), with leaf age accounting for 96.5% of the variance observed in pustule density. A-ANOVA indicated that non-linear estimates were not statistically separated and the analysis was repeated with models in which the non-linear estimates were constant (see appendix). In this case, the relationship between leaf age and pustule density was highly significant ($P<0.001$) with leaf age accounting for 96.1% of the variance in pustule density. A-ANOVA indicated that linear estimates for the interaction between leaf age and strain/isolate of *P. violaceum* were separated statistically. However, although the interaction between leaf age and isolate was significantly different, the change in the effect of isolate on its own was found to be insignificant ($P=0.135$).

Like the three-way comparison of strains described above, all pairwise non-linear regression analyses to strain F15 favoured models in which only linear components were separate and non-linear components were constant. However, when isolates V1 and V2 were compared with one another, A-ANOVA indicated that both isolates shared the same logistic model. The data from isolates V1 and V2 were combined and compared to data from strain F15. Under the assumption that all parameters were separated statistically, non-linear regression analysis revealed a significant logistic relationship between leaf age and pustule density ($P<0.001$), with 96.3% of variance in pustule density accounted for by leaf age. However, under this assumption, A-ANOVA revealed that parameter estimates for non-linear components were not separated statistically ($P=0.096$). When non-linear parameters of the models were made constant during regression analysis, linear parameter estimates for the interaction between leaf age and strain/isolate of *P. violaceum* differed significantly ($P=0.002$). The change in the

effect of strain of *P. violaceum* and leaf age was also found to be significant (P=0.043 and P<0.001, respectively). Thus, models based on the assumption of separate linear parameters were selected (Figure 6.4), one for strain F15 and another for the combined Victorian data set, with both models sharing the same non-linear parameter estimates. These models are presented graphically in Figure 6.5.

The value for leaf age at which the regression lines intersected was determined by equating regression models to each other and solving for leaf age. The regression lines intersected at 11.2 days, the leaf age beyond which the models predict that strain F15 produces greater pustule densities than the Victorian isolates of *P. violaceum*. The model for the Victorian isolates predicts they will not produce pustules on leaves older than 22.7 days, whereas the model for strain F15 predicts that 0.55 pustules per cm^2 will form at a leaf age of 22.7 days.

The area under each curve was approximately 107.7 and 99.0 (pustules.cm^{-2}.days) for the models describing the response of the Victorian isolates and strain F15, respectively. While these estimates indicate that the Victorian isolates caused more disease overall, the difference between the area under each curve appeared to be negligible.

The relationship between the predicted 'rate parameter' of each model and leaf age (Figures 6.6 and 6.7) illustrated that the Victorian isolates, when compared with strain F15, produced a steeper rate of decline in pustule density with leaf age until the inflection point between 8 and 9 days. Beyond the inflection point, the rate of decline in pustule density slowed for both models, although it was more rapid for the Victorian isolates than for strain F15.

$$y_{VIC} = -0.01069 + \frac{16.46}{1+e^{0.4955x-3.91693}}$$

$y_{VIC} = 0$, $x = 22.7$

$$y_{F15} = 0.5370 + \frac{13.12}{1+e^{0.4955x-3.91693}}$$

$y_{F15} = 0.546$, $x = 22.7$

Figure 6.4 Logistic models describing pustule density, y (number of pustules per cm^2) as a function of leaf age, x (days), where y_{VIC} is the response variable for Victorian isolates V1 and V2 of *P. violaceum*, and y_{F15} is the response variable for strain F15 of *P. violaceum*.

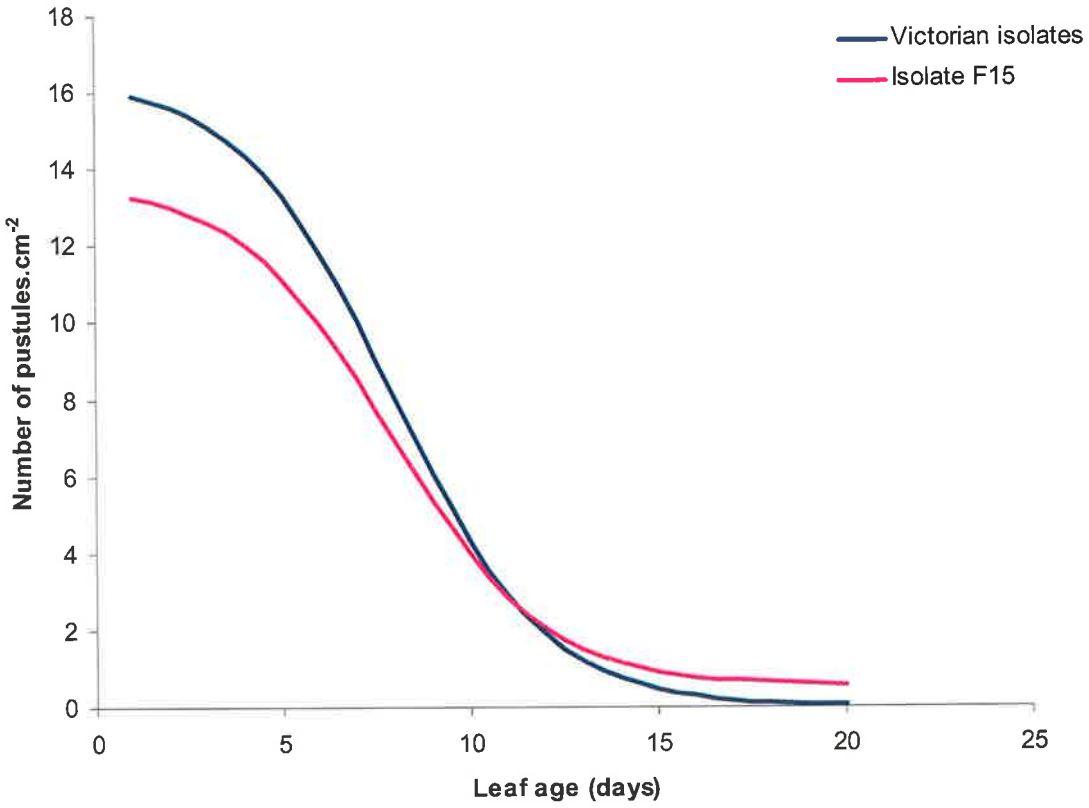

Figure 6.5 Fitted curves for final models showing the logistic relationship between leaf age and pustule density for the Victorian isolates and strain F15.

$$\frac{dy_{VIC}}{dx} = \frac{-8.15593 \times e^{0.4955x-3.91693}}{\left(1+e^{0.4955x-3.91693}\right)^2}$$

$$\frac{dy_{F15}}{dx} = \frac{-6.50096 \times e^{0.4955x-3.91693}}{\left(1+e^{0.4955x-3.91693}\right)^2}$$

Figure 6.6 First derivative of the logistic models for the effect of leaf age (days) on pustule density (number of pustules per cm^2).

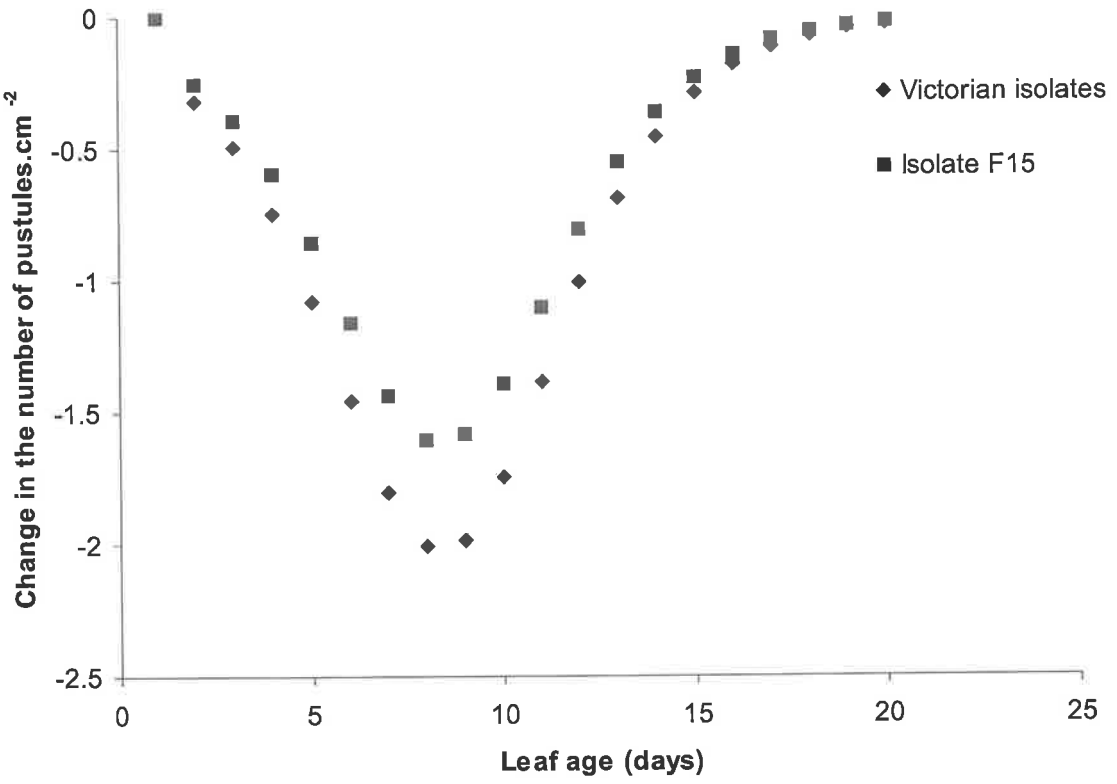

Figure 6.7 The change in pustule density as leaves age, calculated from the first derivatives described in Figure 6.6.

6.4 Discussion

Although there have been studies describing the relationship between leaf age and disease development, this is the first report to show differences among strains of a pathogen in terms of leaf age-related resistance. Previous studies have shown a degree of host specialisation and genetic variation in *P. violaceum* (Evans & Gomez, 2004). While this type of research is important for matching virulent strains of the pathogen to a genetically variable weed population, there has been little empirical research looking at other sources of physiological diversity in *P. violaceum* prior to this study.

The difference between the inverse *logistic* relationship observed in this study and the *exponential* decrease is pustule density demonstrated by Evans & Bruzzese (2003), is likely to be due to the fact that detached leaves were used in the earlier study. In a whole plant assay, leaf area is likely to increase between inoculation and disease assessment, unlike detached leaves that retain more or less the same leaf area after detachment. The increase in leaf area in whole plants would be greatest for younger leaves which had not reached their maximum expansion at the time of inoculation. The effect of leaf expansion is that the ratio of pustules to leaf area is reduced compared to a detached leaf that is the same age at inoculation. This might explain why there was a prominent lag phase in the decrease in pustule density at the younger leaf age spectrum in whole plant assays when compared with data generated using detached leaves.

While the difference in the total amount of disease observed between the Victorian isolates and strain F15 appeared to be negligible across leaf ages assayed, the models predicted that the Victorian isolates, unlike strain F15, would no longer produce pustules as leaf age increased past 22 days. In contrast, strain F15 was predicted to produce greater than 10

pustules per leaf at this leaf age, based on the observation that mature leaves greater than 20 days old had an area greater than 100 cm^2. The existence of pustules on older leaves, although few, ensures the survival of *P. violaceum* as teliospores over the winter when these infected leaves are retained in climates where blackberry is semidormant. Therefore, strain F15 might have a slight advantage over isolates V1 and V2 in the amount of inoculum that is carried over to the next growing season, depending on the extent of defoliation and the zone of the shoot that is defoliated in the current growing season.

The mechanisms contributing to leaf age-related resistance to *P. violaceum* are not clear in European blackberry. However, leaf age-related resistance has been observed in interactions of some woody, perennial plants with biotrophic plant pathogens as a result of a plant's defence response varying with leaf age (Doster and Schnathorst 1985; Reuveni 1998). Increased disease resistance has been associated with increases in peroxidase and β-1,3-glucanase activities as leaves age (Reuveni 1998). The enzyme, β-1,3-glucanase is associated with the defence response of a plant while peroxidase activity is a marker for leaf senescence (Takahama et al. 1999). Enzymes associated with leaf senescence may also be involved in plant defence mechanisms against pathogens (Lamb and Dixon 1997). The differential response among the Victorian isolates and strain F15 may be linked with the comparative ability of pathogen strains to overcome increased levels of plant defence related enzymes in older leaves. Further research is required to elucidate the mechanisms of leaf age-related resistance to *P. violaceum* in the *R. fruticosus* agg.

While glasshouse assays appear to indicate the importance of leaf age in the development of disease, leaf age is only one factor that may affect the epidemiology of blackberry rust. Consequently, the translation of this phenomenon to a field situation remains obscure amidst a

web of environmental, host and pathogen-related factors both limiting and encouraging disease development. The importance of this study comes into effect when considering that the phenology of the host will vary among demes across the landscape.

From metapopulation theory (Levins, 1971; Thompson, 1999), local populations of European blackberry can be described to exist as semi-discrete entities across the landscape, showing heterogeneity in their genetics and climate in the habitats they infest. For this reason, demes of the host will likely vary in phenology, at any one time. While leaf age profiles may vary dramatically among demes as a consequence of this, the variation in genetics and phenology of the host may give rise to other factors which will promote or limit disease. Like leaf age, the likely importance of other factors will vary among local populations of the host.

The challenge for biocontrol is how to best maximise the impact of disease in situations where pathogen habitat will vary markedly across the landscape due to numerous factors. For example, Evans *et al.* (2005) argue that disease levels may also be a function of cane density, which is known to vary among blackberry taxa (Amor, 1975). While dense shoots may result in an increased proportion of leaves that are in an appropriate age profile for significant infection by the pathogen, increased shoot densities may also result in increased humidity in a dense blackberry canopy. As a consequence, this increase in canopy humidity may translate into increased disease levels.

This study shows that physiological variation in infection efficiency within *P. violaceum*, in relation to the phenology of its host, is another tool for characterising rust strains selected for biological control. While searching for appropriate virulence pathotypes is important, the data presented here highlight the importance of elucidating other sources of physiological variation

among strains of *P. violaceum* to promote disease in a variety of environmental and host circumstances. Such information could be used to aid selection of strains of *P. violaceum* to broaden the geographic and the climatic range of the biological control agent in Australia.

7 General discussion

The aims of this project were achieved as follows: 1) The development of SAMPL and AFLP markers was achieved and the relative superiority of SAMPL to AFLP was observed when assessing genetic variation among isolates of *P. violaceum* from Europe, Australia and New Zealand, 2) SAMPL markers were successfully used to determine population structure and dynamics within and among populations of *P. violaceum* in Victoria and 3) Glasshouse assays revealed that three genetically distinct strains of *P. violaceum* varied in their infection efficiency on leaves of *R. anglocandicans* as a function of leaf age.

This study supports a growing body of research that is providing both the conceptual framework for and empirical evidence of the complex nature of obligate biotrophic plant pathogens in a natural ecosystem. While the concepts of metapopulations and the geographical mosaic of coevolution are largely theoretical (Thompson, 1999), recent studies have been successful in detecting varying scales of local and regional adaptation of pathogen demes among natural plant communities (Thrall *et al.*, 2002; Laine, 2005). Recent studies in natural ecosystems have relied mostly on traditional markers for virulence and susceptibility, whereas this study demonstrates the merit of using DNA markers for elucidating population structure and dynamics in rust pathosystems.

The DNA markers developed and applied here have enabled the characterisation of isolates of *P. violaceum* from Australia and Europe and identification of the likely continent of origin of *P. violaceum* in Australia and New Zealand. This study also provided the first insight to the metapopulation dynamics of the pathogen in Australia. In particular, the SAMPL markers allowed observation of how pathogen demes varied genetically from small to large degrees across time and space, which is an indication of varying scales of interaction among these

populations of the biological control agent. The lack of correlation between pairwise differentiation of populations and geographic distance suggested that gene flow among pathogen demes can occur in varying degrees over extensive areas and is an indication that migration and subsequent establishment are largely stochastic processes. Indeed, the migration of spores of the fungus among local populations of the host is likely to be in the direction of prevailing winds, however, variable weather patterns may transport spores in any direction (Aylor *et al.*, 1982). Pathogen strain recruitment will then be a function of habitat quality, which in itself can vary from small to large degrees across the landscape. The potential for long-distance aerial transport of spores in various directions, coupled with the chance nature of pathogen strain recruitment, is the likely reason why gene flow can fluctuate dramatically across time and space.

In this study, sampling was conducted early in the season so that the likelihood of detecting the movement of genotypes (urediniospores) was low. A limitation to the use of SAMPL markers for detection of gene flow is the fact that they are largely dominant. The characterisation of co-dominant loci from SAMPL, or the generation of co-dominant microsatellite markers would lessen the dependence on sampling time because gene flow would then be investigated as a function of allele movement as opposed to the movement of electrophoretic phenotypes among populations. Characterisation of co-dominant SAMPL markers can be achieved by identifying segregating loci resulting from sexual backcrosses. This would necessitate development of an *in vitro* mating system to perform such crosses in the laboratory. Development of an *in vitro* mating system for *P. violaceum* would be of benefit in studies of other characteristics of the pathosystem, for example, identifying optimum environmental conditions required for the initiation of different stages of the pathogen lifecycle (eg. Bruckart & Eskandari, 2002)

Recombination was also found to be an important process in driving change within populations of *P. violaceum*. Although random mating was detected in some populations (KV1 and TE), the detection of multilocus disequilibrium in two populations exhibiting high levels of multilocus variation (KV2 and WR), was likely to be the result of inadequate sampling scale. Further to this argument, the determination of I_A among populations of *P. violaceum* from the Whitfield infestation collected in 2002 (KV1) and 2003 (KV2) suggested that the scale of gene flow among populations may vary markedly between seasons, depending on the comparative life history of adjacent pathogen demes. The population recovered from the Korumburra infestation reflected a clonal structure that was likely to be due to stochastic chance events, such as a founder effect. While recombination may play an important role in maintaining genetic diversity within some populations of *P. violaceum*, stochastic processes such as random genetic drift and migration are likely to be important factors in population divergence.

Although evidence for recombination in the field is strong, it is not known if urediniospores survive over winter, as has been hypothesised for some *Melampsora* species (Pei & Ruiz, 2000; Samils *et al.*, 2001b). If overwintering of urediniospores does occur, the chances for carry over of clonal lineages from season to season will improve substantially (Samils *et al.*, 2001b), as will the likely persistence of introduced genotypes. Given that high amounts of genotypic diversity were observed among the majority of sites and that populations from 2002 and 2003 from Whitfield shared no genotypes, urediniospores of *P. violaceum* are unlikely to survive over winter. However, it may be that for a proportion of pathogen demes climatic conditions are conducive to the overwintering of urediniospores. Further research is required

to confirm whether or not recombination is facultative in *P. violaceum* prior to the clonal phase of the pathogen lifecycle.

DNA genotyping tools and knowledge of the genetic diversity and evolutionary factors driving change and differentiation among populations of *P. violaceum* in Australia can now be used to build upon the theoretical and applied framework for the selection, characterisation and dissemination of rust strains for improved biological control. The application and impact of this research in recent and future phases of the blackberry rust biological program, and relevance to other programs involving Uredinales agents and genetically diverse weeds, will now be explored.

The issue of recombination has important implications for how potential biological control agents are selected, as was reviewed in section 2.5.4. Where recombination is important for maintaining intrapopulation variation, as demonstrated for populations of *P. violaceum* in Victoria, natural selection plays an important role in the maintenance of new allele combinations that are able to survive on susceptible hosts. Thus, the aim for strain selection is to improve the evolutionary potential of the biological control agent, such that the pathogen is able to evolve and adapt to its new environment through natural selection. The most obvious way to do this is to increase the genetic diversity of the agent in the introduced range through the release of numerous strains of *P. violaceum* which are genetically variable and which represent a range of new alleles, such as virulence linked to fitness. While numerous strains of *P. violaceum* were selected in Europe in the 1970s, only strain F15 was eventually released in Australia because of the political and regulatory complications arising from the unauthorised release of *P. violaceum* in Australia *circa* 1984. One implication of this study is that, in the absence of a coordinated release of multiple, characterised strains, there is the risk that

subsequent releases of one or more rust strains will fail to establish because of the 'numbers game' involved in pathogen strain recruitment. That is, the amount of inoculum of a single rust strain upon initial release is small compared to the large effective population size of the well-adapted existing population of *P. violaceum*. Recombination soon after strain establishment would allow gene flow into the existing population, otherwise the clonal genotype released might soon become extinct.

In 1999, researchers from CSIRO Entomology and the Cooperative Research Centre for Australian Weed Management established a garden of *Rubus* clones in Montpellier, France, comprising a representative sample of the *R. fruticosus* agg. from Australia (Scott *et al.*, 2002). These plants were used to trap urediniospores of *P. violoceum* for collection and selection of additional strains with the aim of releasing a subset of them in Australia to increase the genetic diversity of *P. violaceum*. DNA phenotyping, using SAMPL markers (Chapter 4), played a central role in evaluating the capacity of the trap garden to propagate a potentially useful number of diverse strains of *P. violaceum*. Whether or not isolates of *P. violaceum* are collected from infected native plants at multiple sites in the native range or by use of a trap garden, there is usually a cost limitation on the number of strains that can be screened during tests for host specificity. DNA phenotyping, in conjunction with virulence pathotyping, revealed that the trap garden provided a manageable number of genetically diverse and virulent strains for cost-effective selection. The efficiency of the trap garden approach could be tested further by collecting isolates of *P. violaceum* from naturally occurring blackberry in southern France and comparing their genetic diversity with the population isolated from the trap garden. If genotypes of *P. violaceum* beyond those characterised from the trap garden had been available for selection in Europe, then trap

gardens might have been placed in other locations, including England, the likely country of origin of a number of Australian biotypes of the *R. fruticosus* agg. (Evans & Weber, 2003).

Following selection of potential strains used for biological control, characterisation of strains is an important facet of the biological control process as it allows cataloguing (identifying strains with certainty) and the correlation of characters to biocontrol efficacy. Although SAMPL markers were developed primarily as a research tool for population genetic studies, they could also be used for cataloguing, although other marker systems, such as SCARs (Section 2.5.5.3), should be investigated due to their ease of use. Identifying strains with certainty is a basic prerequisite in risk management associated with biological control and DNA phenotyping is extremely valuable when little morphological variation exists among different strains of the agent. Ultimately, the benefit to cost ratio for each marker system will need to be considered prior to adoption as a regular diagnostic/cataloguing tool.

Infection efficiency as a function of leaf age is a previously unidentified phenotype that might be used in characterising strains of *P. violaceum* during the selection phase of a biocontrol program. While virulence is an essential criterion for selection of pathogen strains, this secondary physiological character might be exploited for broadening the geographic and climatic range of the biocontrol agent. Given that the quality of pathogen habitat in the introduced range can vary markedly across the landscape, further research in this area is warranted. Whether or not the difference in infection efficiency between strain F15 and the Victorian isolates would translate to measurable differences in the field is questionable. Further study of the epidemiology of blackberry rust is vital to ascertain if the scale of difference among strains of *P. violaceum* is applicable to biological control.

Following selection and characterisation of pathogen strains, the nature of agent release influences establishment and persistence of genotypes or genes. Numerous species have been introduced into areas outside their original range for biological control. Some of these species have established successfully, whereas many have not and it is becoming apparent that there are a number of general rules that predict the success of establishment (Duncan *et al.*, 2001). The amount of effort employed for introduction of an agent is a likely key to successful establishment (Hopper & Roush, 1993). This statement is especially relevant to *P. violaceum* in Australia, given the fractured and random nature of gene flow, despite the apparent capacity for long distance dispersal. Variation in climate and underlying blackberry taxa leads to dramatic changes in pathogen habitat across time and space. Under these circumstances, there is no guarantee that a pathogen strain released at location A will establish and persist at location B. Clearly, improved establishment and persistence can be achieved through release of agents at numerous sites and over several years. However, this general recommendation needs to be refined and streamlined so that biological control researchers and practitioners have increased confidence in establishment and persistence of strains following dissemination of pathogen strains with limited resources. 'Getting strains to where they are needed' will require the development of targeted release strategies, including targeting individual strains of *P. violaceum* to susceptible blackberry clones and climates conducive to disease development. Further work is required to map blackberry taxa, their clonal composition and disease resistance characters. Environmental mapping using GIS can be used to predict potential areas for release based on historical weather patterns (Pigott *et al.*, 2003). A targeted approach to release highlights the value of strain characterisation in the early stages of selection for efficacy of biological control agents.

Having highlighted the need for targeted approaches for release of biological control agents, further research is required to elucidate the most efficient way of delivering inoculum in the field. In the past, active and passive inoculative approaches have been used for release of agents among a small number of release sites (F. Mahr & L. Morin, personal communication). Given that the blackberry infestation covers a vast area, and that pathogen habitat is variable across this range, inundative release strategies may be a beneficial approach for more expansive infestations. However, such strategies require the mass production of inoculum which is labour intensive.

There has been a lack of research to investigate the success of establishment of pathogen strains. This issue is highly relevant given the release of eight additional strains of *P. violaceum* in 2004 and given that the findings of this study support previous research (Evans *et al.*, 2000) which questioned the establishment of strain F15. The use of DNA markers to trace the fate of genotypes released in the field during the first uredinial phase after release, prior to recombination, may be an important process for determining appropriate methods for inoculum delivery in the field. Assessing strain establishment after this time could be aided by the development of single locus, non-radioactive, PCR-based markers for diagnostic use in rapidly identifying the fate of released strains in the field. However, the original research tool developed here has provided the best resolution of genetic diversity at the subspecific level of *P. violaceum* to date. It should be applied further to characterise the genetic structure of post-release populations of *P. violaceum* and for elucidating co-evolutionary processes in conjunction with appropriate genetic markers in the *R. fruticosus* agg.

Given the time and effort, and to some extent, exasperation, that is invested into biological control, it is understandable that any information that can be used to devise methods by which

to increase the rate of successful establishment will be of use. Even so, all introductions must be evaluated on a case by case basis as parameters for successful biological control are likely to be intrinsic to the host-pathogen interaction being investigated.

While the population dynamics and co-evolutionary aspects of the *P. violaceum* – *R. fruticosus* agg. pathosystem are complicated and not easily quantified, the information presented here has offered insight on how to manipulate selection and release strategies for *P. violaceum* for effective long-term biological control of European blackberry in Australia.

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Appendix

Example of nonlinear regression analysis comparing isolates V1, V2 and strain F15 of *P. violaceum* in their infection efficiency as a function of leaf age (refer to chapter 6)

1. Nonlinear regression analysis – all parameters separate

Response variate: pustule_density
Explanatory: leaf_age
Grouping factor: isolate, all parameters separate
Fitted Curve: A + C/(1 + EXP(-B*(X - M)))

Summary of analysis

	d.f.	s.s.	m.s.	v.r.	F pr.
Regression	11	1019.20	92.6544	96.36	<0.001
Residual	27	25.96	0.9615		
Total	38	1045.16	27.5042		
Change	-4	-7.45	1.8632	1.94	0.133

Percentage variance accounted for 96.5
Standard error of observations is estimated to be 0.981

Estimates of parameters

		estimate	s.e.
B	isolate F15	-0.556	0.155
M	isolate F15	7.096	0.505
C	isolate F15	13.08	1.55
A	isolate F15	1.219	0.595
B	isolate V1	-0.386	0.131
M	isolate V1	7.11	1.27
C	isolate V1	19.67	5.27
A	isolate V1	-0.468	0.973
B	isolate V2	-0.584	0.135
M	isolate V2	8.642	0.396
C	isolate V2	15.04	1.42
A	isolate V2	-0.074	0.705

A-ANOVA

Change	d.f.	s.s.	m.s.	v.r.	F pr.
+ leaf_age	3	995.8330	331.9443	345.23	<0.001
+ isolate	2	4.6047	2.3024	2.39	0.110
+ leaf_age.isolate	2	11.3077	5.6539	5.88	0.008
+ separate nonlinear	4	7.4527	1.8632	1.94	0.133
Residual	27	25.9609	0.9615		
Total	38	1045.1591	27.5042		

2. Nonlinear regression analysis – all linear parameters separate

Response variate: pustule_density
Explanatory: leaf_age
Grouping factor: isolate, all linear parameters separate
Fitted Curve: A + C/(1 + EXP(-B*(X - M)))

Summary of analysis

	d.f.	s.s.		m.s.	v.r.	F pr.
Regression	7	1011.75		144.535	134.09	<0.001
Residual	31	33.41	1.078			
Total	38	1045.16		27.504		
Change	-2	-11.31		5.654	5.25	0.011

Percentage variance accounted for 96.1
Standard error of observations is estimated to be 1.04

Estimates of parameters

		estimate	s.e.
B		-0.4964	0.0514
M		7.908	0.221
C	isolate F15	13.11	
A	isolate F15	0.5391	
C	isolate V1	16.44	
A	isolate V1	-0.05674	
C	isolate V2	16.43	
A	isolate V2	0.04839	

A-ANOVA

Change	d.f.	s.s.	m.s.	v.r.	F pr.
+ leaf_age	3	995.833	331.944	307.97	<0.001
+ isolate	2	4.605	2.302	2.14	0.135
+ leaf_age.isolate	2	11.308	5.654	5.25	0.011
Residual	31	33.414	1.078		
Total	38	1045.159	27.504		

3. Nonlinear regression analysis – constant parameters separate

Response variate: pustule_density
Explanatory: leaf_age
Grouping factor: isolate, constant parameters separate
Fitted Curve: A + C/(1 + EXP(-B*(X - M)))

Summary of analysis

	d.f.	s.s.	m.s.	v.r.	F pr.
Regression	5	1000.44	200.088	147.65	<0.001
Residual	33	44.72	1.355		
Total	38	1045.16	27.504		
Change	-2	-4.60	2.302	1.70	0.198

Percentage variance accounted for 95.1
Standard error of observations is estimated to be 1.16

Estimates of parameters

		estimate	s.e.
B		-0.5217	0.0582
M		7.986	0.226
C		14.80	
A	isolate F15	-0.2089	
A	isolate V1	0.4383	
A	isolate V2	0.5769	

A-ANOVA

Change	d.f.	s.s.	m.s.	v.r.	F pr.
+ leaf_age	3	995.833	331.944	244.94	<0.001
+ isolate	2	4.605	2.302	1.70	0.198
Residual	33	44.721	1.355		
Total	38	1045.159	27.504		

4. Nonlinear regression analysis – all estimates constant

Response variate: pustule_density
Explanatory: leaf_age
Fitted Curve: A + C/(1 + EXP(-B*(X - M)))

Summary of analysis

	d.f.	s.s.	m.s.	v.r.	F pr.
Regression	3	995.83	331.944	235.54	<0.001
Residual	35	49.33	1.409		
Total	38	1045.16	27.504		

Percentage variance accounted for 94.9
Standard error of observations is estimated to be 1.19

Estimates of parameters

	estimate	s.e.
B	-0.5382	0.0929
M	8.027	0.319
C	14.56	1.13
A	0.337	0.472

A-ANOVA

Change	d.f.	s.s.	m.s.	v.r.	F pr.
+ leaf_age	3	995.833	331.944	235.54	<0.001
Residual	35	49.326	1.409		
Total	38	1045.159	27.504		