



GENETIC VARIATION IN
***HYPERICUM PERFORATUM* L. AND RESISTANCE**
TO THE BIOLOGICAL CONTROL AGENT
***ACULUS HYPERICI* LIRO**

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Errata

Pg. 12, line 4: a period should be inserted between 'individuals' and 'However'

Pg. 12, line 5: 'pursued directly' should read 'examined directly'

Pg.13, line 13: 'than' should read 'that'

Pg.22, line 3: should read 'when the beetle is not active'

Pg. 41, line 6: 'Pr >0.039' and 'Pr >0.005' should read 'Pr <0.039' and 'Pr <0.005'

Pg. 42, line 5: 'simplicity' should read 'clarity'

Pg. 44, Table 2.2: 'Time' should read 'Time, in days,'

Pg. 68, line 6: 'reproductive' should read 'reproduction'

Pg. 70, line 14: 'hence' should be omitted

Pg. 82, line 8: 'black fly' should read 'brine fly'

Pg. 83, line 23: 'which' should read 'that'

Pg. 85, line 20: reference to page 27 should be to page 94

Pg. 93, line 6: reference to page 31 should be to page 98

Pg. 132, line 17: omit reference to Feeny (1976)

Pg. 140, Fig. 5.2: should read 'b) increasingly stunted growth as a result of mite infestation'

Pg. 145, line 9 onwards : should read 'It was concluded that increasing inoculum level and spacing of inoculum on the host plant did not result in greater population increase. Higher inoculum resulted in smaller population increases; for example, for inocula of 200, 400 and 800 mites, respective population increases were 8.9, 7.5 and 5.9 times the inoculum level.' rather than 'It was concluded that increasing inoculum level and spacing of inoculum on the host plant resulted in greater population increase.'

Pg. 146: lines 14 and 17: 'Mean number of mites' should read 'mean total number of mites'

Pg. 162, Fig. 5.7: legend should include a last line 'Individuals for whom replicated data was unavailable are marked with an asterisk.'

Pg. 186, line 13: 'the upper leaf' should read 'on the upper leaf'

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Declaration

The research presented in this thesis is my original and independent work, and contains no material that has been accepted for the award of any other degree or diploma in any university. To the best of my knowledge this thesis contains no material previously published or written by another person, with the exception of specific contributions that are referred to in the text.

I consent to the thesis being made available for photocopying and loan if accepted for the award of the degree.

Gwenda Mary Mayo

September 2004

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“The king’s a beggar now the play is done:

All is well ended if this suit be won

That you express content; which we will pay,

With strife to please you, day exceeding day:

Ours be your patience then, and yours our parts;

Your gentle hands lend us, and take our hearts.”

Epilogue from *All’s Well That Ends Well*

William Shakespeare

Abstract

Research on the selection of biological control agents, host specificity and impact assessment is required in order to improve predictions of success in classical biological control of weeds. Perhaps the greatest waste of resources is failure of an agent to establish after detailed testing and considerable release effort. One of the factors significantly affecting establishment is mismatch between agent and weed, as a result of genetic variation in the weed and collection of the agent from a different biotype of the plant in the native range than is present in the introduced range. The eriophyid mite *Aculus hyperici* is the most recently released biological control agent against the toxic weed *Hypericum perforatum* (St. John's wort) in Australia. Although the impact of this agent was initially thought to be extremely promising, failure of *A. hyperici* to establish despite repeated releases was noted at several field sites in New South Wales. Variation in susceptibility of the target weed *H. perforatum* to the mite was suspected to have a genetic basis; therefore studies using multilocus molecular markers, of the plant's reproductive biology and its influence on population genetic structure, formed the basis for an investigation of mite-susceptibility in Australian populations of the weed.

An initial study of the germination characteristics of *H. perforatum* was made because practical problems were encountered with the germination of some accessions. Temperature affected time to initiation of germination of *H. perforatum* seed, but not maximum germination capacity or germination rate. A significant interaction of seed age with temperature resulted in two distinct patterns of germination response to temperature; in older seed high temperature resulted in earlier germination than any other temperature, followed by medium temperature then low temperature, but in younger seed medium temperature resulted in the earliest germination, followed by low temperature then high temperature. The transition between the two patterns appeared to take place towards the

end of the first year and involved a switch in the position of the high temperature response curve relative to the medium and low response. Following this transition, response to temperature seemed to remain stable for the remainder of the lifetime of the seed. An *H. perforatum* seed bank is likely to contain viable seed for at least 13 years, regardless of genotype, but viability may start to decline from seven years of age. It was proposed that the age and temperature-dependent germination response may have evolved to prevent wasteful depletion of seed with the greatest potential longevity.

The mode of reproduction in *H. perforatum* was investigated and results are included as published (Mayo & Langridge, 2003). *H. perforatum* is a facultative pseudogamous apomict whose reproductive biology, in particular the degree of apomixis, was expected to significantly influence spatial and temporal variation in the target host. Reproduction in *H. perforatum* was investigated using markers based on restriction fragment length polymorphism (RFLP) and amplified fragment length polymorphism (AFLP). A novel DNA extraction method was developed because traditional CTAB-based extraction methods were found to be unsatisfactory in this species. Between two Australian populations, the tetraploid plants that were examined displayed 14 polymorphisms from a total of 22 scorable RFLP markers when genomic DNA was probed with M13 bacteriophage, but individuals within each population exhibited identical RFLP fingerprints. Ninety-four percent of the progeny of four crosses made between the two populations exhibited identical fingerprint and ploidy level to the maternal parent, and probably originated apomictically. Seven seedlings with recombinant RFLP or AFLP fingerprints were found from a total of 121 progeny. Both molecular marker techniques detected the same recombinants from a subset of screened progeny. Cytological analysis showed that the seven recombinants comprised three tetraploids ($2n = 4x = 32$), three hexaploids ($2n = 6x = 48$), and one aneuploid ($2n-1 = 31$), which suggested that the level

of normal reduced embryo sacs was only 2.5%. These results are discussed in relation to population genetic structure and the management of invasive populations.

Multilocus RFLP and AFLP techniques also provided improved methods for the identification of genotypes and estimation of genetic diversity within and among Australian populations of *H. perforatum*. Population variation was evaluated from 298 individuals raised from field-collected seed from two transect sites, two quadrat sites, and random samples at 40 other sites. Cytological analysis was made for several individuals of each genotype, in addition to seedlings with unusual leaf morphology. Reproductive biology and genetic variation studies detected variation in fingerprint patterns at different ploidy levels, which indicated that ploidy should be taken into consideration in molecular analyses of this species. Results suggested that the majority of individuals in the field were tetraploid but that a small proportion of other ploidies may also exist. High inter-population variation contrasted with low intra-population variation; 21 genotypes could be identified among the 44 Australian populations, but within-population variation was so low that in most cases populations appeared to be clonal, probably as a result of a high degree of apomixis and introduction via a few founding individuals. Consequently, plant characteristics such as susceptibility to herbivores were expected to be relatively uniform within but not necessarily among populations. The geographic distribution of genotypes was mapped for Australian populations. There was little doubt from the diversity and distribution of genotypes included in the study and from historical evidence in the literature that multiple introductions of the weed have occurred. The RFLP and AFLP datasets provided very different genetic distance measures between profiles. Phylogenetic analysis showed some evidence for the existence of broad-leaved and narrow-leaved groups, but conclusions were not easily drawn with respect to relationships because few nodes were resolved with much confidence.

The correlation between target weed genotypes and susceptibility to *A. hyperici* was examined for evidence of a genetic basis to resistance, in order to estimate potential impact and control success, and to gauge the level and geographical extent of resistance and the risk of replacement of susceptible with resistant genotypes. A novel bioassay method for susceptibility of *H. perforatum* to the mite *A. hyperici* was developed. The level of mite-susceptibility was determined for 230 individuals fingerprinted with multilocus techniques, comprising 20 of the 21 genotypes, among which known histories of field establishment or impact data were available for six genotypes. The results strongly suggested that susceptibility to *A. hyperici* was correlated to *H. perforatum* genotype; consistent susceptibility of individuals belonging to a particular genotype contrasted with variable susceptibility among genotypes. Susceptibility among genotypes ranged from “low”, where mite populations consistently declined from initial inoculum levels, sometimes to the point of complete collapse, through “medium” susceptibility, with relatively slow population increases ranging from 4% to 33% of the most highly susceptible genotype, to “high”, where mite populations could increase as much as 55 times the initial inoculum level in only four weeks. No RFLP or AFLP markers were associated with susceptibility to *A. hyperici*. Given the low intra-population genetic variation, comparison of genotypes of known susceptibility with field establishment history and impact studies led to the prediction that *A. hyperici* would fail to establish at all populations consisting of the three low-susceptibility genotypes in New South Wales, and establish but provide relatively slow or sub-optimal control on the seven medium-susceptibility genotypes dotted across the continent, compared to optimal control of the nine widespread highly susceptible genotypes. It is quite plausible that those genotypes with lower susceptibility will replace highly susceptible ones over time.

Evidence for a possible physical mechanism for mite-resistance was sought. Leaf wax density, wax depth and cell morphology were examined by scanning electron microscopy, using several low and highly mite-susceptible genotypes of *H. perforatum*. Physical impediments to feeding and oviposition were examined for mites fed on low and highly susceptible genotypes of the weed. The results were too inconclusive to exclude hypotheses concerning the influence of physical factors on population dynamics.

The role of hypericin in mite-resistance was investigated. A two- to three-fold difference in production of the toxin hypericin was found among 10 Australian genotypes when flowering stems of 18 individuals were compared. Hypericin-related pigments did not seem to be involved in the mechanism of mite resistance, since genotypes within each mite-susceptibility class displayed a wide range of hypericin values. The hypericin levels of different genotypes were compared to see if existing grazing management recommendations were still appropriate, particularly in relation to resistant genotypes that are not likely to be controlled by *A. hyperici*. Existing grazing recommendations dictated a relatively short grazing period with merino sheep for a high hypericin, narrow-leaved "biotype" (A12 genotype) when compared to a low hypericin, broad-leaved "biotype" (A10 genotype). High hypericin levels were found in the resistant A1 and A2 genotypes and low hypericin in the resistant A10 genotype. The general use of a short grazing season was considered most appropriate, because hypericin levels for the other genotypes fell in the range between the A12 and A10 genotypes.

The relatively low intra-population and high inter-population genetic variation, resulting respectively from a high degree of apomixis and multiple introductions of the weed, has led to sufficient sub-specific variation for agent mismatch to a number of genotypes of the weed to occur. The genetic population structure and its clear influence on agent establishment and efficacy are discussed in relation to hypotheses of biological control

success. Future prospects for control of invasive populations of *H. perforatum* with *A. hyperici* are good, because high agent efficacy on the majority of genotypes of the weed will provide a level of control over the bulk of infestations, and high agent specificity should allow for precise host matching to other mite strain.

1 Introduction, background and aims

1.1 Introduction

The introduction of exotic organisms into a new environment is not a trivial matter (Ewel *et al.*, 1999). We can only be sure that best practice is followed with respect to the two main issues in biological control, safety and efficacy, if our science is sound. Therefore, the Co-operative Centre for Australian Weed Management has identified three key research areas to address those issues; selection of agents, host specificity and impact assessment (Briese *et al.*, 2002). Failure of an agent to establish after detailed testing and considerable release effort has been identified as perhaps the greatest waste of resources in classical biological control (McFadyen, 2002), thus prediction of failure is important if selection of agents is to improve. One of the factors significantly affecting successful establishment is mismatch between agent and weed, as a result of genetic variation in the weed and collection of the agent from a different biotype of the plant in the native range than that present in the introduced range (McFadyen, 2002; Nissen *et al.*, 1995; Sheppard, 1992). The influence of genetic variation in Australian populations of *Hypericum perforatum* (St. John's wort) on successful control with the eriophyid mite *Aculus hyperici* forms the basis for this doctoral research.

H. perforatum is a toxic weed that is estimated to cost around \$22.5 million annually in livestock production losses in New South Wales alone (Bourke, 1997) as a result of animal health problems and reduced carrying capacity in pastures (Bourke, 1997; Campbell & Delfosse, 1984; Parsons, 1957). Over the past seventy years, substantial amounts of time and money have been invested in biological control programs; eleven agents have been released and six have established, but only three provide any level of control (Briese, 1997a). With *A. hyperici*, the most recently introduced biological control agent, significant

effects were observed on the weed and control appeared to be very promising (CSIRO, 1995; Jupp & Cullen, 1996; Wapshere, 1984). However, the mite failed to establish at a number of field sites despite repeated releases, and preliminary experiments demonstrated that variation in susceptibility to *A. hyperici* was responsible (Jupp *et al.*, 1997; Jupp & Cullen, 1996). Variation in leaf morphology and hypericin suggested that two forms of the weed exist in Australia (Campbell *et al.*, 1997), but morphotypes can be difficult to identify visually due to seasonal variation and environmental effects (Campbell *et al.*, 1997; Jupp & Cullen, 1996). Of great concern and a major motivation for this project, was that variation in susceptibility to *A. hyperici* could reduce the impact of this highly promising biological control agent, and might lead to replacement of susceptible with resistant varieties (Jupp *et al.*, 1997). An improved understanding of variation in *H. perforatum* is seen as an integral part of research into control of the weed in Australia (Cullen *et al.*, 1997).

This chapter contains a review of the factors affecting the success of classical biological control of weeds, and a review of the target weed. The remainder of the thesis explores the reproductive biology of the plant and its effect on genetic variation in Australian populations of *H. perforatum*, including variation in mite-susceptibility, germination characteristics and production of the toxin hypericin. Each results chapter consists of an independent introduction and review of the topics addressed, followed by aims, materials and methods, results and discussion. Chapter 3 was accepted for publication in *Genome* (Mayo & Langridge, 2003), and apart from thesis headings has been included as published.

1.2 *Biological control of weeds*

1.2.1 *Classical biological control of weeds*

A 'weed' is an unwanted plant, in most cases alien rather than native. An invasive weed is a weed that has the capacity to establish and spread. Richardson *et al.*(2000), Holzner (1982) and Baker (1991) discuss the categories and characteristics of weeds.

In this review, "biological control" of weeds is taken to mean classical biological control of exotic invasive weeds by the suppression of growth or reduction of plant populations to acceptable levels, following the introduction of exotic herbivores or plant pathogens that are host-specific, self-reproducing, density-dependent natural enemies (Andres & Goeden, 1974; Batra, 1982; Harris, 1993; Wapshere *et al.*, 1989; Wilson, 1964). Other forms of biological control will not be reviewed here. Classical biological control is the most widely used biological method, and has shown phenomenal success in some cases, for example the control of prickly pear (*Opuntia inermis*) by the lepidopteran *Cactoblastis cactorum* in Australia (DeBach, 1974) and, of immediate interest to this project, *Hypericum perforatum* in Canada and the United States of America (Harris, 1993). Traditionally, agents have been phytophagous insects, which individually contribute considerably to plant damage. However, when smaller insects such as aphids and mites are chosen, virulence (the ability to heavily infest a weed) becomes more of an issue, as infestation by many individuals is required to cause sufficient damage (Wapshere, 1982). Biological control offers relatively permanent, low risk, target-specific attack, at non-recurrent cost, thus may be the best option in rangelands and natural ecosystems, for logistical or economic reasons (Andres & Goeden, 1974; Crawley, 1989; Wapshere *et al.*, 1989).

Host specificity is the most important ecological characteristic in successful biological control of weeds, as it reduces the risk of an agent endangering other plant species (Bernays, 1985; Crawley, 1989; Huffaker, 1978; McLaren, 1993; Wilson, 1964). Host range is the main limiting factor in agent choice, as most plants have large associated phytophagous insect faunas that are intrinsically capable of greatly reducing the population densities of their host plants (Wilson, 1964), but only those with a host range limited to species or genera are usually chosen (Wapshere, 1982). Mites and plant pathogens often offer great specificity to forms of a host weed. For example, strains of the rust *Puccinia chondrillina* and of the gall mite *Eriophyes* (= *Aceria*) *chondrillae* specific to *Chondrilla juncea* (skeleton weed) have been used to control different forms of the weed in Australia (Caresche & Wapshere, 1975; Cullen & Moore, 1983; Wapshere *et al.*, 1989).

1.2.2 Successful biological control

General conclusions regarding the success of biological control vary because data are compiled from a variety of sources, and acceptable levels and interpretations of success vary among users (Bruzzese, 1993; Huffaker, 1978) and across geographical scales (Crawley, 1989). Estimates vary from one in six biological weed control attempts giving “satisfactory control” (Crawley, 1989), to one third of agent establishments leading to “substantial success” (Waage & Greathead, 1988). However, Sheppard (1992) considered that worldwide, only 72 biocontrol programs have been underway for long enough to allow for assessment of control, and in 35% of these control was never achieved despite agent establishment.

Success in individual weed control attempts is assessed by comparing population levels before and after release and correlating changes with damage caused by the agent (Wapshere *et al.*, 1989). Authors agree that the estimation of the effect of an agent on the target weed can be difficult to measure objectively (Crawley, 1989; Cromroy, 1976;

Wilson, 1964). Individual release attempts provide a great deal of information on arthropod-plant interactions. However, they must be interpreted cautiously when attempting to guide future control programs, as they are neither replicated nor randomized (Crawley, 1989). Biological control is essentially an empirical discipline, the success of which can be improved through basic research on the systematics, biology and ecology of natural enemies and pests (DeBach & Rosen, 1991; Waage & Greathead, 1988; Waage & Mills, 1992).

1.2.3 Genetic variation and successful control

A great many factors (including climate matching, mate finding, predation or parasitism of biocontrol agents) play a role in the success or failure of biological control programs (Burdon & Marshall, 1981; Nissen *et al.*, 1995; Sheppard, 1992). Among these, limitations to success may result from a high level of genetic diversity in the target weed species and the degree to which natural enemies are adapted to the weed genotype (Barrett, 1982; Burdon & Marshall, 1981; Crawley, 1989). However, there is currently little evidence to distinguish between failures in biological control due to genetic variation or to demographic factors and diseases, and there is a need for evidence of a genetic basis of variation within and between biological control populations, and the relationship of this variation to establishment of agents and control of pests (Hopper *et al.*, 1993).

The importance of plant genetic diversity to the success of biological control has been argued (Burdon & Marshall, 1981; Chaboudez & Sheppard, 1995; Sheppard, 1992). Burdon and Marshall (1981) considered that the importance of genetic diversity and population structure had been underestimated in the failure of biological control of weedy plants. They pointed to the influence of low genetic variation on the vulnerability of crop monocultures to disease, and contrasted this with the high variation in susceptibility found in non-agricultural communities (Burdon & Marshall, 1981). The latter authors recognized

that while considerable variation may occur in natural populations of inbreeding or apomictic species, invasive populations might have a depauperate genetic structure, particularly if they have gone through a genetic “bottleneck” as a result of introduction of only one or a few individuals. However, the relationship between the genetic diversity of weedy species and control success could not be pursued directly because of the lack of quantitative information on population genetic structure. Therefore, reproductive mode was used to approximate genetic diversity in invasive populations, on the assumption that apomictic species will be far less variable on average than sexually reproducing species. Burdon and Marshall (1981) examined the relationship between breeding system and the degree of control achieved in 81 different biological control attempts consisting of 45 species, 24 of which are obligate or facultative apomicts, or clonal, and concluded that plant species that relied on asexual forms of reproduction had a significantly greater chance of being controlled biologically than sexually reproducing species. It was presumed that higher control success was due to genetic homogeneity in the weed, and concluded that sexually reproducing species will usually be more difficult and costly to control biologically than asexually reproducing and genetically uniform weeds, for example by requiring more agents (Burdon & Marshall, 1981). It was argued that the population genetic structure of the target weed should receive greater attention in the selection of target weed species for control. However, it was pointed out that species that have been introduced on multiple occasions present greater control problems, as the gene pool for adaptation to new threats is likely to be greater than for a species introduced only once. Burdon and Marshall (1981) also considered that the narrower the agent’s host range, the more likely there will be resistant or tolerant genotypes in the target weed population, which may make pathogens less effective agents than insects. Burdon and Marshall (1981) admitted that many other factors might play a role in determining the success of biological control programs. The lack of direct data on the population structure

of weeds was acknowledged to be a major shortcoming of the analysis (Burdon & Marshall, 1981).

Even though they have influenced decision-making in biological control, Chaboudez and Sheppard (1995) consider these types of conclusions to be biased. Re-analysis of data from Burdon and Marshall's species list (1981), including new records from Julien (1992) found no evidence that mode of reproduction in weed populations leads to reduced success in biological control (Chaboudez & Sheppard, 1995). The latter authors considered the database too small and unreliable to use in predicting such outcomes, since the difference was due to greater success in control of only two sexually reproducing species compared to two clonal species. Difficulty in such analyses can arise from inaccuracies in the distinction of degree of control, limitations due to poor information on the reproductive biology, and change in taxonomic status (Chaboudez & Sheppard, 1995). The authors considered that genetic diversity may have to be almost nil, as in the case of *Salvinia molesta*, before it may be used in predicting outcome (Chaboudez & Sheppard, 1995). This is not to say that genetic variation will not affect the outcome of biological control, but that success is likely only to be predictable in the case of clones. Environmental factors such as nutrient status and habitat must also be taken into account in order to predict outcome more accurately.

While spectacular success has been demonstrated in control of some clonal weeds, partial success is too often the case with facultative apomicts because limited recombination may result in considerable variation even within a population. The problems encountered in biological control of facultative apomictic weeds are well illustrated by the variable susceptibility of genetically distinct forms of *Rubus fruticosus* L. aggregate (blackberry) to the rust fungus *Phragmidium violaceum* (Evans *et al.*, 1999), and the differential control of three forms of *Chondrilla juncea* L. (skeleton weed) by the agent *Puccinia chondrillina*

(Burdon *et al.*, 1981; Cullen & Moore, 1983). In the latter case, three forms of skeleton weed were shown to exist in Australia (Burdon *et al.*, 1980; Hull & Groves, 1973), and variation in susceptibility to the rust fungus resulted in replacement of a widespread narrow-leaf form by two other resistant forms of the weed (Burdon *et al.*, 1981). Concerns have been expressed that similar situations might easily recur in the case of blackberry (Evans *et al.*, 1998), and the biological control of *H. perforatum* with *A. hyperici* (reviewed below).

An increase in quantitative information is the only way to assess the importance of genetic variation and population structure to successful biological control (Burdon & Marshall, 1981), and detailed studies will assist the move away from generalizations towards informed predictions at least in individual cases (Sheppard, 1992). However, there are only a few examples where DNA-based techniques have been applied to these questions in weed science (Colosi & Schaal, 1994; Evans *et al.*, 1999; Lym *et al.*, 1996; Nissen *et al.*, 1995).

1.3 *Hypericum perforatum*

1.3.1 Distribution of *H. perforatum*

The home range of *H. perforatum* includes Europe, Asia and North Africa (Parsons, 1957; Robson, 1977; Robson, 1981). The plant is listed as a weed in 20 countries and considered a serious weed in Australia and the United States of America (Holm *et al.*, 1979). Interestingly, it is listed as a “principle” (one of the five most troublesome) or “common” (widespread) weed of agriculture in many countries in its presumed natural range (Holm *et al.*, 1979).

It is likely that *H. perforatum* L. has had multiple introductions into Australia (Harris & Gill, 1997), both as a useful medicinal plant and as a contaminant of stock feed, seed,

packing, and sand ballast used for land reclamation (Davey, 1917; Ewart & Tovey, 1909; French, 1905; Harris & Gill, 1997; Parsons, 1957). Early records of *H. perforatum* in Australia are difficult to find, but the plant was under cultivation in the Melbourne and Adelaide Botanic Gardens by 1858 and 1859, respectively (Harris & Gill, 1997). It is generally agreed that the first weedy outbreak occurred about 1880, when seed grown by a German immigrant at Bright, Victoria, escaped from a garden and spread to the local racecourse (French, 1905; Harris & Gill, 1997; Parsons, 1957). It appears that its introduction to South Australia coincided with this, as a herbarium specimen dating to 1881 was collected from Coromandel Valley, near Adelaide. The earliest verbal record of the weed in New South Wales is from Mudgee in around 1890 (Harris & Gill, 1997), then from the 'Mannus' homestead near Tumbarumba in 1899, either transported from Bright or as a separate introduction by an English or German family (Calvert, 1932). Within a few years of the Bright escape, slopes along the Ovens Valley from Harrietville to Myrtleford were infested, and the weed had moved via stock feed throughout the state to other gold mining areas (Parsons, 1957). Gold miners reputedly spread the plant over the Great Dividing Range to Wonnangatta and Grant, in Gippsland (Calvert, 1932), and via stock routes into NSW before the end of the last century (Davey, 1919). *H. perforatum* now occurs in all States in Australia (Harris & Gill, 1997), but it is considered a serious problem only in Victoria, New South Wales and South Australia (Fig. 1.1), with small populations of little economic importance in Western Australia, Queensland and Tasmania (Campbell & Delfosse, 1984).

Harris and Gill (1997) estimated the spread of St. John's wort from Bright to have averaged about two kilometers per annum. Mode of spread to new areas is most likely to have occurred either deliberately or via accidental movement as seed in contaminated fodder or soil, or on the feet or coats of domestic and wild animals (Harris & Gill, 1997).

Figure 1.1. *H. perforatum* infestation in the south-east of South Australia.



Herbarium records suggest that the Australian range of the weed is still expanding (Harris & Gill, 1997), although populations evidently wax and wane over time due to disturbances such as fires, drought, cultivation and mowing (Harris & Gill, 1997; Shepherd, 1983). From estimates of 175,195 ha in Victoria (Shepherd, 1983) and 188,000 ha in New South Wales (Campbell, 1977), the total area infested with St. John's wort in those States was at least 350,000 ha by 1982 (Cullen *et al.*, 1995). The area of infested land in New South Wales alone has since been revised to more than 250,000 ha (Campbell & Watson, 1994), but infestations are said to have been increasing and figures of up to a million ha have been quoted (CSIRO, 1995).

1.3.2 Description and general biology of *H. perforatum*

Hypericum perforatum L. (Clusiaceae) is one of 400 species in the genus *Hypericum* (Robson, 1977; Robson, 1981). The plant is a glabrous herb with perennial crowns (Campbell *et al.*, 1995). A single taproot grows approximately 70 cm vertically, and lateral roots grow 5-8 cm under the soil surface, from which adventitious buds produce new

crowns in spring and autumn (Clark, 1953b). In winter, rosettes of procumbent non-flowering stems are produced (Fig. 4.16, Chapter 4), which sometimes exceed 30 cm in length. Winter growth may develop roots where stems are in close proximity to the soil, and can form a close carpet on the ground and exclude other plants (Clark & Clark, 1952). Summer growth is erect and up to 30 to 120 cm high, often reddish, with two opposite longitudinal ridges. Flowering stems branch into broad corymbs near the top (Figs. 4.17 and 4.18, Chapter 4), with up to 30 fertile stems on a large crown. Dried summer stems may persist for several years. Leaves are small, sessile and opposite, oblong-ovate, entire, with obscurely reticulate venation, darker green above, dotted with many oil or "pellucid" glands and edged with marginal black nodules (Fig. 5.2, Chapter 5). The translucent oil glands, from which the plant received the specific epithet *perforatum* (from the Latin perfor, to pierce through), are reported to contain protohypericins, and the black nodules to contain hypericin, the photodynamic pigment responsible for the toxic effect of the plant (Southwell & Campbell, 1991). Flowers are 1-2 cm diameter, bisexual, with five bright yellow petals containing black nodules on the margins, and occasional black streaks. The calyx has five lanceolate lobes, black nodules are either present or absent, and is sometimes streaked brown. Stamens number 50 to 100, with filaments united at the base into three bundles. The anther bears black nodules, and is bisporangiate with 2-celled pollen grains (Mártonfi *et al.*, 1996). The style is trifurcate, united along the lower region, with each bearing a terminal red-tipped stigma. The ovary is three-celled, and ovules are anatropous and tenuinucellar (Mártonfi *et al.*, 1996). The fruit is a brownish-red, resinous, three-celled capsule that splits on ripening. Seed is approximately 1 mm long x 0.5 mm wide, and a single plant can produce 15,000 to 30,000 seeds (Davey, 1919; Parsons, 1957). The seed coat is hard, dark brown or occasionally pale grey, with a reticulate testa.

H. perforatum is a facultative pseudogamous apomict, whose reproductive biology is reviewed in Chapter 3. The hybrid origin, and cytology of *H. perforatum* are reviewed in Chapter 4 (Sections 4.2.4, and 4.2.7, respectively). Several “varieties” of St. John’s wort have been recognized in Australia, on the basis of plant morphology and hypericin content (reviewed in Chapter 4, Section 4.2.5)

1.3.3 Toxicity and medicinal uses of *H. perforatum*

H. perforatum contains hypericin, which can cause photosensitization of skin exposed to visible light, depression or an agitated mental state, and can affect temperature regulation and sensation (Bourke, 1997; Southwell & Campbell, 1991). Ingestion by stock results in weight loss or failure to gain weight, reduced milk and wool production, abortion, lower fecundity and survival rates, and even death if animals are not removed from the source (Bourke, 1997; Campbell & Delfosse, 1984; Dodd, 1920). The toxicity of St. John’s wort to stock and variation in hypericin concentration are reviewed in more detail in Chapter 7 (Sections 7.2.1, 7.2.2 and 7.2.3).

St. John’s wort has also been introduced deliberately for medicinal purposes (Groves, 1997; Harris & Gill, 1997), and has been recommended in herbals as a remedy for wounds, ulcers, burns, respiratory and urinary tract infections, and for hysteria and depression (Grieve, 1982; Hoffman, 1990; Low & Rodd, 1994; Wren, 1980). The active principle hypericin is thought to inhibit human immunodeficiency virus (Mitich, 1994) and tuberculosis bacterium (Low & Rodd, 1994). The use of St. John’s wort has increased in the past decade, with preparations commercially available in Australia recommended for the treatment of mild anxiety, depression and pre-menstrual stress.

1.3.4 Control of *H. perforatum* in Australia

Difficulties encountered in the control of St. John's wort in Australia have necessitated a shift in attitudes to control, from reliance on either chemical, cultural or biological control, to a more integrated approach. The Australian integrated weed management (IWM) system for St. John's wort draws on more than 90 years of research into chemical, cultural and biological controls (Campbell & Watson, 1994). IWM offers the potential to control St. John's wort in many agricultural situations (Cullen *et al.*, 1997), mainly by the combination of herbicides with competitive pastures and stock management (Campbell, 1997; Campbell & Nichol, 1997; Campbell & Watson, 1994), but also by agroforestry, mowing, cultivation, cropping, or fire (Calvert, 1932; Campbell *et al.*, 1975; Carn, 1936; Clark & Clark, 1952; Clark, 1953b; Davey, 1919; Davey, 1922; Ewart, 1910; Moore & Cashmore, 1942; Stening, 1933). However, in southeastern Australia the weed remains a problem on non-arable land and poorly managed pasture, in natural ecosystems, forestry reserves, and non-agricultural situations such as water catchments, easements and roadsides, because most of the recommended cultural and chemical techniques are not feasible or costs are too high (Campbell & Delfosse, 1984; Cullen *et al.*, 1997; Harris & Gill, 1997; Shepherd, 1985). Therefore, in many situations biological control remains the only cost-effective option. The remainder of this review covers biological control of *H. perforatum*. Other control methods for *H. perforatum* are not covered, with the exception of grazing management (reviewed in Chapter 7, Section 7.2.4).

Successful biological control of St. John's wort has been achieved in California, parts of Canada, and South Africa (Gordon & Kluge, 1991; Harris & Peschken, 1971). More than 600,000 adult beetles of *Chrysolina quadrigemina* and *C. hyperici* were sent from Australia to California in 1945-47, where the former species has been largely responsible for one of the classical biological control success stories. Phenomenal reduction in weed

densities were seen, from 70% to 15% in one year, and ultimately to less than 3% of its original density (Briese, 1997a; Harris, 1993; Huffaker & Kennett, 1959).

Eleven biological control agents have been introduced into Australia in 15 separate release attempts, and six of those species have established (Table 1.1). However, only *Chrysolina* spp. (in particular *C. quadrigemina*) have contributed significantly to the control of St. John's wort so far (Briese, 1997a), and despite more than 70 years of effort the weed still causes unacceptable economic damage. Heavy predation was thought to be the major factor responsible for failure of the moths *Anaitis efformata*, *A. plagiata* and *Actinotia hyperici* to establish (Briese, 1986; Briese, 1997a). Unfavorable physical conditions may have been involved in the failure of *Chrysolina varians* and *C. brunsvicensis*. The fly *Zeuxidiplosus giardi* and the aphid *Aphis chloris* established and dispersed readily, but cause only localized damage (Briese, 1997a). *Agrilus hyperici* was considered to be one of the most important natural enemies of *H. perforatum* in Europe (Wapshere, 1984; Wilson, 1943), but in Australia it appears to be restricted to several timbered areas due to competition by *Chrysolina* beetles (Briese, 1991a).

Table 1.1 Natural enemies of *H. perforatum* released or considered for release in Australia (adapted from Briese, 1997).

Species	Mode of action	Origin	Release year	Relative occurrence	Reference
Phase 1: 1928-1939					
Coleoptera: Chrysomelidae					
<i>Chrysolina varians</i> (Schall.)	leaf defoliator	England	1930-33	no establishment	a
<i>C. brunsvicensis</i> (Grav.)	leaf defoliator	England	1930-34	no establishment	a
<i>C. hyperici</i> (Forst.)	leaf defoliator	England	1930-34	occasional**	b
Lepidoptera: Geometridae					
<i>Anaitis efformata</i> L.	leaf defoliator	England	1933-38	no establishment	b,e
<i>A. plagiata</i> Guenée	leaf defoliator	England	1933-38	no establishment	b,e
Lepidoptera: Eucosmidae					
<i>Lathronympha hypericana</i> Hubn	leaf webber	England		not released	a
Lepidoptera: Oecophoridae					
<i>Depressaria hypericella</i> Hubn	leaf webber	England		not released	a
Hemiptera: Aphididae					
<i>Aphis chloris</i> Koch (holocyclic)	sap sucker	England		not released	a
Phase 2: 1935-1942					
Coleoptera: Chrysomelidae					
<i>Chrysolina quadrigemina</i> (Suffr.)	leaf defoliator	France	1938-39	common**	c,d,e
Coleoptera: Buprestidae					
<i>Agrilus hyperici</i> (Creutzer)	root borer	France	1939-40	rare*	c,d
Lepidoptera: Noctuidae					
<i>Actinotia hyperici</i> Schiff.	leaf defoliator	France		not released	c
Lepidoptera: Gelechiidae					
<i>Aristotelia morphochroma</i> Wals.	stem/seed capsule borer	France		not released	c
Diptera: Cecidomyiidae					
<i>Zeuxidiplosus giardi</i> Kieff.	leaf-bud galler	France		not released	c
Phase 3: 1940-1956					
Diptera: Cecidomyiidae					
<i>Zeuxidiplosus giardi</i> Kieff.	leaf-bud galler	California ex France	1953-55	common*	e
Phase 4: 1978-present					
Coleoptera: Chrysomelidae					
<i>Chrysolina hyperici</i> (Forst.)	leaf defoliator	France	1980	uncertain	f,g
<i>C. quadrigemina</i> (Suffr.)	leaf defoliator	France	1981	uncertain	f,g
Coleoptera: Buprestidae					
<i>Agrilus hyperici</i> (Creutzer)	root borer	France	1981, 1985, 1992	uncertain	g,h,l
Lepidoptera: Geometridae					
<i>Anaitis efformata</i> L.	leaf defoliator	France	1981-83	no establishment	i
Lepidoptera: Gelechiidae					
<i>Aristotelia morphochroma</i> Wals.	stem/seed capsule borer	France		not released	
Lepidoptera: Noctuidae					
<i>Actinotia hyperici</i> Schiff.	leaf defoliator	France	1985-86	no establishment	g
Hemiptera: Aphididae					
<i>Aphis chloris</i> Koch (anholocyclic)	sap sucker	France	1986-87	common*	j
Acarina: Eriophyidae					
<i>Aculus hyperici</i>	sap sucker	France	1991	common*	k

* = has caused localized damage, ** = has caused heavy damage at a number of sites

a = Currie and Garthside (1932), b = Currie and Fyfe (1938), c = Wilson (1943), d = Wilson and Campbell (1943), e = Wilson (1960), f = Delfosse and Cullen (1981), g = Wapshere (1984), h = Briese (1991b), i = Briese (1986), j = Briese and Jupp (1995), k = Jupp and Cullen (1996), l = Jupp (1993b).

Control of St. John's wort with *Chrysolina* beetles appears to be limited to Mediterranean climates such as South Australia and Western Australia, due to the ability of the plant to regenerate in areas with high rainfall during summer when the beetle is active (Briese, 1997a), and to sucker in shallow soils (Clark & Clark, 1952). Reluctance of the insects to colonize the weed in shaded areas (Clark, 1953a) and the absence of competitive plant species (Clark, 1953b) may have compounded the problem of asynchrony of beetle and weed life history stages. However, further attempts to control the weed with collections of *Chrysolina* spp. that were more suited to summer rainfall areas were unsuccessful (Briese, 1997a).

The eriophyid mite *Aculus hyperici* was found to be a major factor in control of *H. perforatum* in southern France (Wapshere, 1984) and did not appear to be inhibited by shade (Briese, 1997a). It was hoped that *A. hyperici* would provide more consistent control because of its rapid population growth and dispersal, and impact via depletion of the large nutrient reserves in the root system, thereby reducing the ability of the weed to regenerate after drought, fire and defoliation, and leading to progressive dwarfing and eventual death of the plant (Briese, 1997a).

In 1991, *A. hyperici* was released at 120 nursery sites across New South Wales and Victoria, via a network of Weed Officers, Catchment Management Officers, and Landcare groups (CSIRO, 1995). The prospect of control was very promising, and a significant impact of *A. hyperici* on plant vigour has been demonstrated (Jupp *et al.*, 1997; Jupp & Cullen, 1996). However, at several sites mites repeatedly failed to establish, and variable susceptibility was demonstrated among four known morphological forms (Jupp *et al.*, 1997). Failure to establish at two sites in northern New South Wales could not be explained by climate or release site conditions, and was most likely a result of low host-plant susceptibility since laboratory experiments showed that mite populations did not

increase on plants collected from those sites, compared with significant increases on plants from four other sites with successful establishment (Jupp *et al.*, 1997). A significant negative relationship of mite density with root and shoot growth was demonstrated, indicating that reduction in plant growth and vigour could only be expected in plants from the latter sites (Jupp *et al.*, 1997). The impact of and variation in susceptibility to *A. hyperici* is reviewed in more detail in Chapter 5 (Section 5.1.4.3).

At present, the greatest threat to successful control of *H. perforatum* with *A. hyperici* is the existence of resistant forms of the weed, which would not only reduce the initial impact of this highly promising biological control agent, but might lead to replacement of susceptible with resistant varieties (Jupp *et al.*, 1997). The well-documented case of biological control of only one of three forms of *Chondrilla juncea* (skeleton weed) by the rust *Puccinia chondrillina* (Cullen & Groves, 1977) serves to emphasize the importance of genetic variation to the success of weed control programs. Subsequent to the spectacular control of skeleton weed form A by the rust, forms B and C invaded and expanded their range (Burdon *et al.*, 1981). There is some justification in predicting a parallel in the case of mite-resistant St. John's wort, due to the potential of the weed to spread.

The biological control of *H. perforatum* provides good examples of the need to understand the ecology of a system with respect to biological and geographical variation in both plant and herbivore populations, and to appreciate the complexity of interactions between agents, their physical environment, and other organisms (Briese, 1986; Briese, 1997a).

1.4 Aims

Accurate estimates of the occurrence and distribution of genetic variation among individuals, populations and species are essential to understanding phenomena in ecological studies of any kind (Schaal *et al.*, 1998). Assessing genetic variation in Australian populations of St. John's wort was identified as one of the top priorities for improving control of the weed (Cullen *et al.*, 1997). Research conducted into the population structure and distribution of genetic variants was expected to contribute to control of the weed in general (including IWM programs, distribution and modeling studies, and weed impact assessments), and lay the groundwork for investigations into the factors affecting successful biological control with *A. hyperici* (Cullen *et al.*, 1997). The influence of genotype on other phenotypic variation, including germination characteristics and hypericin level, was also explored. A molecular approach was chosen because morphology-based identification of genotypes of *H. perforatum* is problematic (Campbell *et al.*, 1997; Jupp & Cullen, 1996). For the sake of simplicity, DNA phenotypes discovered using molecular techniques are referred to simply as "genotypes" in the following text.

Research into the germination characteristics of various accessions of *H. perforatum* is presented first (Chapter 2), because in order to continue the project, practical problems stemming from an effect of seed age and germination temperature had to be solved. The role of environment and genotype on variation in germination at a range of temperatures and seed ages was investigated, by recording time to initiation of germination, germination rate, maximum germination capacity, seed longevity and viability. An interaction between seed age and temperature, resulting in two distinct germination patterns, was discovered in this species, and the transition between patterns was examined.

In Chapter 3, proportions of reproductive pathways in *H. perforatum* were investigated and the results are included as published (Mayo & Langridge, 2003). *H. perforatum* is a

facultative pseudogamous apomict whose reproductive biology, in particular the degree of apomixis, was expected to significantly influence spatial and temporal variation in the target host. Tetraploid parents from two genetically dissimilar Australian populations were cross-pollinated and 121 progeny screened at random with either or both of two multilocus methods developed for the purpose. Initial samples were examined with an RFLP fingerprinting technique in which genomic DNA was restricted with HaeIII and probed with M13, and subsequently AFLP was used for larger sample sizes. Cytological methods were adapted and used to determine ploidy of parents and progeny. A novel DNA extraction method was developed because traditional CTAB-based extraction methods were found to be unsatisfactory.

The aims of research presented in Chapter 4 were to identify genetic variants among the 298 individuals examined, and assess inter- and intra- population variation of 44 populations located in five Australian States, and two overseas populations. The RFLP and AFLP multilocus methods developed during the current project were fundamental to the investigation, and the genotypes discovered were analyzed for relatedness. Population variation was evaluated in detail from transects and quadrats made at four sites, and for a smaller number of samples from most other sites. Variation in ploidy was investigated by cytological methods for a subset of individuals, including several with unusual leaf morphology. The geographic distribution of genotypes was mapped for Australian populations, and examined with respect to hypotheses of multiple introduction and spread.

Chapter 5 presents a novel method developed for bioassay of mite-susceptibility in *H. perforatum*. Level of susceptibility to *A. hyperici* was determined for 230 individuals, including 20 of the 21 genotypes fingerprinted with multilocus techniques in Chapter 4. The degree to which mite-susceptibility correlated with variation in plant genotype was examined for evidence of a genetic basis to the plant-arthropod interactions involved, and

related back to impact studies and records of mite establishment in the field, where available. Variation in susceptibility was mapped onto the distribution of genotypes of *H. perforatum* in order to gauge the level and geographical extent of resistance to *A. hyperici*, and examined in relation to potential impact and success of biological control and the risk of replacement of susceptible with resistant genotypes.

Several low and high mite-susceptible genotypes were examined by scanning electron microscopy, for physical factors in *H. perforatum* (leaf wax density, wax depth, and cell morphology) and physical impediment to feeding in *A. hyperici*, to determine whether these factors could account for variation in mite-susceptibility and contribute to resistance mechanisms (Chapter 6).

The influence of *H. perforatum* genotype on hypericin concentration was examined in Chapter 7. Production of the toxin hypericin was determined for flowering stems of 18 individuals consisting of ten genotypes (confirmed in Chapter 4). The relationship between hypericin concentration and susceptibility to *A. hyperici* was examined to see if hypericin might play a biochemical role in variable host-plant susceptibility among genotypes. Toxicity to stock and control of St. John's wort genotypes by grazing management was reappraised in relation to the hypericin levels of different genotypes.

2 Variation in germination characteristics

2.1 Introduction

Characteristics such as dormancy and longevity in *Hypericum perforatum* (St. John's wort) have contributed significantly to spread of and difficulty in controlling the weed (Campbell, 1985; Clark, 1953b). Consequently, a number of studies have investigated the effect of light, chemical inhibitors, seed age and temperature on dormancy of *H. perforatum* seed (reviewed below). Of major concern for this project was the common experience of extremely low germination in young seed (Campbell 1985, Cashmore 1939, Clark 1953, Brian Sindel *pers. comm.*), and confusion in the literature regarding the effect of seed age and temperature on germination. Inconsistency in past methodology (for example accession locality, age and wash time, Table 2.1) has contributed to this confusion, and prevented any specific conclusions about genetic or environmental influences on germination characteristics. Initially the aim of this project was to maximize germination of the limited quantities of young seed available from crosses, and successfully germinate seed accessions of a variety of origins and ages. A range of genotypes was investigated in early studies on the effects of seed age and germination temperature, which were later expanded to include a report on longevity. Evidence was uncovered for a greater influence of environment than genetics on variation in germination characteristics of field-collected seed. The results are discussed in relation to the evolution of germination traits and the implications for control of *H. perforatum*. Genotype numbers prefixed with an "R" or "A" refer to RFLP or AFLP fingerprints presented in Chapter 4, and RFLP number was used unless unavailable. Fingerprint numbers are given for the most likely genotype, based on evidence of low within-population variation (Chapter 4), since not all seedlings were examined with molecular techniques.

2.2 Review

2.2.1 Light

Light was shown to increase germination in *H. perforatum* seed from zero to between 12 and 81% for seed ranging from one to six months old, from five field sites (Campbell, 1985), and from zero to around 30% in one month-old seed originating from the field but grown together in a common garden (Campbell *et al.*, 1992) (Table 2.1). However, light was not necessary for germination of nine year-old seed from Tuena. Campbell (1985) concluded that there was an interaction of age with light, the response to light persisting for six months or more but less than nine years.

Table 2.1. Percentage germination of *H. perforatum* seed in the presence and absence of light, from a range of seed ages and wash times (*d* = days, *m* = months, *y* = years, *C* = raised in a common garden).

Reference	Locality	Seed age	Wash time	Percentage germination after (time)		
				in light	in dark	(time)
Cashmore (1939)	Tumbarumba, NSW	23 m	5 min	76	-	2 m
"	"	12 m	"	81	-	"
"	"	10 m	"	68	-	"
"	"	10 m	"	77	-	"
Clark (1952)	Bright, Vic (?)	12 m	3 d	79	-	28 d
Campbell (1985)	Tuena, NSW	2 m	5 x 3 min	68	20	87 d
"	"	9 y	"	70	65	87 d
Campbell (1985)	Tuena, NSW	2-3 m	5 min	81	4	41 d
"	Coolah, NSW	2-3 m	"	49	3	"
"	Mudgee, NSW	5-6 m	"	46	1	"
"	Captains Flat, NSW	1-2 m	"	17	0	"
"	Orange, NSW	1-2 m	"	12	0	"
Campbell (1990, 92)	Tuena, C	1 m	15 min	28	1	111 d
"	Mudgee, C	"	"	31	0	"
"	Orange, C	"	"	32	0	"

2.2.2 Chemical Inhibition

Cashmore (1939) concluded that seed from Tumbarumba required a four to six month after-ripening period before becoming fully germinable. Clark (1953b) compared germination of 12 month old seed at 28 days, with seed unwashed (77%), washed (79%), and washed plus seed capsule extract (23%), and suggested that this apparent dormancy may be the result of a germination inhibitor in capsule exudate, which could be removed by washing or a gradual drying and wearing away of the exudate. Campbell (1985) concluded that this inhibitor could persist under laboratory storage, since washing improved germination of both 9 year old and 1-6 month old seed from Tuena. There was considerable variation in washing time used in investigations, from five minutes (Cashmore, 1939) to three days (Clark, 1953b) (Table 2.1).

2.2.3 Seed age

Cashmore (1939) germinated bi-monthly samples from several accessions, and observed a trend of low germination in 2 month-old seed, increasing to around full capacity by 10 months. These results cannot be explained by washing or leaching of inhibitor, and are not likely to be due to reduced response to inhibitor with increasing seed age, because Clark (1953) showed a marked effect of capsule exudate on 12-month old seed. Campbell (1985) concluded there was an effect of seed age, but unfortunately, in two of his experiments, this factor was confounded with genotype and environment. Environmental factors may influence rate of germination, and seed batches collected at different times from the same localities may differ in dormancy characteristics (Thurston, 1960). In Campbell's first 1985 experiment, old seed was collected during a favourable season and young seed in a drought year, from sites 500m apart at Tuena, and thus genotype and environmental effects cannot be discounted. In his second experiment, Campbell (1985) noted a difference in time to initiation of germination for seed from Tuena and Mudgee, and supposed that these

results might be explained by “varietal” differences. However, seed ranged in age from one to six-months old and was collected from separate field sites, and Campbell (1990) later concluded, from one-month old seed grown in a common environment, that “ecotype” had no effect (28-32% germination, Table 2.1).

Maximum seed longevity in *H. perforatum* has been estimated to range from six years (Clark, 1953b) and 10 years (Ewart, 1910), to 36 years in an old un-thinned *Pinus ponderosa* plantation (Harris & Gill, 1997), but no formal study has been undertaken.

2.2.4 Temperature

Clark's (1953b) field study at Bright indicated that mean temperature in the field must exceed 11°C for germination, and that most germination occurred during the six warmest months. Clark also noted that temperatures above 27°C seemed to inhibit germination in the laboratory, although Cseresnyes and Baleanu (in Campbell, 1985) found optimal temperature for germination to be 30°/20°C (8h/16h). Campbell (1985) concluded that high temperature inhibited germination of young but not old seed, and suggested that the former discrepancy might be explained by an interaction between age and temperature. However, Campbell (1985) did not formally test temperature or an interaction of age with temperature, but simply dropped the temperature at 48 days in an experiment with young and old seed, from 30°/20°C (8h/16h, 8h day) to a constant 15°C. No control was maintained for young seed at the higher temperature. Following this, he observed onset of germination in young seed, but no increase in old seed, since most old seed had already germinated. Unfortunately, the design confounds temperature not only with time, but also with the effect of environment and possibly genotype, since new seed was produced in a drought and were collected 500m from old seed. To confuse matters further, Campbell (1990) later could not increase germination in one month-old seed by dropping the temperature to 15°C some time into an experiment, as he had previously done. Instead, he

achieved 30% germination at 30°/20°C (8h/16h), an identical regime to his 1985 experiment, and one that he concluded inhibits germination in young seed!

Logic dictates that for young seed there is no clear indication that temperatures >27°C completely inhibit germination, or support for the conviction that low temperature is optimal for germination (Campbell *pers. com.*). It is worth noting that for the experiment on the effect of light on germination of one to six-month old seed, Campbell (1985) chose a germination temperature of 23°/15°C (8h/16h), and that Cashmore (1939) germinated seed of a range of ages at 20°C.

2.2.5 Other factors

In addition to the above factors, gibberellic acid (GA) plus potassium nitrate (KNO₃) have been shown to substitute for light in breaking dormancy (Campbell, 1990) (Campbell *et al.*, 1992), and hot water immersion has been shown to reduce germination of St. John's wort seed from one locality but not another (Campbell, 1985). In his investigation of seed dispersal, Cashmore (1939) showed that sodium chlorate or arsenic pentoxide sheep dips increased rate of germination but did not affect total germination capacity.

2.3 Aims

The overall aim of the experiments undertaken in this project was to examine the role of environmental and genetic factors on seed germination at a range of temperatures and seed ages, given the confused state of past literature. During this process, the opportunity existed to concurrently investigate seed viability and longevity.

Experiment 1 examined germination, at three temperatures, of twelve accessions of a range of ages and origins, and was designed as a brief exploration in the course of routine germination. Experiment 2 further investigated the effects of temperature and age on germination, using two morphological variants that were required as parents, collected in

consecutive seasons and observed over an extended time period. Experiment 3 investigated the transition between the two characteristic age-dependent germination patterns of response to temperature, which were revealed by Experiments 1 and 2, for four genotypes that had exhibited a “young” pattern in those experiments.

During the project, it was noted that some older accessions either had very low germination (eg., 18%, 7% and 9% respectively in nine year-old “Orange-2” and seven year-old “Talmalmo” and “Tanunda”) or failed to germinate at any temperature, even over long periods of time (eg., eight year-old “Wellington”). Experiments 4 and 5 examined seed viability and longevity for accessions for which germination data had previously been collected.

2.4 Method

It was often not possible to determine exactly when seed collected in the field had matured, so maximum possible seed ages are presented unless otherwise indicated. All seed was stored dry in paper envelopes, in the dark at room temperature.

2.4.1 Experiment 1. Germination temperature in diverse accessions

Germination over time at low, medium and high temperature was determined for twelve accessions. The accessions (Appendix A) consisted of seed grown in a common garden by Campbell *et al.* (1992) (seven year-old “Orange-1”, “Tuena”, “Mudgee Short”, “Captains Flat”, “Bemboka” and “Coolah”), and field-collected seed (two year-old “Cowra”, 9-12 months-old “Cassilis”, 1-3 months-old “Mudgee-L” and 0.2-3 months-old “Scott Creek-1” and “Scott Creek-2”). For each accession, sixty seed were prepared as follows and divided equally among temperature treatments. Seed was separated from debris, washed for 24 hours with three changes of water, surface sterilized for 5 minutes in 70% ethanol, rinsed in sterile water, soaked for 5 minutes in 1% sodium hypochlorite, then rinsed three times in

sterile water before air drying in a laminar flow. Dry seed was sprinkled onto sterile 1% water agar plates and sealed with Parafilm[®]. Seed was germinated in EnvironAir cabinets (SRJ Cabinet Sales, Greenacre, NSW), at low (15°C constant), medium (23°C:16°C) and high (25°C constant) temperatures, all with 12 hour days. Seed germination was measured as a percentage of total seed at nine time points over 44 days.

Seed surface sterilization and germination on sterile 1% water agar (as described above) was more convenient and less prone to fungal contamination (data not presented), than germination of non-sterile seed on damp filter paper or sand (Campbell, 1985; Cashmore, 1939; Clark, 1953b).

2.4.2 Experiment 2. Seed age and germination temperature in two genotypes

Seed of *H. perforatum* was collected in bulk, in consecutive years (“new” and “old” seed), at Turon, NSW (broad-leaf genotype) and Scott Creek, SA (narrow-leaf genotype). Seed was surface sterilized as described in Experiment 1, and germinated at low (15°C:10°C), medium (22°C:18.5°C) and high (30°C:25°C) temperatures in controlled environment rooms with 12 hour day. Three replicates of approximately 100 seeds per plate were prepared for each of the 12 treatments (2 localities x 2 ages x 3 temperatures). Germinated seed was calculated as a percentage and averaged over replicates, at each of four time points (Turon seed at 10, 14, 22 and 87 days, Scott Creek seed at 13, 17, 25 and 90 days). A three-way ANOVA was performed using Genstat 5 release 4.1.

2.4.3 Experiment 3. Temperature response pattern transition

The four seed lots that had exhibited characteristic “young” temperature response patterns in Experiment 1, and all four seed lots from Experiment 2 were germinated again at three temperatures, 6.4 and 5.3 years later, respectively. Seed was prepared and germinated as described in Experiment 1, with the exception that three replicates, each of approximately

100 seed, were prepared per temperature treatment, and seed was not soaked prior to surface sterilization. Germinated seed was calculated as a percentage of total seed and averaged over replicates, for each of 16 time points over 55 days.

2.4.4 Experiment 4. Decline in viability

Twenty-two accessions (Fig. 2.5) ranging in age from approximately two to twelve years were germinated at medium temperature (23°C:16°C), and the percentage germination calculated at six time points over 39 days, or until no more germination was occurring. Germination data collected previously for 14 of the accessions at this temperature, either at a single or for multiple time points, were used for comparison of viability. Seed surface sterilization was as described in Experiment 1, with an extension of time in ethanol and hypochlorite to nine minutes. Seed were not washed prior to this, because the combination of washing for long periods (24-48 hours) with surface sterilization reduced germination in 2-12 year-old seed by between 5 and 95%, compared to untreated controls or surface sterilizing alone, in 19 of the 22 accessions investigated in this experiment (data not presented). Seed germination was measured as a percentage of total seed at six time points over 39 days.

2.4.5 Experiment 5. Seed longevity

Twenty-seven accessions, for which previous estimates of maximum germination capacity were available, were germinated at three temperatures. Seed was prepared and germinated as described in Experiment 1, except that it was not soaked prior to surface sterilization. In the case of accessions tested previously in Experiment 1 and 2, three replicates, each of approximately 100 seed, were prepared per temperature treatment. Due to time constraints, accessions used previously in Experiment 4 were un-replicated within temperature treatments. Germination was calculated as a percentage of total seed at 16 time points over

59 days. Where relevant, germination percentages were averaged over replicates for each temperature treatment.

2.5 Results and discussion

2.5.1 Experiment 1. Germination temperature in diverse accessions

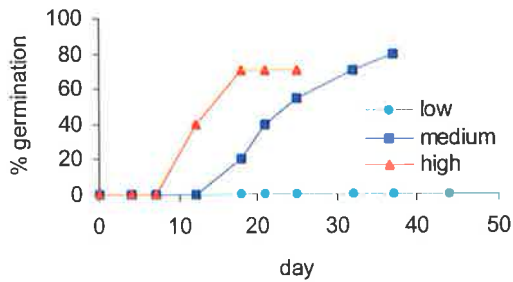
2.5.1.1 Temperature and seed age effects

Response to temperature fell into two distinct and contrasting patterns, depending on seed age. Although medium temperature resulted in earlier germination than low temperature in a similar manner in seed of all ages, there was a striking contrast in response to high temperature. All samples two years and older displayed a pattern of earliest germination at high temperature followed by later germination at medium then low temperatures (Fig. 2.1 a-h), while germination in seed less than 12 months old was inhibited at high temperatures over a 44-day period (Fig. 2.1 i-l). In the following discussion, the former will be referred to as the “older” age group, and the latter as “younger”. Campbell’s conclusion that high temperature inhibits the germination of young seed (Campbell, 1985) was supported for the three genotypes in the younger group. However, his conviction that low temperature is optimal for the germination of young seed (Campbell *pers. com.*) is not supported by this study, since in all younger seed samples medium temperatures led to the highest germination. Further, the present results suggest that low temperature may be inhibitory in older seed, something that Campbell did not observe (Section 2.2.4).

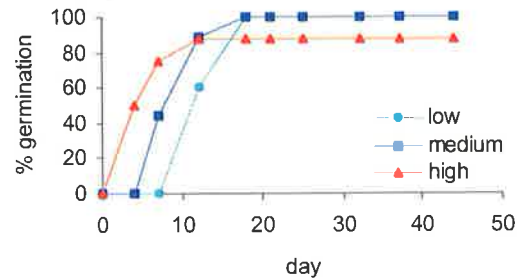
The results show that the temperature response pattern in younger seed can last up to a year at least in the R1 genotype. The “older” response pattern was present in at least one genotype by two years of age and had persisted for at least seven years in five other genotypes.

Figure 2.1. Percentage germination of *H. perforatum* over time, at low, medium and high temperatures of (a-g) seven year-old seed from plants grown in a common garden, (h) two year-old field-collected seed, and (i-l) field-collected seed less than 12 months old. Accession names are followed by RFLP fingerprint codes in brackets, then seed age.

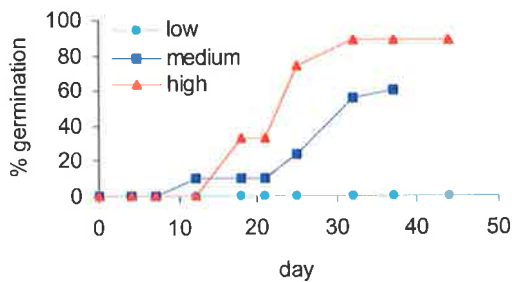
a. Tuena (R12), 7 years



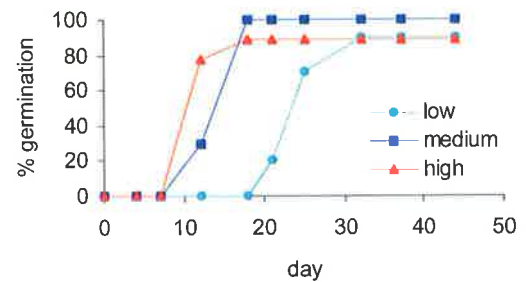
b. Orange-1 (R10), 7 years



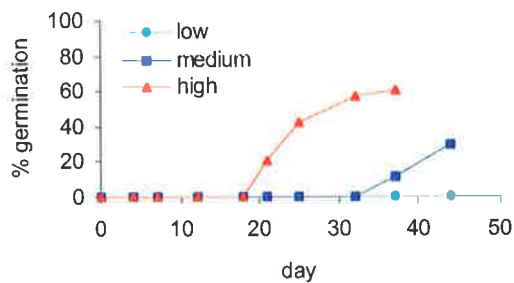
c. Mudgee Short (R1), 7 years



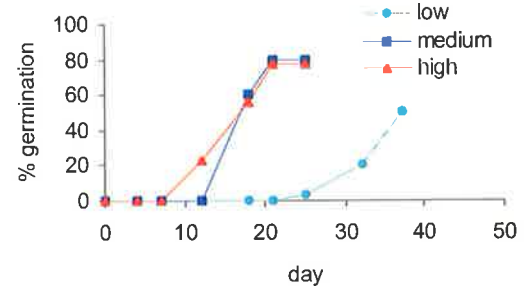
d. Captains Flat (R7), 7 years



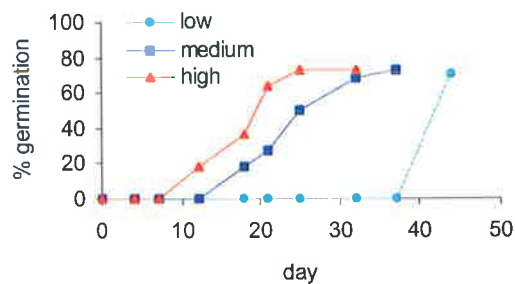
e. Coolah (R1), 7 years



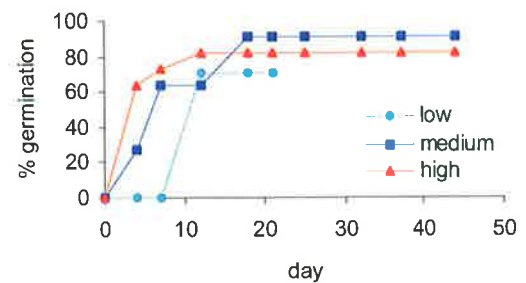
f. Bemboka (R7), 7 years



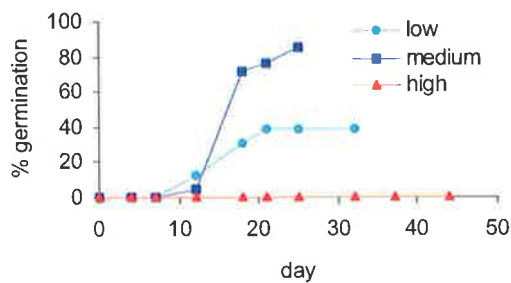
g. Adelong (R3), 7 years



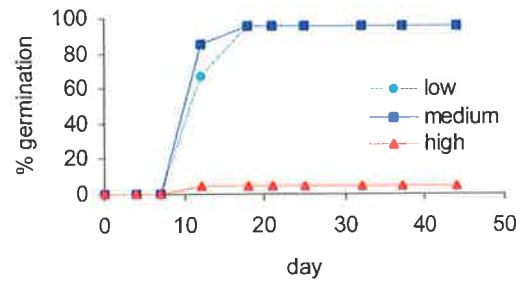
h. Cowra (R13), 2 years



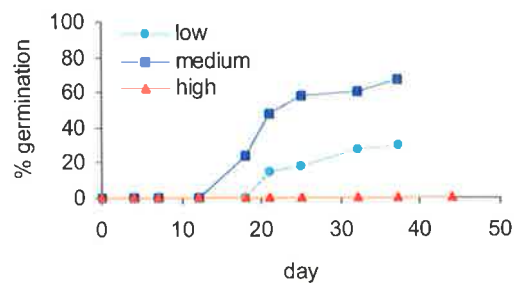
i. Cassilis (R1), 9-12 months



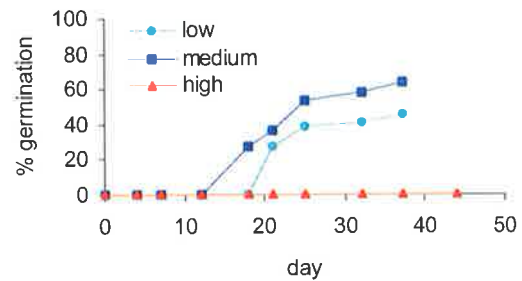
j. Mudgee L (R4), 1-3 months



k. Scott Creek 1 (R6), 0.2-3 months



l. Scott Creek 2 (R6), 0.2-3 months



These results suggest that age group had a larger effect on response to temperature than either genotype or environment, because the contrasting patterns of response were consistent within groups irrespective of genotype and origin, and small variations in age. For example, despite their varied genotypes and origins, note the similar responses of field-collected Cowra seed (Fig. 2.1 h) and common garden samples (Fig. 2.1 a-g) within the older group, and those of Cassilis (Fig. 2.1 i), Mudgee L (Fig. 2.1 j) and Scott Creek (Fig. 2.1 k, l) within the younger group. In particular, compare the young sample from Cassilis (Fig. 2.1 i) to the two older accessions with which it shared an identical genotype (Fig. 2.1 c, e).

2.5.1.2 Genetic variation

While age group seems to have been the major factor contributing to the alternative response patterns, genotype also appears to have contributed some minor variation. Among seven year-old seed originating from a common garden (Fig. 2.1 a-g), there was variation in time to initiation of germination and rate of germination, at all temperatures. Compare

the lack of germination at 15°C over a 44 day period for Tuena, Mudgee Short and Coolah (Fig. 2.1 a, c, e), with delayed germination relative to other temperature treatments for Orange-1, Captains Flat, Bemboka and Adelong (Fig. 2.1 b, d, f, g). Note that two pairs of genotypes (Fig. 2.1 c and e, d and f) shared similar rates and times to initiation of germination within pairs. The most likely explanation of variation in seven year old accessions is an effect of genotype, since environmental and age effects in this seed, harvested at the same time from a common garden, are expected to be minimal. However, such conclusions are presented cautiously because treatments were not replicated. Minor variation in response patterns in the younger group cannot be attributed directly to genotype, since environmental and small age differences were also present in this group. The question of the effect of environment cannot be resolved, since the experiment did not include accessions of the same age and genotype from different field sites.

Seven year-old seed from Tuena, Orange-1 and Mudgee Short were the original seed batches used by Campbell (1990, 1992) to test the effect of light and $\text{KNO}_3 + \text{GA}$ on one month-old seed. Campbell concluded that there was no effect of genotype at 104 days in the presence of light at 30°C:20°C (see Section 2.2.3). However, analysis of percentage germination at 104 days (and presumably at plateau) may have obscured variation during germination. In the same accessions seven years later, it is interesting to note minor variation in time to initiation of germination and rate of germination that, as discussed above, may be an effect of genotype (Fig. 2.1 a, b, c).

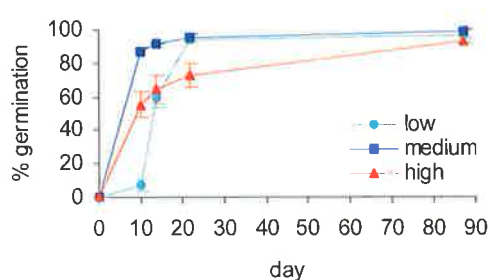
2.5.2 Experiment 2. Seed age and germination temperature in two genotypes

Temperature had a highly significant influence ($\text{Pr} < 0.0001$) on percentage germination over the first three weeks at 10, 14 and 22 days but not at 87 days ($\text{Pr} = 0.40$), for seed of both genotypes and ages. Temperature clearly influenced rate of germination, but not germination capacity over this period (Figure 2.2). There was a highly significant

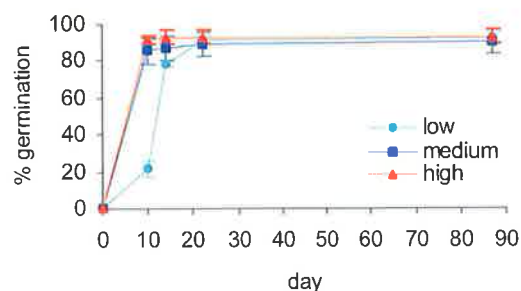
interaction between temperature and seed age ($Pr < 0.0001$), for all time points prior to 87 days, in both accessions. There were also significant differences for and an interaction between genotype and seed age at 87 days. However, in Year 1 of the project, seed was available only from field sites, so it was not possible to design the experiment to differentiate genotype or age from environmental effects. In particular, the significance of age may reflect the collection of old Scott Creek seed in a very dry year. Had time permitted, the experiment would have been repeated with seed produced from the same plant under controlled conditions.

Figure 2.2. Mean percentage germination (\pm SD) up to 90 days at low, medium and high temperatures (see text for details), for two genotypes of *H. perforatum*. Accession names are followed by RFLP fingerprint codes in brackets, then minimum and maximum possible seed ages.

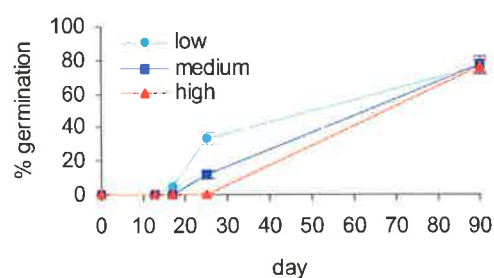
a. Turon "new" (R10), 2-5 months



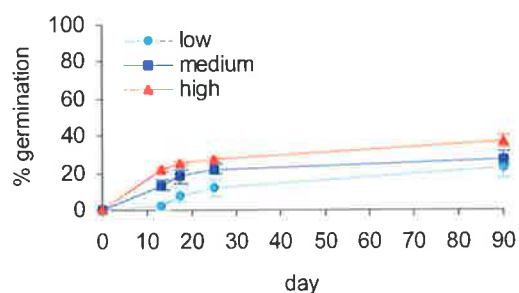
b. Turon "old" (R10), 14-17 months



c. Scott Creek "new" (R6), 0.3-5 months



d. Scott Creek "old" (R6), 12-17 months



It is clear that temperature affected germination differently among the four collections (Fig. 2.2). Old seed of both genotypes (Fig. 2.2 b, d) exhibited the same pattern of response

to the range of temperatures as the “older” group discussed in Experiment 1, that is, time to initiation of germination was reduced as temperature increased. Recall that young seed in Experiment 1 followed a pattern of earliest germination rate at medium, followed by low temperature, but that high temperature was inhibitory up to 44 days. Interestingly, new seed from Turon seems to be transitional between the two patterns (Fig. 2.2 a). The true pattern of response in new Scott Creek seed (Fig. 2.2 c) may have been obscured as a result of late germination, because no information was available on percentage germination between day 25 and 90, but it does appear that high temperatures resulted in the slowest germination. There is an indication that in this seed, low temperature resulted in the earliest germination, which was not the case in young Scott Creek seed in Experiment 1 (Fig. 2.1 j, k). It is possible that young Scott Creek seed undergoes a complete reversal in response to temperature with age (Fig. 2.2 c and d), and that the different patterns in the two young Scott Creek accessions reflect slight variation in age, given that the exact age of field-collected seed could not be determined. These results show that the “older” temperature response pattern was in place prior to 17 months of age in both genotypes, and that the transition can occur as early as 5 months in at least the R10 genotype. A comparison of samples sharing the R10 fingerprint, Turon (Fig. 2.2 a, b) and seven year-old Orange-1 (Fig. 2.1 b), suggests that in this genotype the “older” pattern is exhibited from around 6 months to at least seven years of age.

The results also showed that seed could reach full germination capacity regardless of temperature, although it might take 2-3 months to do so. What appeared to be an exception in old Scott Creek seed may be because germination had not reached a plateau by 90 days at any temperature.

2.5.3 Experiment 3. Temperature response pattern transition

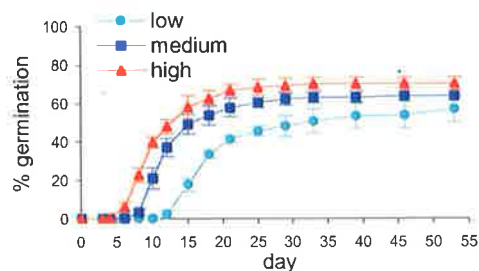
2.5.3.1 Temperature and seed age

Germination in all samples had reached a plateau by 55 days. There was no significant difference among temperature treatments for Mudgee L, Scott Creek-2, Turon “old” and “new”, and Scott Creek “old” and “new”, a small but significant difference for Cassilis ($P > 0.039$) and a significant difference for Scott Creek-1 ($P > 0.005$).

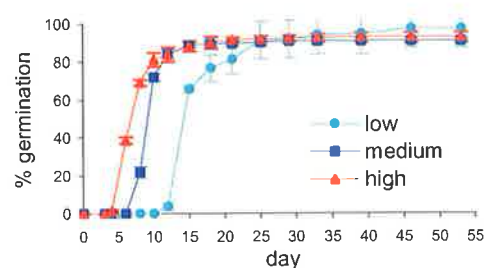
Six of the samples represented young seed batches from Experiments 1 and 2, germinated under identical conditions more than five years later. The comparison of response to temperature with increasing age for these samples enabled a more stringent analysis of germination response transition with increasing age. All six samples exhibited a typical “old” germination response to temperature, in which high temperature resulted in the earliest germination, followed by medium then low temperatures (Fig. 2.3). This “old” response pattern (Fig. 2.3 a-d) replaced the “young” pattern exhibited by all four samples from Experiment 1 (Fig. 2.1 i-l), which strongly supports the hypothesis that seed samples undergo a transition from a “young” to “old” temperature response pattern with increasing age. The results for samples from Experiment 2 support this argument. The young Turon sample from Experiment 2 had changed from a putative “transitional” response to an “old” response (Fig. 2.2 a and 2.3 e). The Scott Creek sample from Experiment 2, which when young appeared to respond most slowly to high temperature, had also changed to an “old” response with increasing age (Fig. 2.2 c and Fig. 2.3 e).

Figure 2.3. Germination response to temperature in six *H. perforatum* accessions from (a-d) Experiment 1 and (e, f) Experiment 2. Mean percentage germination (\pm SD) over time is presented for low, medium and high temperatures (see text for details). Accession names are followed by RFLP fingerprint codes in brackets, then maximum possible seed ages.

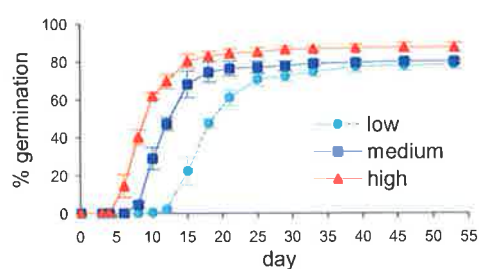
a. Cassilis (R1), 7.4 years



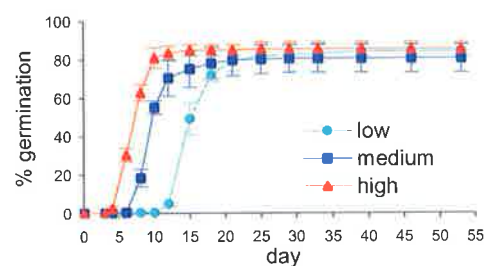
b. Mudgee-L (R4), 6.7 years



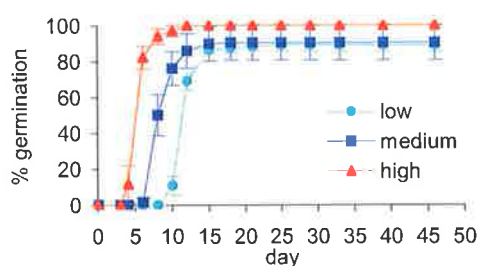
c. Scott Creek-1 (R6), 6.7 years



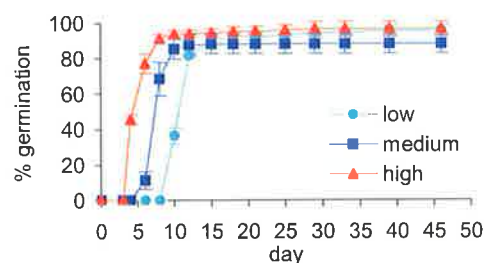
d. Scott Creek-2 (R6), 6.7 years



e. Turon "new" (R10), 5.7 years



f. Scott Creek "new" (R6), 5.7 years



2.5.3.2 Variation among accessions

For all eight accessions and for each temperature, sigmoid curves were fitted using the formula $Y = \text{bottom} + (\text{top} - \text{bottom}) / (1 + 10^{(\log EG50 - \log X) \times \text{hillslope}})$, with R^2 ranging from 0.959 to 0.998 (GraphPad Prism 3.02, 2000), and are presented without standard errors for the sake of simplicity (Fig. 2.4 a-c). Time to 50% final germination (T50) and slope of curve around T50 are presented together with accession age and genotype (Table 2.2).

Figure 2.4. Non-linear regressions on germination response to (a) low, (b) medium and (c) high temperatures in eight *H. perforatum* accessions from Experiments 1 and 2. Accession identifiers are followed by RFLP fingerprint codes in brackets.

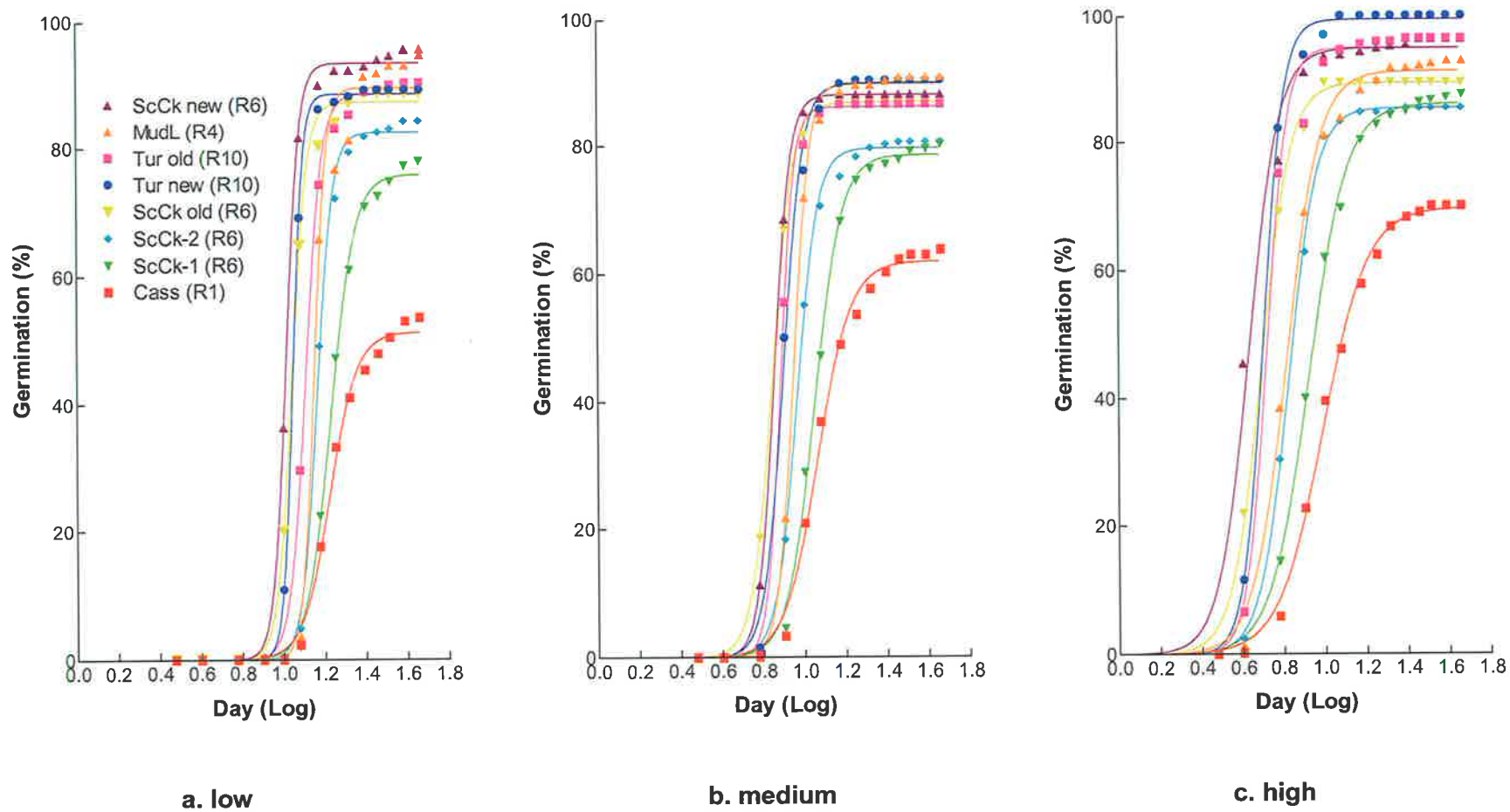


Table 2.2. Time to 50% final germination (T50) and slope from non-linear regression on germination at low, medium and high temperatures, for *H. perforatum* accessions presented in Fig. 2.4 a-c.

Accession	Age (years)	RFLP genotype	Low		Medium		High	
			T50	slope	T50	slope	T50	slope
Cassilis	7.4	R1	16.8 ^a	6.6 ^a	11.5 ^a	5.4 ^a	9.7 ^a	4.0 ^a
Scott Ck-1	6.7	R6	17.0 ^a	7.4 ^a	11.2 ^a	6.5 ^{ab}	8.4 ^b	4.6 ^{ab}
Scott Ck-2	6.7	R6	14.6 ^b	11.4 ^b	9.2 ^b	8.5 ^{abcd}	6.7 ^c	6.2 ^{cd}
Mudgee L	6.7	R4	14.2 ^b	14.8 ^b	8.9 ^b	11.0 ^d	6.5 ^c	5.3 ^{bc}
Turon old	6.7	R10	12.9 ^c	11.0 ^b	7.7 ^c	13.0 ^{abcd}	5.2 ^d	8.6 ^{de}
Turon new	5.7	R10	11.1 ^d	17.6 ^c	7.9 ^c	9.1 ^{abcd}	5.0 ^d	8.7 ^e
Scott Ck old	6.7	R6	11.1 ^d	11.9 ^b	7.0 ^d	8.5 ^{bc}	4.9 ^d	6.0 ^{cd}
Scott Ck new	5.7	R6	10.4 ^e	13.5 ^b	7.1 ^d	11.0 ^{cd}	4.3 ^e	5.8 ^{bcd}

Note: different letters indicate statistically significant differences.

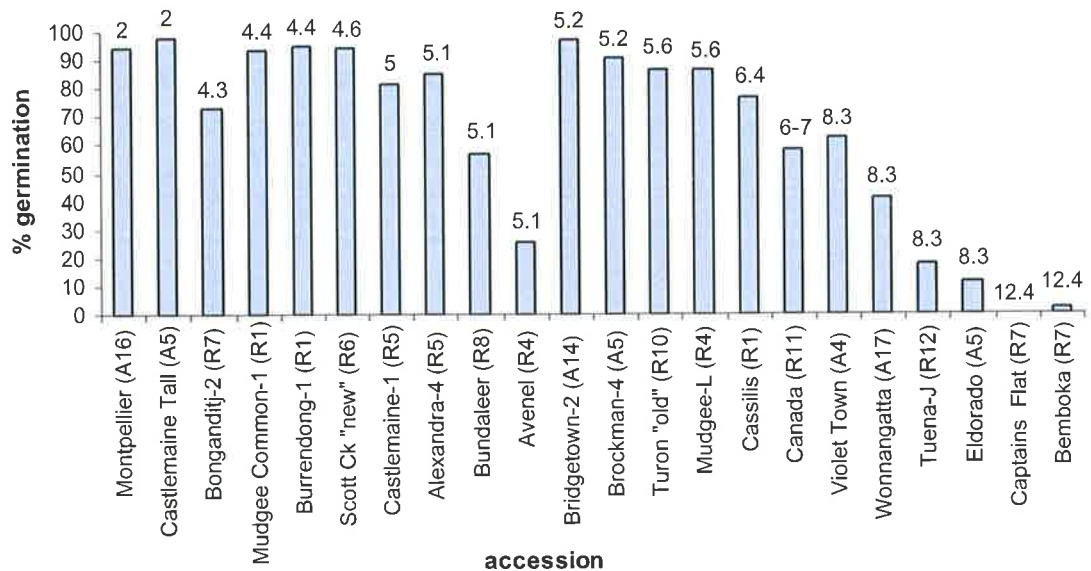
Variation in time to 50% germination (T50) and germination rate (slope) were not readily explained by genotype. Among the two Turon (R10 genotype) and four Scott Creek samples (R6 genotype), T50 and/or slope differed significantly for at least one temperature. This occurred despite the fact that two of the latter (Scott Creek-1 and 2) had been collected from individual plants approximately 20 m apart on the same date (Appendix A). Although there is a possibility that undetected genetic variation existed between the two individuals, the effect of microclimate, positional effects of seed capsules or residual chemical inhibitors may also explain the differences. Variation among other samples may be an effect of small differences in seed age or environmental conditions in the field. Certainly, a comparison with previous experiments indicated that germination initiated earlier with increasing age in some cases (for example, for Scott Creek seed compare Fig. 2.1 k and l with Fig. 2.3 c and d respectively, and Fig. 2.2 c with Fig. 2.3 f). Additional evidence for a non-genetic effect is provided by several other younger, unreplicated samples of the R1 genotype (Burrendong-1 at 5.4 years, and Mudgee Common-1 at 5.3 years old) that appeared to germinate earlier and faster than the Cassilis sample

above, at these temperatures and under identical conditions (from Experiment 5, data not presented). However, in a comparison among accessions it was generally not possible to discriminate between the effects of age or field environment without appropriate controls.

2.5.4 Experiment 4. Decline in viability

Maximum germination was reached between 8 and 22 days, and is presented at 39 days with accessions arranged in order of increasing age (Fig. 2.5). Disregarding the apparently low germination in three samples of around 5 years old (Bonganditj, Bundaleer and Avenel, discussed below), there was a consistent trend of lower germination with increasing age in samples over seven years of age.

Figure 2.5. Percentage germination for 22 H. perforatum accessions arranged in order of increasing age from two to approximately twelve years, at 39 days and medium temperature. Age is given in years at the top of each histogram. Accession names are followed by RFLP or AFLP fingerprint codes in brackets.



Decline in viability could be assessed by comparison with earlier data for the same seed lots, in the following three of the latter samples. Germination in 12.4 year-old Captains Flat and Bemboka seed (Fig. 2.5) was very low compared with identical seed lots used in Experiment 1 (Fig. 2.1 d, f). Viability in 8.3 year-old Tuena-J was also very low,

compared to germination at 3 years (70%, left-hand column Fig. 2.6). Previous data were not available for the other samples over seven years of age (Canada, Violet Town, Wonnangatta and Eldorado), but the suggestion is made that an age-related decline was responsible for the low germination seen in these samples. Decline in viability was also assessed for samples 6.4 years of age and younger. Germination was similar or had increased rather than declined, in samples from Bonganditj, Mudgee Common-1, Burrendong-1, Castlemaine-1 and Alexandra-4 (Table 2.3), and in Scott Creek “new” (Fig. 2.2 c), Turon “old”(Fig. 2.2 b), Mudgee-L (Fig. 2.1 j) and Cassilis (Fig. 2.1 i). The relatively low germination in Bonganditj (Fig. 2.5) therefore did not indicate a decline. Whilst there appeared to be a reduction in germination of Bundaleer seed, results for Experiment 5 showed that this sample had not declined by 6.1 years (Fig. 2.5), a discrepancy that may be explained by the presence of fungal contamination in the former un-replicated sample. However, Avenel showed a reduction compared to germination at 10 months of age (Table 2.3), which indicated that in some samples germination might start to decline as early as five years of age. These results indicate that in the majority of cases the potential for maximum germination was maintained for at least 5.6 to 6.4 years (Fig. 2.5).

Table 2.3. Germination percentages at 15 days and at plateau (39 days) for seven accessions in Experiment 4, compared to data obtained 4.25 years previously at medium temperature. Maximum possible age is given in months (m) or years (y).

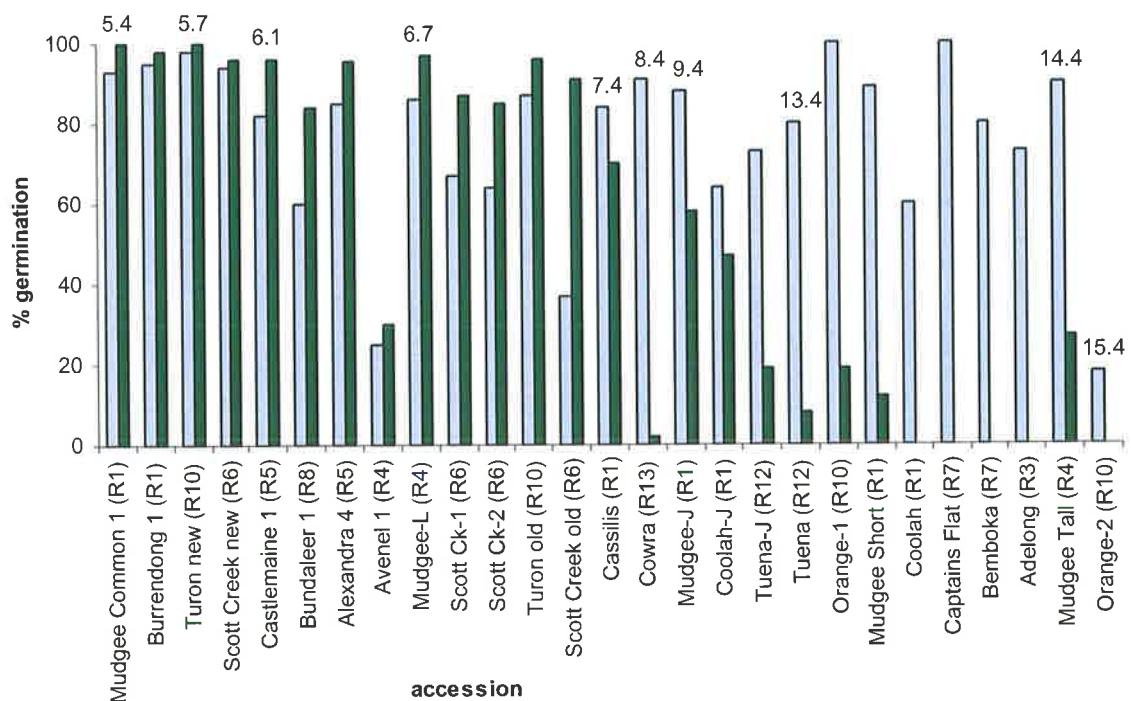
Accession	Age (m)	Germination %	Age (y)	Germination %
	11.5.97	at 16 days	Experiment 4	at 15 (39) days
Bonganditj-2 (R7)	1	32	4.3	73 (73)
Mudgee Common-1 (R1)	1.5	82	4.4	91 (93)
Burrendong-1 (R1)	1.5	77	4.4	88 (95)
Castlemaine-1 (R5)	9	63	5	78 (82)
Alexandra-4 (R5)	10	95	5.1	85 (85)
Bundaleer (R8)	10	87	5.1	44 (57)
Avenel (R4)	10	53	5.1	25 (25)

2.5.5 Experiment 5. Seed longevity

2.5.5.1 Relative decline in viability

The measure of viability considered was the maximum germination obtained, irrespective of temperature. For each accession a comparison was made to the maximum percentage germination achieved in any previous experiment, at plateau and at any temperature (Fig. 2.6, left-hand column). The average maximum germination achieved was 86% (SD = 13%), calculated for 26 samples and excluding Orange-2, for which the only previous data was at 9 years of age. The age of seed at the time of previous germination was not considered, because only relative viability at the time of the Experiment 5 was of interest. Samples were arranged in order of increasing age at the time of Experiment 5 (Fig. 2.6).

*Figure 2.6. Pairwise comparisons for *H. perforatum* accessions, of maximum percentage germination achieved at plateau at any temperature, using data obtained previously (left-hand column) and during Experiment 5 (right-hand column). Samples are arranged in order of increasing age, with age at the time of Experiment 5 given in years at the top of each histogram (a blank indicates the same age as the accession to the left). RFLP fingerprint codes are given in brackets.*



There was no decline in viability in accessions up to and including 6.7 years of age, but an increase or the same percentage germination compared with previous maximum germination capacity (Fig. 2.6). By contrast, germination in every sample 7.4 years of age and over had declined when compared with previous capacity. Decline in Cassilis seed germination by 7.4 years (Fig. 2.6) is interesting, because at 6.4 years of age no reduction in viability was detected (Fig. 2.5). Viability continued to decline for two samples with reduced germination in Experiment 4 (Tuena-J and Bemboka), or was stable (Avenel).

2.5.5.2 Longevity

It is clear that a proportion of seed of some accessions may be viable for at least 13-14 years (Fig. 2.6). The conclusion was reached that five of the 13 accessions 13.4 years of age and over had died, since they failed to germinate by 53 days. An estimation of longevity was made from the window of time between the age at which failure to germinate was first recorded in either Experiment 4 or 5, and the most recent age at which any germination was achieved (Table 2.4). Note that some of the latter data may not have been presented thus far, or may differ to that used in Fig. 2.6 for comparison of maximum possible germination.

Table 2.4. Range of longevity for six accessions that had died by Experiment 5. Penultimate germination percentages and ages (y = years) are followed by maximum possible age at death (the age at which failure to germinate was first recorded).

Accession	Germination	Age	Maximum possible age at death
Coolah (R1)	60%	7 y	13.4 y
Captains Flat (R7)	100%	7 y	12.4 y
Bemboka (R7)	2%	12.4 y	13.4 y
Adelong (R3)	73%	7 y	13.4 y
Orange-2 (R10)	18%	9 y	15.4 y

Seed from Bemboka (R7) died between 12.4 and 13.4 years of age, and it is likely that seed from Captains Flat, which shared an RFLP fingerprint, also died closer to 12.4 than seven years old, given that viability was 100% at the younger age. The Coolah, Adelong and Orange-2 samples offer only a rough guide to upper and lower limits, given the large time gap. It is worth noting that seed from Wellington was dead by eight years (see Aims, Section 2.3), although there was no previous data available from which to make comparisons. It is clear that at least annual sampling over an extended period of time would be necessary in order to estimate longevity for remaining live samples.

2.5.5.3 Variation in viability and longevity

Examination of the 10 genotypes included in Experiment 5 did not reveal any striking correlation between genotype and decline in viability, since all samples over 7.4 years of age had reduced viability, irrespective of genotype. Average maximum germination of all accessions up to and including seven years of age was 86% (SD = 13%, range = 53-100%, N = 25). Neither did genotype appear to greatly influence maximum germination capacity, since that parameter varied considerably within several genotypes prior to the onset of decline at 7.4 years of age. For example, maximum germination ranged between 60 and 100% in R1 genotype samples seven years old or less (Mudgee Common, Burrendong, Cassilis, Mudgee-J, Coolah-J, Mudgee Short and Coolah). It is likely that environmental effects contribute greatly to variation in viability in this field-collected seed, since seed was stored under identical conditions. In order to draw conclusions on the influence of genetics or environment, it would be necessary to re-design the experiment to control for the effect of environment.

The only samples for which the effect of genotype on longevity could be compared with any confidence were the common garden samples from Experiment 1. By 13.4 years of age four failed to germinate (Coolah (R1), Captains Flat (R7), Bemboka (R7) and Adelong

(R3)), and three showed extremely low germination (Tuena (R12), Orange-1 (R10) and Mudgee Short (R1)). Of these, R1 genotype samples Mudgee Short and Coolah obviously varied in longevity, as did the R7 genotype (Captains Flat and Bemboka, see discussion above), although probably not by much. However, the within-genotype level of variation in longevity is not likely to be any lower than the variation among genotypes (Fig. 2.6).

2.5.5.4 Temperature response pattern

All 27 samples exhibited the typical “old” seed response pattern (for example Fig. 2.3), regardless of whether there was a decline in germination, which indicated that this response could last up until the end of the life of the seed.

2.6 Conclusion

2.6.1 Temperature and seed age effects

Temperature had a highly significant influence on germination of *H. perforatum* seed, but although temperature affected time to initiation of germination, in almost all cases it did not substantially alter maximum germination capacity over time or the germination rate characteristic of each sample. There was a significant interaction of temperature with seed age, resulting in two distinct patterns of germination response to temperature. While medium temperature (23°C) resulted in earlier germination than low temperature (15°C) in seed of all ages, response to high temperature (25°C) varied with age. In the “older” temperature response pattern, high temperature resulted in earlier germination than any other temperature. In the “younger” response, at high temperature seed germinated later than at any other temperature, and in many cases failed to germinate over the first 44 days. Plainly, high temperature had the opposite effect on old and new seed, and the position of the high temperature response relative to either of the other temperatures can be said to be diagnostic for seed age. The two patterns described were stable for these age groups, irrespective of the fact that material was sourced from a wide range of genotypes and field

conditions. In total, 26 samples aged 2 years and older, and comprising 10 genotypes, were tested at three temperatures (some more than once), and all viable seed demonstrated the “older” pattern. Six samples under 12 months old were tested at the three temperatures, of which five showed the “young” pattern (although one was unclear), and one seemed to be transitional between response patterns. The transition between the two patterns, involving a switch in the position of the high temperature response curve relative to the medium and low response, probably takes place towards the end of the first year, but may occur as early as 5 months (Fig. 2.2 a). The “older” temperature response pattern appeared to last for the remainder of the lifetime of the seed.

In answer to the original questions posed in the introduction, medium temperature was considered optimal for germination of young seed from crosses or accessions. However, in the youngest seed batches, it was noted that time to initiation of germination could be up to two weeks longer even at medium temperature, and seed may only reach maximum germination capacity by 6-8 weeks. For this reason, fresh seed from crosses was stored for a month prior to germination, and surface sterilization to control fungal contamination over this extended time was considered important. High temperature was optimal for germination of older seed from accessions, which varied in age and genotype and had been produced under diverse environmental conditions. Older seed had often germinated by 3 days and reached maximum capacity by 10-12 days.

2.6.2 Viability and longevity

The average maximum germination was 86% (SD = 13%) for 26 samples from Experiment 5. For all samples tested, viability started to decline at around seven years of age, to between 0% and 20% by 13-14 years. Longevity ranged from 13.4 to more than 14.4 years, with more than half the samples over 13.4 years of age having died. No clear evidence was discovered for genetic variation in viability or longevity.

2.6.3 Variation

Age or environmental effects were more likely than genetic variation to be responsible for variation in time to initiation of germination and rate of germination, despite the fact that genetic variation appeared to be responsible for some minor variation in common garden samples. There was a general shift to earlier initiation of germination with increasing age, in some samples, although this did not alter the basic temperature response pattern. Whatever the cause, variation among collections in germination characteristics could have added to the confusion that existed in the literature.

2.6.4 Evolution of germination traits

The age and temperature-dependent germination response may have evolved to ensure staggered germination of *H. perforatum* seed of different ages from a long-lived seed bank. In seed of all ages, low temperatures over the colder winter months would retard but not necessarily prevent germination, provided other germination requirements were met. The age-temperature interaction would result in a peak of young seed germination in optimal spring temperatures, while in the first season restricting unproductive germination of young seed over summer, when high temperatures might compromise seedling survival. This strategy could prevent wasteful depletion of young seed with the greatest potential longevity. In addition to this, chemical inhibition and the light requirement of young seed would secure a large proportion of young seed within the seed bank, buried under leaf litter or in shallow soil, even if other conditions were optimal. Seedling recruitment from the older portion of the seed bank would be expected to extend from spring into summer, provided other conditions were suitable for germination. These strategies combine an almost continuous potential for germination over much of the year, with maximum seed bank longevity. Even with no further replenishment, a *H. perforatum* seed bank is likely to contain seed that would be viable for 13 years or more, regardless of genotype.

2.6.5 Weed control

The continuous production of seedlings each year makes targeting herbicide applications on emerging seedlings more difficult, and is compounded in the case of such long-lived seed. For effective control, seedlings would have to be removed before maturity, over a considerable number of years. In areas where infestations are inaccessible or chemical control is uneconomical, the persistence of biological control agents offer the best hope of a long-term solution to the control of this plant.

2.7 Summary

Temperature affected time to initiation of germination of *H. perforatum* seed, but not maximum germination capacity or germination rate. There was a significant interaction of temperature with seed age, resulting in two distinct patterns of germination response to temperature. In older seed, high temperature resulted in earlier germination than any other temperature, followed by medium temperature then low temperature. In younger seed, medium temperature resulted in the earliest germination, followed by low temperature, then high temperature. The transition between the two patterns, involving a switch in the position of the high temperature response curve relative to the medium and low response, appeared to take place towards the end of the first year, after which response to temperature seemed to remain stable for the remainder of the lifetime of the seed. Environmental effects were more likely than genetic variation to be responsible for variation in time to initiation and rate of germination. The age and temperature-dependent germination response may have evolved to prevent wasteful depletion of seed with the greatest potential longevity. Regardless of genotype, an *H. perforatum* seed bank is likely to contain seed that would be viable for 13 years or more, even though viability may start to decline from seven years of age. These germination characteristics would undoubtedly have contributed to past difficulties in control of this weed.

3 Modes of reproduction in Australian populations of *Hypericum perforatum* L. (St. John's wort) revealed by DNA fingerprinting and cytological methods

Genome 46: 573-579 (2003)

3.1 Abstract

Hypericum perforatum L. (St. John's wort) is widely used in homeopathic medicine, but has also become a serious weed in Australia and many other countries. Reproduction in *H. perforatum* was investigated using markers based on restriction fragment length polymorphism (RFLP) and amplified fragment length polymorphism (AFLP). Between two Australian populations, plants displayed 14 polymorphisms from a total of 22 scorable RFLP markers when genomic DNA was probed with M13 bacteriophage, but individuals within each population exhibited identical RFLP fingerprints. Ninety-four percent of the progeny of four crosses made between the two populations exhibited identical fingerprint and ploidy level to the maternal parent, and probably originated apomictically. Seven seedlings with recombinant RFLP or AFLP fingerprints were found from a total of 121 progeny. Both molecular marker techniques detected the same recombinants from a subset of screened progeny. Cytological analysis showed that the seven recombinants comprised three tetraploids ($2n = 4x = 32$), three hexaploids ($2n = 6x = 48$), and one aneuploid ($2n-1 = 31$), which suggested that the level of normal reduced embryo sacs was only 2.5%. These results are discussed in relation to the management of invasive populations, and the implications for plant breeding and production of St. John's wort for medicinal purposes.

Key words: *Hypericum perforatum*, apomixis, DNA fingerprint, RFLP, AFLP

3.2 Résumé

Hypericum perforatum L. (le mille-pertuis) utilise la médecine homéopathique, mais en Australie et dans beaucoup de pays, elle est aussi devenue une très mauvaise herbe. La reproduction du *H. perforatum* a été examinée au moyen des polymorphismes sur la longueur des fragments de restriction (RFLP) et du polymorphisme de longueur des fragments amplifiés (AFLP). Entre deux populations Australiennes, 22 marqueurs RFLP au total ont montré 14 polymorphismes, quand l'ADN génomique a été hybridé au bactériophage M13. De chaque population pourtant, toutes les plantes ont manifesté empreintes RFLP les mêmes. De la progéniture des hybrides entre les deux types génétiques différents, l'analyse moléculaire et cytologique a montré 94 pour cent de types maternelles, qui sont provenus probablement de l'apomixie. Chaque de marqueurs techniques moléculaire a pu distinguer des recombinants identiques, parmi une sélection de la progéniture. Des 121 progénitures, sept semis se sont manifestés des empreintes RFLP et AFLP recombinants. L'analyse cytologique a montré que les sept recombinants résultent de trois tétraploïdes ($2n = 4x = 32$), trois hexaploïdes ($2n = 6x = 48$), et une aneuploïde ($2n-1 = 31$), ce qui suggère que le pourcentage de sacs embryonnaire réduit normaux a été 2.5%. Les résultats et ses conséquences ont été discutées pour la gestion des populations envahissantes, et pour la reproduction et la production médicinale du mille-pertuis.

Mots clés: *Hypericum perforatum*, apomixie, empreinte l'ADN, RFLP, AFLP

3.3 Introduction

Hypericum perforatum (St. John's wort) is used in the production of several homeopathic remedies, but it is also considered a weed in 20 countries (Holm *et al.*, 1979). Increasing our understanding of reproductive pathways in St. John's wort could not only assist researchers interested in breeding superior hybrid genotypes and the commercial production of uniform cultivars (Arnholdt-Schmitt, 2000), but could also aid the control of the plant where it behaves as an aggressive weed (Mayo & Roush, 1997).

Apomixis is of particular interest because the entire maternal genotype is transmitted via a stable seed propagated clone (Vielle-Calzada *et al.*, 1996). *H. perforatum* is a facultative pseudogamous apomict, which produces bipolar *Hieracium*-type aposporous initials resembling the sexual *Polygonum*-type (Noack, 1939). The species is thought to be of allopolyploid origin (Mártonfi *et al.*, 1996) and most commonly occurs as a tetraploid ($2n = 4x = 32$). Embryo sacs may be either reduced (meiotic) or unreduced (aposporic, of nucellar origin) and may be either double fertilized or pseudogamous (only the endosperm must be fertilized in order for the embryo to develop) in a facultative apomict of this type, resulting in four possible categories of progeny (Savidan, 2000). With rare exceptions, seed progeny of such a tetraploid demonstrate distinct DNA complement ratios of embryo to endosperm (respectively, shown in brackets below): reduced, double fertilized (sexually produced) "B_{II} hybrids" (4C: 6C); unreduced, pseudogamous (apomictically produced) maternal types (4C: 10C); reduced, parthenogenetic haploid types (2C: 6C); or unreduced double fertilized "B_{III} hybrids" (6C: 10C) (Matzk *et al.*, 2001; Nogler, 1984).

Noack (1939) performed crosses of five *Hypericum* species ($n = 8$ or 9) with tetraploid *H. perforatum* ($n = 16$), and concluded from the proportion of triploid seedlings that a normal reduced embryo sac occurred in only 3% of ovules. Self-pollination of tetraploid *H. perforatum* and cross-pollination with *H. quadrangulum* ($2n = 16$) produced quite different

proportions of tetraploid and hexaploid embryos (73 and 32% tetraploids, 28 and 67% hexaploids, respectively), with no evidence from endosperm chromosome number of reduced egg sacs from either cross. Despite this, one embryo produced from the cross was clearly the result of normal sexual reproduction. The possibility exists that a proportion of the tetraploid embryos were also derived in this manner, since embryo and endosperm data were independent in the majority of cases. Noack (1939) did not report any reduced parthenogenetic (diploid) progeny of any *H. perforatum* crosses, but the cross with *H. quadrangulum* indicated one case of autonomous endosperm development. A higher level of sexuality was proposed after single locus RFLP analysis (Halušková & Cellárová, 1997). However, sample sizes were small, ploidy levels were not presented, and calculations appeared to take into account only those families in which recombinants occurred. Brutovská *et al.* (1998) found 7% triploid (sexually produced), 12% tetraploid (apomictically produced), and 81% pentaploid (B_{III} hybrids), but no diploid progeny (reduced parthenogenetic) in a study of tetraploid in-vitro regenerated plants cross-pollinated with reduced pollen from diploid regenerants. This contrasted with the absence of B_{III} hybrids in progeny of self-pollinations, all of which were tetraploids (but for which sexual or apomictic origin could not be discriminated). Arnholdt-Schmitt (2000) made a preliminary investigation of RAPD fingerprint variation in progeny for which parental genotypes were undetermined, and concluded that “non-identical reproduction was present as a minor effect”. Ploidy levels were not evaluated, although the latter author suggested cytological evaluation as a complementary technique. Matzk *et al.* (2001) pointed out that apart from limited sample sizes and inadequate methods in previous studies, the “high plasticity of reproduction” in *H. perforatum* could have rendered these molecular tools inefficient at differentiating between sexual and apomictic plants. Flow cytometric seed screen (FCSS) analysis was used to evaluate DNA content ratios of embryo to endosperm nuclei, for 113 *H. perforatum* accessions (Matzk *et al.*, 2001). Pseudogamy was the rule,

although a few cases of autonomous endosperm development apparently occurred in tetraploids (Matzk *et al.*, 2001). Eleven different routes of reproduction were identified among accessions (Matzk *et al.*, 2001). However, part of this complexity might be explained by the variation in ploidy evident within some accessions and even individual plants, given that the production of and capacity for parthenogenesis in meiotic or aposporic egg sacs appeared to depend on parental ploidy. Matzk *et al.* (2001) found that tetraploids were most common and most often facultative apomicts, reproducing by a combination of sexuality and apomixis, and frequently also producing reduced parthenogenetic and (or) unreduced double fertilized egg sacs. Less commonly, tetraploids were obligate apomicts. Diploid accessions were uncommon, and reproduced either by obligate sexuality alone, or additionally by the unusual route of chromosome doubling, followed by production of reduced or unreduced egg sacs, then double fertilization or pseudogamy (as in tetraploids). Hexaploids either reproduced by obligate apomixis or were sterile. Proportions of pathways varied widely with accession and (or) individual (Matzk *et al.*, 2001). It is not only clear that the degree of apomixis may vary widely in *H. perforatum*, but also that the contribution of alternative pathways should not be ignored in an investigation of reproductive biology in this species.

Proportions of reproductive pathways in cross-pollinated *H. perforatum* were investigated in this study. Initially, a multilocus RFLP fingerprinting technique was used to characterize genetic variants of the weed in Australia, in which genomic DNA restricted with *HaeIII* was probed with M13 bacteriophage DNA (Mahr *et al.*, 1999), although AFLP was subsequently found to be more suitable for analysis of large sample sizes. The progeny of genetically dissimilar, cross-pollinated tetraploid parents were screened at random, and cytological methods were used to determine ploidy. Traditional cetyltrimethylammonium bromide (CTAB) based DNA extraction methods resulted in sufficient contamination in

the final pellet to affect PCR-based fingerprint reliability, and commercial extraction methods were both costly and inclined to shear DNA; therefore, an alternative, relatively low cost, CTAB-free extraction method suitable for both AFLP and RFLP is presented.

3.4 Materials and methods

3.4.1 Plant material

Seed collection was made from plants of two Australian populations of *H. perforatum* at Cassilis, New South Wales (32°00'30"S, 149 °59'00"E), and at Scott Creek, South Australia (35°05'18"S, 138°40'26"E). Individual plants from the same locality are represented numerically (e.g., "Cassilis 1"). Seed was washed for 24 h with several changes of water, surface sterilized, and germinated on sterile 1% w/v water agar in a growth cabinet (23°C: 16°C, 12 h day), then grown in pots under natural light in a glasshouse.

3.4.2 Cross pollination

Reciprocal crosses Scott Creek 1 × Cassilis 1 (cross 1) and Cassilis 1 × Scott Creek 1 (cross 2), and unidirectional crosses Scott Creek 2 × Cassilis 1 (cross 3) and Scott Creek 3 × Cassilis 1 (cross 4), were made in the following manner. Individual recipient buds were emasculated the evening before anthesis by removal of stamens and petals. Cross-pollination involved brushing the androecium of the donor against the stigma of emasculated recipient flowers immediately post emasculation, and again on the following day. The risk of accidental pollination was minimized by enclosing each inflorescence in a glassine bag and practicing strict hygiene, and assessed by including 10 unpollinated, emasculated controls per inflorescence (which set no seed).

3.4.3 Small-scale DNA extraction

Approximately 0.2 g fresh shoot tips were placed into 2-mL microcentrifuge tubes with three stainless steel ball bearings (3 mm diameter), and held in liquid nitrogen. Tissue was roughly crushed with a cold eppendorf grinder, then intermittently vortexed for 15 s and returned to N₂ until ground to a fine powder before removal of ball bearings with a magnet. Per sample, 50 µL 20% w/v SDS and 5 µL RNaseA was mixed with 1 mL of extraction buffer (pH 9) (500 mM NaCl, 100 mM sodium acetate, 10 mM EDTA and 3% w/v polyvinylpyrrolidone (PVP-40)) just before use and held at 37°C. Without allowing tissue to thaw, 1 mL pre-mixed buffer was added per tube, vortexed, and mixed slowly at room temperature for 30 min. An equal volume of chloroform - isoamyl alcohol (24:1) was added, shaken, mixed gently for 5 min, then centrifuged at 10 000 x g for 10 min. Supernatant was transferred to a clean tube, then 0.25 times the volume of supernatant of 5 M potassium acetate was added, mixed, and incubated on ice 30 min. Precipitate was removed by centrifugation at 10 000 x g for 15 min, supernatant transferred to a tube containing 3 µL of 20 mg proteinase K/mL, and incubated at 37°C for 30 min. Chloroform - isoamyl alcohol extraction was repeated as above, followed by centrifugation at 15 000 x g for 10 min. Supernatant was transferred, to which 0.4 volumes of 4 M ammonium acetate was added and mixed, followed by 0.6 volumes cold isopropanol, gentle mixing, and incubation on ice for at least 1 hour. Centifugation at 10 000 x g for 10 min and careful removal of supernatant was followed by a 70% v/v ethanol + 10 mM magnesium acetate wash and repeated centrifugation before drying pellets briefly in a Speedyvac and dissolving in 40 µL TE (10 mM Tris-HCl, 1 mM EDTA (pH 8.0)) overnight at 4°C. Extractions for RFLP were increased by a factor of five. Minipreps yielded up to 6 µg DNA, whereas larger scale extractions yielded 30 µg, estimated against a standard marker on 1% w/v agarose.

3.4.4 RFLP procedure

Restriction fragment length polymorphisms (RFLPs) were generated from 3 µg genomic DNA, restricted with *Hae*III, and probed with (α -³²P)dCTP random-labeled bacteriophage M13mp18RF DNA, as per Evans *et al.* (1998).

3.4.5 AFLP procedure

The following method was modified from Vos *et al.* (1995) and Powell *et al.* (1997). Adaptor and primer sequences (all Gibco-BRL, Carlsbad, Calif.) were as given in Powell *et al.* (1997), preamplification primer extensions were *Pst*I+A and *Mse*I+C, and selective primer extensions are given in Table 3.1. All PCRs were performed in a PTC-100 Programmable Thermal Controller with Hot Bonnet (MJ Research, Waltham, Mass.). Restriction and ligation were performed simultaneously on 1 µg genomic DNA with 6 µL 10x restriction-ligation buffer (100 mM Tris-acetate, 100 mM magnesium acetate, 500 mM potassium acetate, 50 mM dithiothreitol (pH 7.5)), 1 µL each of 5 µM *Pst*I and 50 µM *Mse*I double-stranded adaptors, 1.2 µL 10 mM ATP, 5 U *Pst*I (Roche Diagnostics, Basel, Switzerland), 5 U *Mse*I (New England Biolabs, Beverly, Mass.), and 1 U T4 DNA ligase (Roche Diagnostics) in a final volume of 60 µL at 37°C for 3 h. Pre-amplification was performed in a final volume of 25 µL with 75 ng each of *Mse*I+C and *Pst*I+A primers, 0.2 mM each dNTPs (Pharmacia), 1.5 mM MgCl₂, 1 U *Taq* DNA polymerase (Promega) and 1x *Taq* buffer, and 4 µL of the restricted and ligated DNA mix. The pre-amplification thermal cycle involved denaturation at 94°C for 30 s, annealing at 56°C for 1 min, and extension at 70°C for 1 min for 20 cycles. After pre-amplification, the product was diluted by the addition of 200 µL H₂O to be used as template DNA for selective amplification. End-labeling was performed in 10 µL volume with 25 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 5 mM DTT, 5 mM spermidine, 10 µCi (α -³²P)ATP, 2 U T4 polynucleotide kinase (Roche Diagnostics), and 50 ng *Pst*I+2 primer. Selective amplification was carried out on 5

μL template DNA in 20 μL total volume containing 5 ng labeled plus 25 ng unlabeled *Pst*I+2 primer, 30 ng *Mse*I+3 primer, 0.2 mM each dNTPs, 1.5 mM MgCl_2 , 0.5 U *Taq* DNA polymerase and 1x *Taq* buffer. The selective amplification thermal cycle involved 94°C for 30 s, 65°C for 30 s, 72°C for 1 min, followed by 9 cycles over which the annealing temperature was decreased by 1°C per cycle, then 25 cycles of 94°C for 30 s, 56°C for 30 s, 72°C for 1 min. Equal volumes of formamide loading buffer (98% formamide, 10 mM EDTA (pH 8), 0.05% w/v bromophenol blue, 0.05% w/v xylene cyanol) were added, samples were denatured 3 min at 90°C, and 2 μL of each was loaded on 6% denaturing polyacrylamide gel (Bresatec Sequagel-6) pre-run for 10 min at 40 W on an SQ3 80-6301-16 sequencer (Hoefer Pharmacia Biotech Inc., San Francisco, Calif.). Samples were electrophoresed for 2.5 h at 40 W. Gels were transferred to Whatman 3MM paper and dried for 40 min at 80°C on a Bio-Rad 583 gel drier (Bio-Rad, Hercules, Calif.), then exposed to X-ray film (Fujifilm Super HR-G30). Autoradiograms were scored for presence or absence of a given band.

Table 3.1. Selective amplification primer extensions used in AFLP analysis of *H. perforatum* and resulting proportions of polymorphic markers.

	No. of polymorphic markers/total scorable markers			
	<i>Pst</i> I+AG	<i>Pst</i> I+AA	<i>Pst</i> I+AT	<i>Pst</i> I+AC
<i>Mse</i> I+CAC	10/42	16/67	10/63	4/30
<i>Mse</i> I+CCA	4/47	12/65	10/67	7/40
<i>Mse</i> I+CGT	6/30	8/50	10/42	7/23
<i>Mse</i> I+CAA	6/51	10/79	7/79	6/52
<i>Mse</i> I+CAG	9/48	6/62	15/83	—

3.4.6 Cytology

The following shoot tip method, modified from Thompson (1995), provided abundant mitotic metaphase cells throughout the season. Actively growing 10- to 20-mm-long shoot

tips were placed in water, on ice, for 24-48 h. Tissue was fixed for 24 h in at least ten times its own volume of Farmer's fluid (absolute ethanol – glacial acetic acid, 3: 1) and rinsed with and stored in 70% v/v ethanol at 4°C. Shoot tips were blotted on tissue paper, the apex excised into 1 M HCl and heated in a heat block at 60°C for 12 min, rinsed three times in water, and stained for 30 min in aceto-orcein. The meristem was dissected into aceto-orcein, covered with a coverslip, warmed, gently tapped, and squashed. At least two (three in the case of parents and recombinants) unambiguous chromosome counts were made for each of two shoot tips. A Zeiss Photomicroscope II with a 63x oil-immersion objective lens was used to examine and photograph prepared slides.

3.5 Results

3.5.1 RFLP analysis

Of 22 scorable RFLP bands, 14 (63.6%) were polymorphic between the two populations (Fig. 3.1). Plants within each population shared identical RFLP fingerprints (N = 13 for Scott Creek and N = 20 for Cassilis, data not presented), therefore crosses were restricted to one or a few parent plants for which tetraploidy had been confirmed, from each population. From a total of 16 seedlings selected from cross 1 at random, none had recombinant RFLP fingerprints. From 15 randomly screened progeny of reciprocal cross 2, two displayed recombinant RFLP fingerprints. Both recombinants possessed all six polymorphic markers present in the recipient Cassilis parent. One recombinant displayed four bands, and the other had three bands, of eight possible donor markers. On cytological examination, both recombinants were found to be hexaploids ($2n = 6x = 48$) (Fig. 3.2). RFLP on nine randomly selected progeny of cross 3 clearly identified two recombinants, with two and three bands, respectively, from a possible six donor bands. Cross 3 recombinant progeny, and all non-recombinant progeny screened with RFLP, were shown by cytological examination to be tetraploid ($2n = 4x = 32$) (Fig. 3.2).

Figure 3.1. RFLP fingerprints produced by Southern hybridization of M13 DNA to *HaeIII*-digested *H. perforatum* DNA of individuals from Cassilis (lane 1) and Scott Creek (lane 2). Arrowheads indicate dominant polymorphic markers for each genotype, respectively.

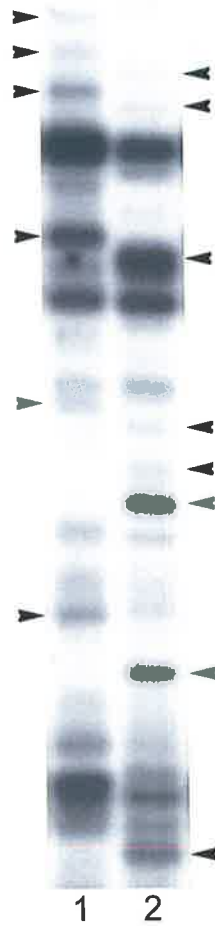


Figure 3.2. Metaphase plates of *H. perforatum* terminal-bud meristem cells of (a) tetraploid $2n = 4x = 32$ and (b) hexaploid $2n = 6x = 48$. Bars represent $1 \mu\text{m}$.

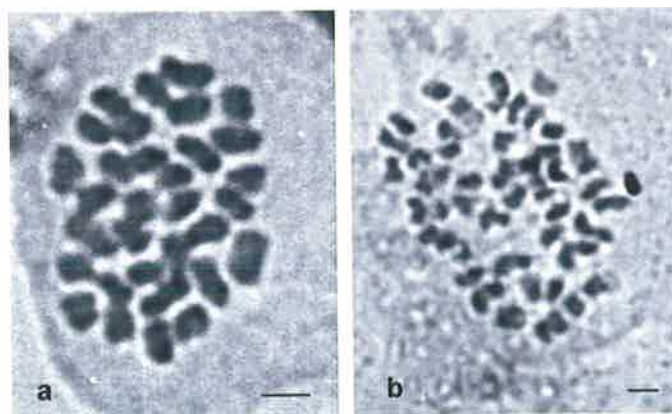
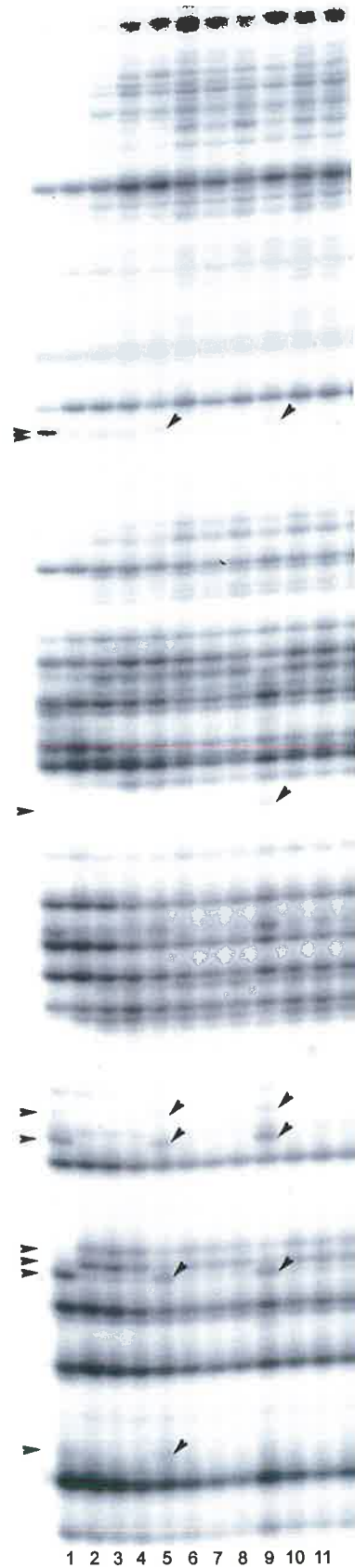


Figure 3.3. AFLP of *H. perforatum* genomic DNA amplified with primers PstI+AG and MseI+CAC, from nine progeny of cross 3 (lanes 3-11), with Cassilis 1 as donor parent (lane 1) and Scott Creek 2 as recipient (lane 2). Donor markers are indicated with arrowheads for two recombinants (lanes 5 and 9). One polymorphic recipient band with fast mobility is not shown.



3.5.2 AFLP analysis

Analysis of Cassilis and Scott Creek parent DNA with 19 primer combinations produced 16% polymorphic markers (87 and 76 bands dominant for each parent, respectively, from a total of 1020 scoreable bands), with an average of 8.6 polymorphic bands per primer set (Table 3.1). Primer set *Pst*I+AG-*Mse*I+CAC was chosen to screen the same 9 progeny from cross 3, plus 27 randomly selected additional seedlings, on the basis of the percentage of polymorphism exhibited and ease of band scoring. From this total of 36 progeny, recombination was indicated only in the same two progeny identified by RFLP. Each of the recombinants had five out of six possible donor bands, although actual AFLP bands differed in each case (Fig. 3.3). Two recombinant progeny from cross 4, from a total of 54 randomly screened with the *Pst*I+AG-*Mse*I+CAC primer combination, comprised a tetraploid and a hexaploid. A third variant progeny from cross 4 had a fingerprint missing four common bands and three polymorphic recipient bands, contained no bands from the donor, and was shown to belong to an aneuploid ($2n-1 = 31$). All non-recombinant progeny screened with AFLP were found to be tetraploid.

3.6 Discussion

Both AFLP and RFLP fingerprinting were able to discriminate between parental genotypes of *H. perforatum*, and were able to distinguish the same recombinant progeny within the subset of nine progeny screened with both techniques. These techniques offer the ability to rapidly identify genetic diversity in cultivars, collections and wild populations. For example, among 40 Australian weed populations, at least 18 distinct AFLP profiles could be distinguished (data not presented). Although M13 RFLP fingerprinting revealed good levels of polymorphism, the AFLP technique combined with miniprep DNA extraction proved more time efficient because of their shorter processing times and larger sample capacity. AFLP and cytological analysis offered an early, non-destructive progeny screen

to determine reproductive origin, and was particularly effective in differentiating between sexually and apomictically derived tetraploids with similar morphologies. Potential applications of the method include paternity analysis, estimation of genetic diversity, genome mapping and the identification of markers for traits of interest.

Results of AFLP and RFLP screens of four crosses indicated that seven progeny from a combined total of 121 had fingerprints differing from that of the maternal parent. The remaining 94.2% comprised tetraploid progeny with maternal fingerprint patterns, and are most likely to have arisen apomictically from unreduced pseudogamous embryo sacs. Of the seven recombinant progeny, cytological examination indicated three tetraploid, three hexaploid and one aneuploid. The tetraploid recombinants are presumed to have resulted from the fertilization of reduced egg sacs, thus it can be estimated that the level of normal reduced embryo sacs was 2.5%, very close to that reported by Noack (1939). The aneuploid might be explained by the occurrence of lagging chromosomes, which have been reported in this species (Hoar & Haertl, 1932), and probably arose via an aposporic embryo sac, given that its fingerprint contained no donor bands. Hexaploids arise from the fertilization of unreduced egg cells and, as expected, their DNA fingerprints exhibited all bands from the maternal parent and approximately half from the paternal parent. A much lower proportion of B_{III} hybrids were seen in the present study (2.5%) compared with cross-pollination studies by Noack (1939) and Brutovská *et al.* (1998). Timing of pollination has been suggested to influence the fate of aposporic embryo sacs in this species (Brutovská *et al.*, 1998). The earlier maturity of aposporic relative to meiotic egg sacs means that the former are likely to have passed the stage of receptivity by the time of pollen tube penetration, thus artificially earlier pollination produces a higher proportion of B_{III} hybrids, while delayed pollination may induce the parthenogenetic production of haploids (Savidan, 2000). Accordingly, variation in levels of B_{III} hybrids among studies

may be an artefact of differing methodology. Additionally, factors such as the choice of male parent, photoperiod, temperature, plant age, nutrition, competition, and chemical exposure are thought to affect the balance between apomixis and sexuality in some facultative apomicts (Grimanelli *et al.*, 2001; Mordhorst *et al.*, 1997). More research is needed to assess the contribution of environmental and genetic factors to the range of variation in mode of reproductive observed by Matzk *et al.* (2001).

The influence of reproductive system on population genetic structure and consequent effects on the success of weed control programs has been emphasised (Burdon & Marshall, 1981). While population structure cannot be directly deduced from knowledge of mode of reproduction, a reasonable assumption is that a high degree of apomixis will result in low within-population variation. It has been proposed (Burdon & Marshall, 1981) that biological control agents are more effective against asexually reproducing weeds because of their tendency towards genetic homogeneity, which may be compounded by genetic “bottlenecks” where introductions are limited to a few individuals. While spectacular success has been demonstrated in control of some clonal and apomictic weeds, partial success is too often the case with the latter. The problems encountered with facultative apomicts are well illustrated by the differential control of genetically distinct forms of *Rubus fruticosus* L. aggregate (blackberry) by the rust fungus *Phragmidium violaceum* (Evans *et al.*, 1998), and of three forms of *Chondrilla juncea* L. (skeleton weed) by the agent *Puccinia chondrillina* (Cullen & Delfosse, 1990).

The lack of within-population variation in field-collected seed from two Australian *H. perforatum* accessions is not unexpected given the high degree of apomixis at these sites and that populations have probably arisen from only a few founders, but it must be stated that neither of these field sites was sampled with a population study in mind. However, at two other Australian field sites not included in the present study, transect sampling

suggested genetic uniformity in populations sharing the AFLP fingerprint of the Cassilis parent. Lack of intra-population variation contrasted with significant diversity in fingerprints among 41 Australian field sites (data not presented). Inter-population variation may reflect multiple introductions of *H. perforatum* to Australia (Harris & Gill, 1997), but as this study demonstrated, it may also be expected as a result of occasional sexual recombination, particularly given the proposed hybrid origin of *H. perforatum* (Mártonfi *et al.*, 1996) and high heterozygosity that is characteristic of apomicts (Nogler, 1984). During this study, significant inter-population variation was also seen in hypericin level (data not presented), which may influence grazing management strategies for sheep and other animals susceptible to photosensitization (Southwell & Bourke, 2001). It is proposed from the current results that plant characteristics such as hypericin level or response to herbicides are likely to be relatively uniform within a given population, but that variation may be experienced among populations. The low level of sexuality suggests that such traits would not be expected to spread rapidly among populations. However, natural reproductive behaviour and the level of gene flow in the field require further research.

It is concluded that while AFLP was more time-efficient than RFLP, both fingerprinting methods, in combination with cytological techniques, constitute useful tools for plant breeding and population studies, identification, paternity analysis and estimation of genetic variation and thus have great potential with respect to production or control of *H. perforatum*.

4 Genetic variation in *Hypericum perforatum*

4.1 Introduction

Hypericum perforatum has been introduced into Australia a number of times (Harris & Gill, 1997), and significant variation has been demonstrated in morphology and hypericin content among seven populations of the weed (Campbell *et al.*, 1992). Variation in susceptibility to the mite *Aculus hyperici* was demonstrated among four of the forms characterized by Campbell *et al.*, and strong evidence was presented for the influence of variable host plant susceptibility on establishment success of the mite at two sites in northern New South Wales (Jupp *et al.*, 1997). Of major concern was that variation in susceptibility to *A. hyperici* might reduce the impact of this highly promising biological control agent (Jupp *et al.*, 1997). Morphological identification of variation based on leaf form is problematic. However, due to a considerable level of overlap in leaf-size between forms (Jupp & Cullen, 1996) and the effect of seasonal variability and environmental effects (Campbell *et al.*, 1997), hence more accurate methods for evaluating the level and distribution of variation in Australian populations were considered essential to this study. As discussed in the previous chapter, the reproductive biology of *H. perforatum* was expected to significantly influence spatial and temporal variation in the target host.

The following review covers variation in facultative apomicts and agamic species complexes, variation in *H. perforatum*, and choice of marker system. Two multilocus methods, RFLP and AFLP, were compared in investigations of intra- and inter-population variation and genetic relationships, and variation and distribution patterns were considered in relation to biological control. Ploidy level was examined, and discussed with respect to the use of multilocus methods, taxonomic issues, and the evolution of the *H. perforatum* polyploid complex.

4.2 Review

4.2.1 Agamic species complexes

Stebbins (1950) considered *H. perforatum* to consist of a relatively small and simple agamic complex. Agamic complexes include distinct strains connected by intermediary hybrids that reproduce partially or wholly by apomixis (Asker & Jerling, 1992; Nogler, 1984). Such complexes arise by hybridization and polyploidy, and contain an abundance of polymorphic forms which may be sexual (usually diploid) or facultatively apomictic (usually polyploid). Although diploids are almost always sexual, very low levels of apospory (unreduced egg sacs from somatic cells) have been demonstrated (Quarin *et al.*, 2001), and rare diploid apomicts may in fact be segmental or paleopolyploids (diploidized polyploidy with chromatin from multiple genomes) (Carman, 1997; Grimanelli *et al.*, 2001). Tetraploid is the commonest level in apomicts but other levels such as triploid, pentaploid, and hexaploid may also exist (Asker & Jerling, 1992; Nogler, 1984). Apomictic polyploid complexes are common, and typically include distinct strains connected by intermediate hybrids with various levels of ploidy, some of which may build up large distributions of one homogeneous cytotype, as in sexual diploid and apomictic tetraploid representatives of *Paspalum* species (Quarin *et al.*, 2001). Sexual species in polyploid complexes are usually geographically and genetically isolated from one another (de Wet, 1968; Nogler, 1984).

Variation in base levels of ploidy in agamic species complexes may occur via “diploid-tetraploid-dihaploid” cycles, with the production (depending on the ploidy of the maternal parent) of reduced and unreduced egg cells, the fate of which will be determined by fertilization and pollen source. Such events may occur not only among the same or closely related individuals via selfing or crossing, but also by hybridization among more distantly related individuals within and between taxa, and may result in further polyploidization.

Detailed reviews of the terminology, mechanisms and evolutionary significance of such processes can be found in Nogler (1984) and Asker and Jerling (1992).

The taxonomy of genera that include hybrids, polyploids and apomicts is fraught with difficulties (Asker & Jerling, 1992). Even the species concept itself is a topic of controversy, and has provoked an enormous amount of disparate opinion and a plethora of new concepts and publications, particularly in respect of higher plants, whose genetic systems and reproductive modes preclude a universal theory (Claridge *et al.*, 1997; Coyne, 1992; Rieseberg & Burke, 2001; Templeton, 1989). The taxonomic concept of species, where groups are recognized by overall similarity, is preferred over the biological species concept by botanists (Gornall, 1997). However, the taxonomic species concept is subjective, and among other difficulties does not cope well with facultative apomixis, where the production of new genotypes can lead to apomictic lineages of common origin that are morphologically differentiated slightly from one another (variously termed “agamospecies” or “microspecies”), and may inhabit the same area but be reproductively isolated (Gornall, 1997). This, and the lack of equivalence of sexual and asexual species, causes problems in defining taxa (Hörandl, 1998). Agamic complexes are therefore regarded as a task for specialists, who tend to work on their own particular group, but as a consequence, even between agamic complexes in different genera different approaches to classifying species have been utilized. According to Weber (1996), taxonomists dealing with apomictic groups such as the *Rubus* complex can only solve these difficulties by pragmatism. In the case of *H. perforatum*, infraspecific variation and hybridization probably render subspecific status useless (Campbell *et al.*, 1997; Mártonfi *et al.*, 1996).

4.2.2 Evolutionary significance of apomixis

Darlington (1958) stated that “with the loss of sexual recombination the apomict, like the permanent hybrid, is cut off from ultimate survival. Apomixis is an escape from sterility, but it is an escape into a blind alley of evolution”. This view has been radically revised on several counts since then. Firstly, apomicts are rarely, if ever, obligate, therefore variation generated by sexual reproduction is accessible to most apomicts. Facultative apomixis does not prevent variation, but multiplies certain products. Secondly, sources of genetic variation exist, other than those generated by sexual reproduction. Thirdly, the quality and quantity of genetic variation needed in order to survive in different environments is not known. Several authors (Grant, 1981; Marshall & Weir, 1979) support the view that *obligate* apomixis spells extinction in the long run, but the former showed that if an apomictic species retains any capacity at all to self- or out-cross, it is possible to maintain variation and such forms do not necessarily represent evolutionary dead-ends. Facultative apomixis therefore combines the advantages of occasional sexuality with clonal perpetuation of successful gene combinations, together with seeds for long distance dispersal and the possibility of colonizing new areas with a single individual.

4.2.3 Variation in apomicts

Causes of polymorphy in agamic complexes result from several ongoing processes; first, hybridization and allopolyploidy between the original sexual ancestors, second, self-pollination or hybridization between facultative apomicts with resulting segregation, and third, chromosomal and genic changes within clones themselves (Stebbins, 1950).

4.2.3.1 Hybridization, polyploidy, and heterozygosity

High heterozygosity appears to be characteristic of most apomicts, very likely because apomixis is inextricably linked to hybridization and polyploidy, and related sexuals are often cross-fertilizers and polymorphic (Asker & Jerling, 1992; Nogler, 1984; Pamilo,

1987). Hybridization among closely or distantly related forms may result in large increases in heterozygosity that can be fixed by apomictic reproduction, and consequently apomixis is of great interest in agricultural production (Savidan, 2000; Vielle-Calzada *et al.*, 2000).

4.2.3.2 *Reproductive biology and population variation*

A surprising amount of variation has sometimes been detected within apomictic species (Asker & Jerling, 1992). Despite the extreme heterozygosity characteristic of apomicts, a high degree of apomixis is expected to result in genetically uniform populations by comparison with outcrossing species, where frequency differences account for most of the genetic divergence (Morell *et al.*, 1995; Nogler, 1984). However, even in highly apomictic plants, there is still the possibility of a level of sexuality, which together with high heterozygosity creates the potential for gene recombination and variability (Nogler, 1984). Facultative apomicts may produce sexual seed by strict out-crossing, obligate self-pollination or a combination of both (Nybom, 1993), in the same manner as sexually reproducing species. Breeding system greatly influences heterozygosity; obligate outcrossing increases heterozygosity and results in individual-specific genotypes in the offspring, while repeated selfing increases homozygosity, with the result that selfing species show considerably less intra-population variation than their outcrossing counterparts (Nybom, 1993). The relative frequencies of selfing and outcrossing largely determine the level of heterozygosity of a population. In obligate sexual species practicing only outcrossing, Hardy-Weinberg equilibrium will be reached in one generation, while selfing species will be composed entirely of homozygotes at equilibrium. Mixed selfing and outcrossing populations attain an equilibrium level of heterozygosity that is determined by the relative magnitude of selfing and outcrossing (Marshall & Weir, 1979). The same authors showed that while apomixis slows the rate of approach to equilibrium in all three cases, it does not alter equilibrium values. This is not unexpected given a lower

frequency of sexual recombination events in facultative apomicts than in obligate sexuals (Asker & Jerling, 1992).

Complex population structures in facultative apomicts develop as a result of degree of apomixis and sexual breeding system (Nybom, 1993). Resulting hybrid forms may maintain heterosis and expand through apomictic or vegetative reproduction (Nogler, 1984), and can be extremely long-lived as long as the habitat remains suitable (Nybom, 1993). Therefore, apomictic species may contain genotypes that are thousands of years old occurring side-by-side with those more recently evolved (Nybom, 1993). Any study of population structure in an apomictic species benefits greatly if clones are first identified and their spatial distribution analyzed (Nybom, 1993).

4.2.3.3 *Intra-individual variation in apomicts*

Intra-individual or somaclonal variation may arise in the heterozygous state by point mutations, chromosomal rearrangements (duplications, inversions and deletions), somatic crossing-over, autosegregation, amplification or loss of DNA, mobile genetic elements and viral infections (Asker & Jerling, 1992). Where any of these occur in a meristem, mutations may be passed on to modules within an individual, including those taking part in reproduction. Functional heterozygosity due to mutation is expected to increase with time in apomicts, since in the absence of recombination the affected loci remain heterozygous and the number of heterozygous loci per individual increases (Pamilo, 1987). Recessive mutations, even those that are lethal in the homozygous state, are likely to accumulate in apomicts (Asker & Jerling, 1992). Apomicts, with few exceptions, are polyploids, and this seems to have conferred advantages in the accumulation of variation; mutation rates in apomicts are frequently higher compared with related sexuals (Asker & Jerling, 1992), and it has been proposed that polyploidy increases the mutation rate per individual (Manning & Dickson, 1986). Polyploids also appear to be quite tolerant of such single chromosome

deviations, which may occur in somatic cells or gametes as a result of disturbances in mitosis and meiosis (Asker & Jerling, 1992).

4.2.4 Hybrid origin of *H. perforatum*

H. perforatum displays apomixis, polyploidy, and occasionally sterile pollen grains, all of which indicate a hybrid origin (Noack, 1939; Robson, 1981). On morphological and geographic grounds *H. perforatum* is most likely an allopolyploid resulting from hybridization between diploid *H. maculatum* Crantz subsp. *maculatum* ($2n=16$) and diploid *H. attenuatum* Choisy ($2n=16$) followed by chromosome doubling (Robson, 1981).

4.2.5 Ploidy in *H. perforatum*

Although tetraploidy is the most common level (Robson, 1981), euploid and aneuploid variation (exact multiples of the basic haploid chromosome set, and deviations from those, respectively) is not uncommon in this species and may arise within reproductive (Chapter 3) or somatic tissue (Brutovská *et al.*, 1998).

Hoar and Haertl (1932) and Robson (1981) agreed that chromosomes were difficult to count satisfactorily in the genus owing to excessive clumping and their small size. Chromosomes in *H. perforatum* are the smallest in the genus, and have been estimated to range in size from 0.78 to 1.52 μm (Brutovská *et al.*, 2000), and 1 to 1.7 μm (Reynaud, 1986). A comprehensive karyological study by Brutovská *et al.* (2000) showed that the diploid set contains one pair of long median centromeric, nine median centromeric and six submedian chromosomes.

4.2.6 Variation in *H. perforatum*

In its home range in Europe, a number of forms, varieties and subspecies of *H. perforatum* have been recognized, based on width of leaves and sepals, length of capsules, and by the presence or absence of black glands on petals (Mártonfi *et al.*, 1996). However, there

appears to be no morphological discontinuity between wide-leaved forms (var. *perforatum*) in the north and the United Kingdom, and narrow-leaved or small leaved forms (vars *angustifolium* and *microphyllum* de Canulle, respectively) in the south (Robson, 1968). Significant overlap of different forms may result from environmental effects, which can lead to difficulty with identifications. For example, broad-leaved specimens from dry areas in the north of Britain have been misidentified as var. *angustifolium*, in which the leaves are narrower, and the leaf margins roll under towards the midrib.

In the past, Australian material has been recognized as var. *angustifolium* de Canulle, and in New South Wales three morphotypes, “broad”, “narrow” and “intermediate”, were recognized on the basis of leaf length and width, plant height, flowering time, stem diameter, capsule size, pellucid gland density, and hypericin content (Campbell, 1987; Campbell *et al.*, 1989; Campbell *et al.*, 1992). Three of the narrow-leaved accessions also varied in stem, bud and/or sepal colour. However, morphotypes were difficult to distinguish in the field unless the flowering stems were growing vigorously, and the conclusion was drawn that all forms in New South Wales would be better considered as belonging to the same variable taxon, *H. perforatum* (Campbell *et al.*, 1997). Investigation of the potential biological control agent *Colletotrichum gloeosporioides* identified variation in susceptibility in *H. perforatum* to this anthracnose fungus among the three characterized morphotypes identified by Campbell *et al.*, as well as seven other Australian accessions and three Canadian ‘ecotypes’ (Shepherd, 1995). Susceptibility to the mite *Aculus hyperici* was extremely low in the intermediate morphotype compared to the broad and narrow morphotypes characterized by Campbell *et al.*, and low susceptibility was demonstrated in seedlings from two sites at which the mite failed to establish, compared to seedlings from four sites with medium and high susceptibilities corresponding to successful establishment (Jupp *et al.*, 1997). However, considerable overlap in leaf-size

between different forms prevented identification of morphotypes (Jupp & Cullen, 1996), and improved methods for the identification of genetic variants were considered critical to the success of future studies (Jupp *et al.*, 1997).

Variation in the weed in Australia is consistent with documented evidence of multiple introductions from the home range over the last century (Harris & Gill, 1997). A reasonable assumption, based on the investigation of reproductive biology (Chapter 3), was that a high degree of apomixis in the facultative apomict *H. perforatum* would result in low intra-population variation; however, the possibility existed that a low level of recombination since introduction may also have occurred.

4.2.7 Marker system

Biometrical analyses of morphological traits (e.g. leaf, flowers and fruit characters, pathogen susceptibility), secondary compounds and isozymes have traditionally been used to estimate genetic variation in population studies, and for taxonomic or cultivar identification (Asker & Jerling, 1992; Hughes & Richards, 1988). However, molecular methods offer a number of advantages over other methods for demonstrating distinctness, because DNA sequence is independent of environmental conditions or management practices, and almost any plant tissue can be used provided that DNA of sufficient purity can be isolated (Morell *et al.*, 1995). At the inception of this project, no marker systems or DNA extraction methods had been published for *H. perforatum*.

It is important to choose an appropriate genetic marker system for the questions being asked, on the basis of the resolution (how much information is provided by the marker) and attributes of the system (Morell *et al.*, 1995; Sunnucks, 2000). An appropriate methodology was required that with a high level of certainty would allow identification of individual genotypes of *H. perforatum* in the field. A higher level of inter- compared to

intra-population variation was expected in Australian populations on the basis that variation in morphology and susceptibility had been demonstrated among populations (Section 4.2.6), within which a high degree of apomixis was likely (Chapter 3). Whilst an estimation of intra-population variation was desirable, there was not sufficient reason to target the marker system specifically at the level of fine evolutionary processes within populations.

The most sensitive genetic signals are genotypic arrays, which reshuffle at each generation in sexual species, and are useful at the level of individual identification, parentage and relatedness. Such arrays can be recognizable for longer in organisms without frequent genetic recombination (Sunnucks, 2000). Multilocus markers were considered because they are relatively easy to develop in a new organism, simultaneous visualization of a large number of loci is technically convenient, and they tend to have high overall variability (variation per marker). There are, however, some disadvantages with such markers. A fundamental limitation is dominant inheritance, which means that there are only two states for a polymorphism, present or absent, and heterozygotes are rarely detected (Morell *et al.*, 1995; Sunnucks, 2000).

The sensitivity of two multilocus techniques was considered. Restriction fragment length polymorphism (RFLP) with genomic DNA restricted with *Hae*III and probed with M13 bacteriophage DNA (Evans *et al.*, 1998) was examined, because this method had demonstrated appropriate resolution in past spatial distribution studies on clonal plants (Nybom, 1993). Subsequently, a decision was made on pragmatic grounds to transfer to amplified fragment length polymorphism (AFLP) markers (see discussion). Other studies with AFLP markers (Becker *et al.*, 1995; Powell *et al.*, 1997; Reineke *et al.*, 1998) indicated that extensive genome coverage by AFLP primers offered the greatest chance of

detecting small genetic differences, since a larger component of the genome could be scanned than in other systems.

4.3 Aims

The primary aims were to identify genetic variants and to assess the degree of genetic variation among populations of *H. perforatum* across the range of the weed in southern Australia. Within-population variation was evaluated for a small number of samples at most sites, and in more detail from transects and quadrats, at four sites. Multilocus RFLP and AFLP techniques were developed for this purpose. Individuals were examined from populations located in five Australian States and two overseas accessions, and the genotypes discovered were analyzed for similarity. Variation in ploidy was also investigated among populations, including several individuals with unusual leaf morphology. A geographic distribution of genotypes was compiled and examined with respect to hypotheses of multiple introduction and spread. Genotype distributions were also applied to investigations of the extent of resistance to *A. hyperici* (Chapter 5), and of hypericin level variation (Chapter 6).

4.4 Materials and methods

4.4.1 Accessions

Accessions of *Hypericum perforatum* seed were obtained from five Australian states and two additional overseas localities. Populations and individuals included in this study are given with corresponding collection methods in Table 4.1 (page 27), and details of collection locations, co-ordinates, dates, and collectors are summarized in Appendix A. Seed was stored in paper bags or in plastic microfuge tubes, in the dark at room temperature. Seed collection was made either by bulking from multiple plants, by collection of separate seed lots from individuals randomly distributed throughout the

population, by collection of separate seed lots from individuals approximately 10m apart along a transect through the population, or by collection of separate seed lots from individuals defined by quadrat co-ordinates. Fresh leaf collected from the field was the only material available for three accessions.

Each accession was given a four-letter identifier reflecting its origin, and separate individuals within accessions were given additional numerical and/or alphabetical suffixes that reflected collection method. Numerical suffixes indicate seed or fresh material from separate individuals by random or transect collection (for example, Burr 1, Burr 2), and alphabetical suffixes indicate seedlings within a single seed lot; for example, bulked seed (Cass a, Cass b) or seed from one individual (Aven 1a, 1b, etc). Exceptions included the quadrat samples for which the collector supplied co-ordinates, and four bulk seed-derived Cass and ScCk individuals used in crosses (Cass x1 and ScCk x1, x2, x3).

Consultation with collectors revealed the fact that several accessions had been obtained from the same field site, consequently these are considered to have come from the same population (Table 4.1). Three pairs of collections made from the same site but in a different year include the Coolah field site (CooJ and Cool), Mudgee Common (MuCo and MudJ), and Bonganditj Native Forest Reserve (SESA and Bong). Two pairs of collections made from the same site at the same time, but collected separately on the grounds of morphological dissimilarity, included Turon River (TurB and TurN), and Mudgee (MuSh and MuTa), and at Castlemaine, seed from a particularly tall plant (CasT) was collected from the same site but in a different year to seed previously collected at that site (Cast).

4.4.2 Plant culture

Seed was germinated as described in Chapter 2. Seedlings were transferred into trays until large enough to plant into pots. Seed-derived individuals were maintained in 4" pots over

the duration of the project, by division of the crown and repotting at 6-monthly intervals. Plants were grown in a general purpose potting mix, and maintained in an unheated glasshouse (<30°C) with Osmocote® (Scott Australia) fertilizer applied at three monthly intervals. The fungicide Benlate® (DuPont) at 1g/L applied as a dip to control *Botrytis* sp., also controlled the mite species *Tetranychus urticae* (two-spotted mite) and *Polyphagotarsonemus latus*, most probably by acting as an ovicide and inducing sterility in adult females (Roush & Plapp, 1982). Ambush (Crop Care Australasia Pty Ltd) was similarly applied to control black fly larvae (Ephydriidae), whitefly (*Trialeurodes vaporariorum*) and St John's wort aphid (*Aphis chloris*). Plant material for DNA analysis was collected from actively growing shoot tips, free of pests and pesticide residue.

4.4.3 DNA isolation

The final extraction method, modified from Hodgson (1998), was relatively low cost, produced very clean DNA suitable for both AFLP and RFLP, and appears in detail in Chapter 3. No isolation methods had been published for *H. perforatum*, and a considerable amount of time was expended on the development of a technique for extraction of DNA of sufficient purity. Details of investigations will not be discussed here, except for the following brief notes. Traditional CTAB-based DNA extraction methods, for example Doyle and Doyle (1990) resulted in sufficient contamination with carbohydrate and/or protein in the final pellet to affect resolution of RFLP bands and PCR-based fingerprint reliability, and commercial extraction methods were both costly and inclined to shear DNA. Unpredictable and often insufficient yields were finally attributed to low pH of the initial slurry from leaves, and the pH of the buffer was adjusted accordingly; slurry pH > 6.5 prevented degradation of DNA, and required a buffer with pH \geq 9.

4.4.4 RFLP

RFLP profiles were generated by hybridization of 3 µg of digested total genomic DNA, to α -³²P-dCTP random-labeled M13 bacteriophage probe (Evans *et al.*, 1998) as follows. Restriction enzyme digests were fractionated for 42 hours unless otherwise specified, by electrophoresis in 1% agarose using TAE, with two buffer replenishments. Marker DNA (*Hind*III-digested λ) was run in outside lanes, and DNA from a standard *H. perforatum* sample (see below) was run in two or three widely separated lanes of each 20-well gel. Fractionated DNA was transferred to Hybond-N (Amersham) nylon membrane according to the method of Southern (1975) and cross-linked to the membrane using UV light (GS Gene Linker, Bio-Rad). Hybridization with the bacteriophage M13 DNA probe (M13 mp18, Biolabs, New England) followed the two-step hybridization procedure of Weihe *et al.* (1990). Briefly, this involved an initial hybridization step with unlabeled single-stranded M13 DNA at 20 ng/ml, followed by double-stranded M13 DNA random-labeled with α -³²P-dCTP (Ready-To-Go™ DNA labeling beads, Pharmacia Biotech), separated from unincorporated nucleotides (Microspin™ column, Pharmacia Biotech), then denatured and hybridized at 10 ng/ml to target DNA at 60°C overnight. Membranes were washed with 2x SSC + 0.1% SDS, at 63°C and exposed to X-ray film (X-Omat AR, Kodak) for <4 hours (“short exposure”) or >48 hours (“long exposure”) at -80°C.

*Hae*III, *Hin*fI and *Eco*RI were investigated separately for usefulness in generating polymorphisms. *Hae*III produced clear bands ranging from 2-10 kbp, and was chosen for subsequent RFLP analysis. *Hin*fI and *Eco*RI produced fragments too short or long for easy analysis (<3 kbp in the former, and a smear from 10-23 kbp in the latter).

A standard was chosen which contained an even spread of many common bands (Cass x1), for which relative band positions and band intensity for identical and repeat DNA extractions were verified on the same and separate gels. A number was assigned to each

standard band, and to additional bands present in other fingerprints. Two master gels representing unique fingerprint patterns were made, which incorporated standard Cass x1 samples in left-hand, right-hand and center lanes of each gel. Where any ambiguity existed, samples on the master gels were compared side-by-side on additional gels.

RFLP profiles were scored manually, directly from the autoradiographs, by assigning a value of 1 for band presence and 0 for band absence. In the few samples where very high intensity bands occurred, early exposures were used to score bands in close proximity on either side, or in the event that bands were completely obscured they were recorded as missing data. Very faint or smeary bands were not scored.

The relative front (RF) was used to generate graphs of RFLP profiles, and was determined as the ratio of the distance from the origin to the band of interest, to the distance from the origin to the shortest scored band, expressed as a percentage. Discrepancies were experienced in RF calculated among some gels, even for identical run times, so for graphing purposes RF values from one master gel with the majority of bands represented were used, and positions of the remaining four bands were approximated.

Scores of band presence or absence were used to calculate a pairwise genetic distance matrix using the formula of Nei (1978). A dendrogram based on unweighted pair-group method using an arithmetic average (UPGMA) cluster analysis of the genetic distance matrix was prepared to investigate genetic relationships (Morell *et al.*, 1995).

4.4.5 AFLP

AFLP profiles were generated as described in detail in Chapter 3. The primer combination chosen was *Pst*IAG/*Mse*ICAC, on the basis of the percentage of polymorphism exhibited, ease of band scoring, and a preliminary screen of several accessions. Transect samples were additionally screened with *Pst*IAG/*Mse*ICAG. Controls without DNA template were

made for restriction-ligation, pre-amplification PCR, and radiolabeled PCR steps. Cassilis x1 was chosen as a standard and loaded at least four times, evenly spaced, across each gel. A commercial size-standard marker (kb DNA ladder #201115, Stratagene) was included next to the Cassilis standard on two gels. Profiles were scored manually, directly from the autoradiographs, by assigning a value of 1 for band presence and 0 for band absence. Pairwise genetic distance matrices and dendrograms were constructed as for RFLP profiles (Section 4.4.4).

4.4.6 Cytology

The cytological method, modified from Thompson (1995), appears in detail in Chapter 3. Shoot meristems were available throughout the season, produced superior preparations, and were technically simpler than roots or archesporial cells. For each individual analyzed, at least two unambiguous chromosome counts were made for each of two shoot tips. A Zeiss Photomicroscope II with a 63 \times oil-immersion objective was used to examine and photograph prepared slides.

4.5 Results

4.5.1 RFLP

4.5.1.1 *HaeIII/M13 profiles and population variation*

RFLP profiles from *HaeIII*-digested DNA probed with M13 were scored between 9.4 kbp and approximately 3 kbp. The 42 individuals analyzed were a subset of the 295 analyzed with AFLP (indicated with an asterisk in Table 4.1, page 27). Fourteen RFLP profiles were distinguished, and assigned numbers prefixed with an "R". Examples of an autoradiograph containing 11 profiles (Fig. 4.1), and a schematic representation of all 14 profiles, compiled from band values for relative front (Fig. 4.2), are presented. All 44 scorable RFLP bands were informative (i.e. polymorphic) among profiles.

Twenty-nine geographically separated populations were represented by the 42 individuals analyzed (Table 4.1). In only three cases was more than one profile found within a population. At two sites in NSW, at Mudgee, and Turon River near Sofala, a mixture of genotypes were already thought to be present on the basis of morphological dissimilarity, and separate bulked collections of morphotypes had been made. Heterogeneous populations were confirmed at both sites. The Mudgee site included short (MuSh) and tall (MuTa) forms previously characterized by Campbell *et al.* (1992) on the basis of biometric analysis; two individuals of the short form had an R1 profile, and one tall form individual displayed an R4 profile. The Turon River site contained broad (TurB) and narrow leaved (TurN) morphotypes (Paul Lutschini, pers. comm.); one broad-leaved individual had an R10 profile, and a narrow-leaved individual displayed an R1 profile. A third heterogeneous population near the border of South Australia and Victoria, for which there was no a-priori reason to expect variation, was found in Bonganditj Native Forest Reserve (SESA and Bong accessions); one SESA individual was R5, and one Bong individual was R7. Of the remaining populations where more than one individual was sampled, individuals within populations shared RFLP profiles (see asterisks, Table 4.1). It is worth noting that because this was an initial screen, within-population samples sizes were very small ($N = 1$ to 3). Intra-population variation was investigated further with AFLP techniques.

One case of RFLP profile variation among populations is particularly noteworthy. When Flow 2b was examined with RFLP, its R14 profile could be distinguished from all other R5 individuals by the presence of a faint band at approximately 10 kbp (compare Alex 4b and Flow 2b, Fig. 4.1). However, on the basis of AFLP, Flow 2b could not be differentiated from other Flow individuals or other A5 samples (Section 4.5.2.1).

Figure 4.1. Southern hybridization of M13mp18RF DNA to *Hae*III-digested *H. perforatum* DNA. RFLP profile numbers are followed by the identifier of each sample in brackets. *Cass x1* was used as a standard.

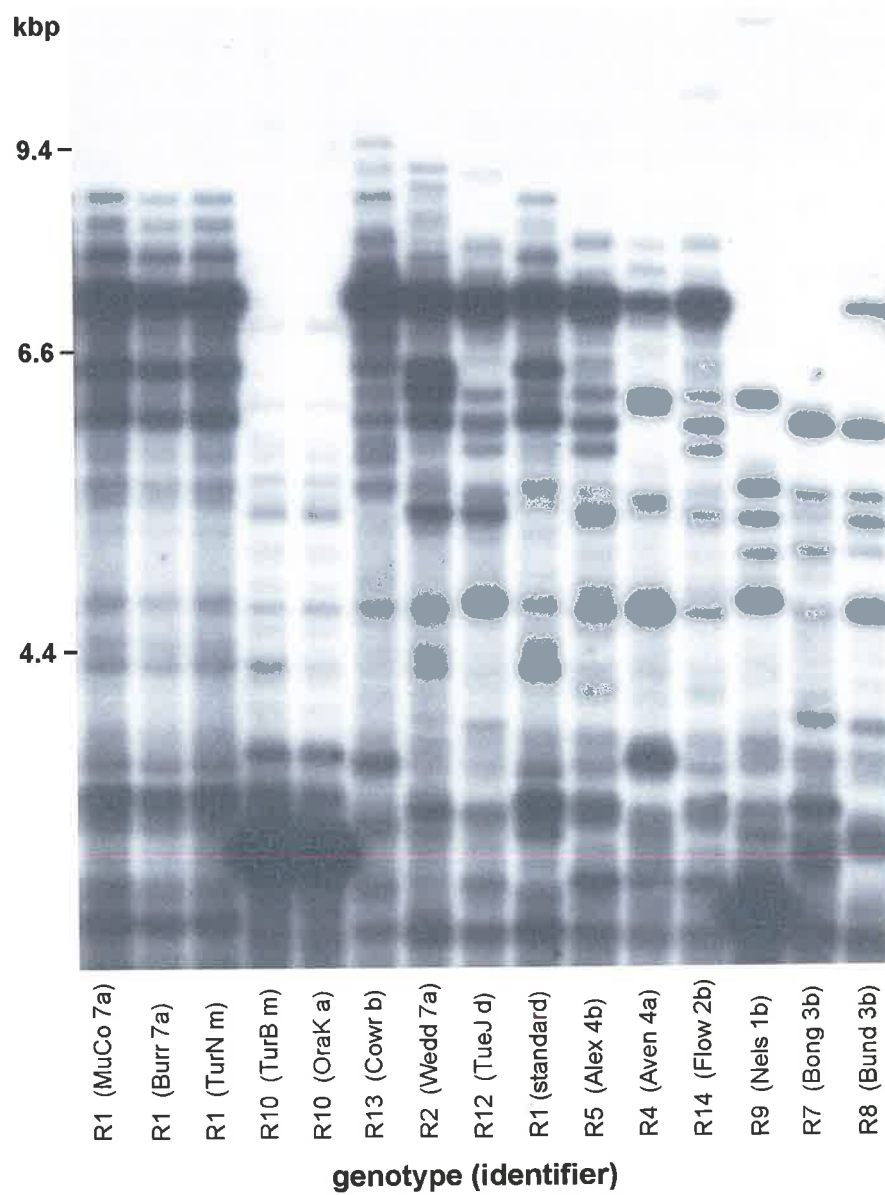
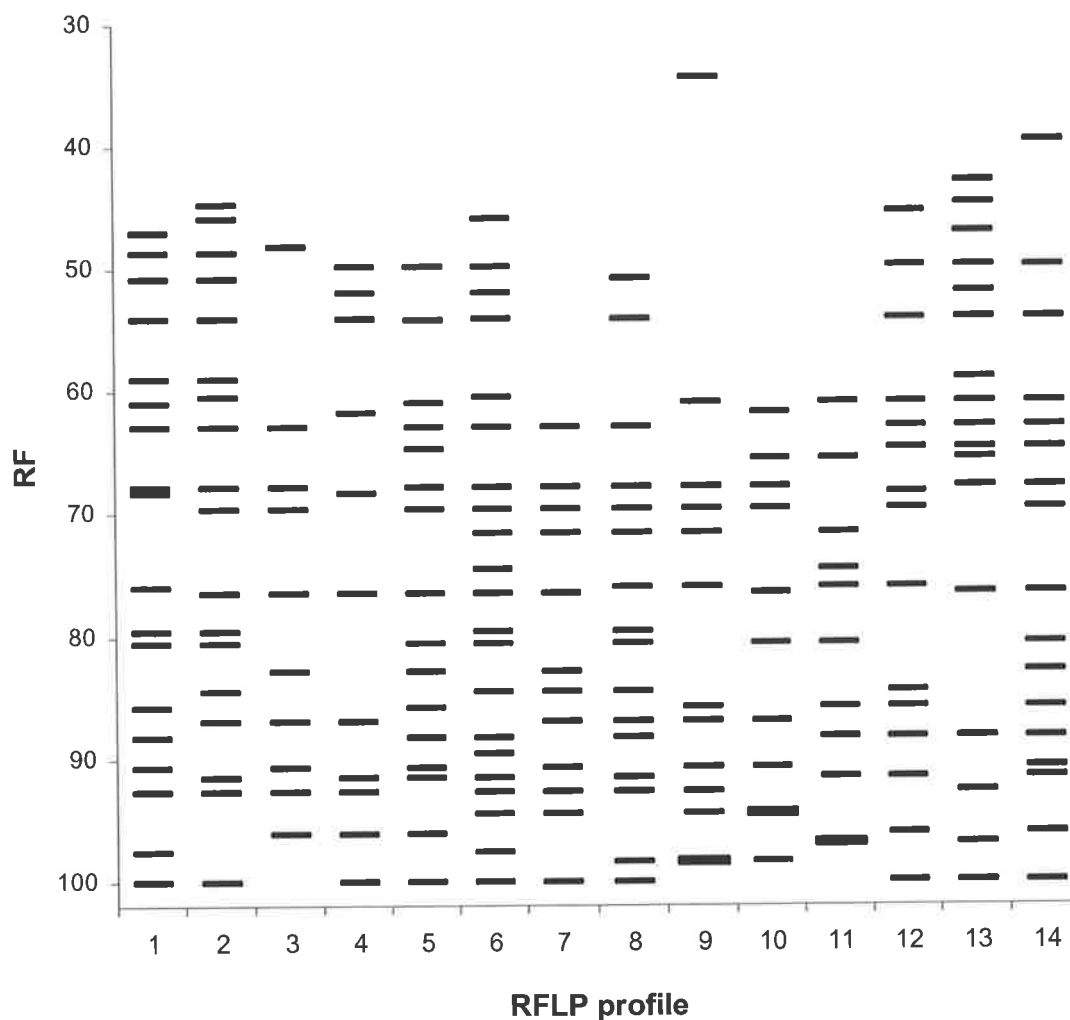


Figure 4.2. Schematic representation of 14 RFLP fingerprint profiles for *H. perforatum* digested with *HaeIII* and probed with *M13*, generated from RF values as described in Section 4.4.4. Bold lines indicate very high intensity bands.



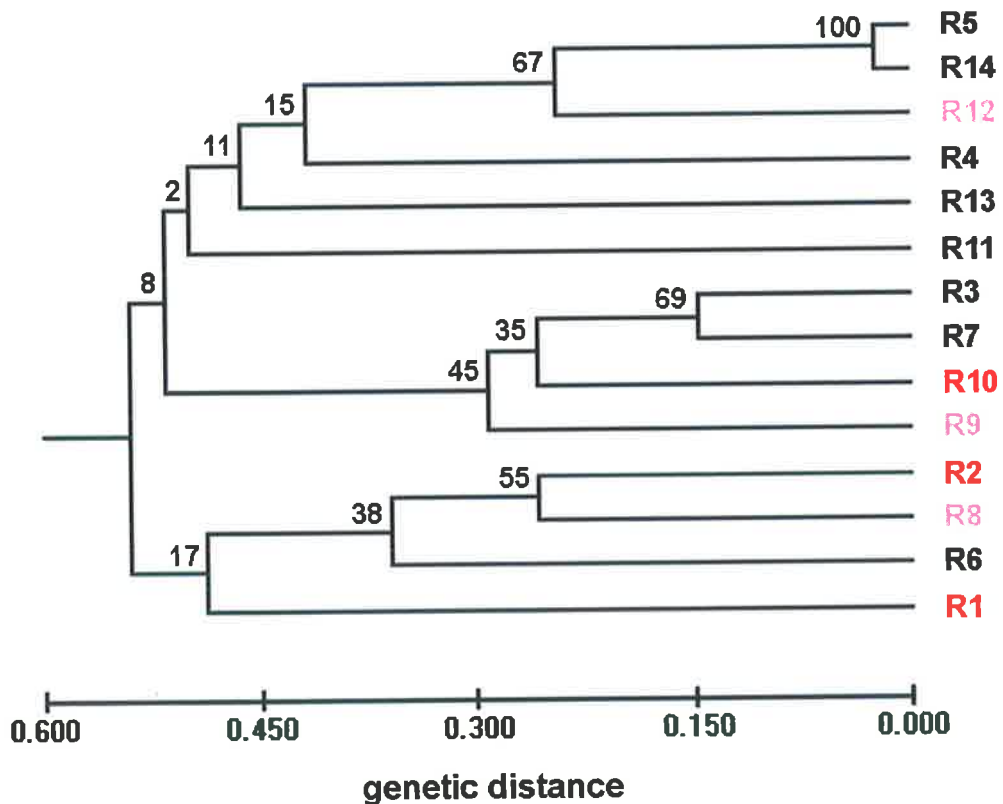
4.5.1.2 Genetic relationship based on RFLP

A pairwise genetic distance matrix using Nei's unbiased (1978) distance (Fig. 4.3) was constructed from the 14 RFLP profiles presented in Section 4.5.1.1, using Tools For Population Genetic Analysis (TFPGA) software (Miller, 1997). UPGMA analysis revealed three tied trees, and 81% of bootstrap replicates produced trees containing ties. A dendrogram based on UPGMA cluster analysis of the genetic distance matrix (Fig. 4.4) is presented for comparison with the corresponding 13 AFLP profiles (Section 4.5.2.4).

Figure 4.3. Genetic distance matrix for 14 RFLP profiles, based on Nei's (1978) unbiased distance.

RFLP Profile	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	0.000													
2	0.452	0.000												
3	0.606	0.452	0.000											
4	0.649	0.488	0.350	0.000										
5	0.452	0.526	0.319	0.417	0.000									
6	0.626	0.360	0.670	0.542	0.465	0.000								
7	0.606	0.383	0.147	0.417	0.383	0.429	0.000							
8	0.383	0.258	0.452	0.488	0.452	0.360	0.319	0.000						
9	0.495	0.718	0.346	0.578	0.536	0.693	0.248	0.381	0.000					
10	0.693	0.526	0.258	0.417	0.452	0.670	0.258	0.452	0.280	0.000				
11	0.519	0.847	0.602	0.560	0.442	0.623	0.647	0.480	0.393	0.480	0.000			
12	0.488	0.649	0.565	0.383	0.229	0.583	0.565	0.417	0.578	0.740	0.406	0.000		
13	0.488	0.565	0.565	0.452	0.417	0.583	0.565	0.649	0.718	0.649	0.602	0.526	0.000	
14	0.488	0.565	0.350	0.452	0.023	0.503	0.417	0.488	0.578	0.488	0.480	0.258	0.452	0.000

Figure 4.4. Dendrogram constructed from the distance matrix presented in Fig. 4.3. The proportion of similar replicates obtained from bootstrapping is given above nodes. Genotypes with low mite-susceptibility are indicated in red (R1, R2 and R10), medium mite-susceptibility in pink (R8, R9 and R12) and all others were highly mite-susceptible (see Chapter 5).

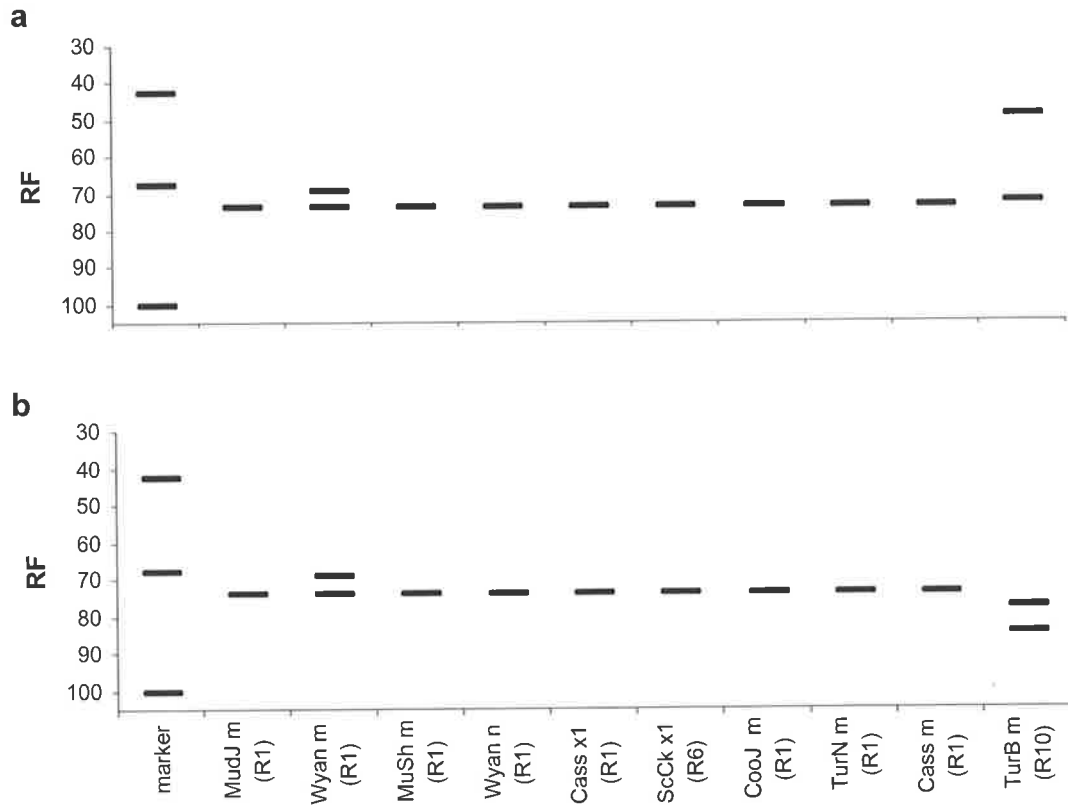


AFLP methods were not able to distinguish between R5 and R14 profiles (Section 4.5.2.1), therefore re-analysis of RFLP data without profile R14 was made for direct comparison of multilocus methods. Results are not presented because re-analysis produced identical relationships among the 13 profiles and similar bootstrap results to those presented above.

4.5.1.3 High intensity M13 bands

Early screens with the M13 probe indicated that DNA from 10 individuals, digested with *HaeIII*, *EcoRI* or *HinfI*, also contained polymorphisms in fast-migrating bands with very high intensity (for example R9 and R10 profiles, Fig 4.1). The extreme intensity of these bands most likely indicated repeated M13 sequence between restriction sites. With *HaeIII* as the restriction enzyme, in most samples high intensity bands were located between 2 kbp and 564 bp (Fig. 4.5 a), outside the area previously scored with the M13 probe, with the exception of bands found between approximately 3 and 4 kbp in R9, R10 and R11 profiles (Fig. 4.2). Note that RF values in Figs. 4.2 and 4.5 are not equivalent because they are ratios of distance to the shortest scored fragment. With the exception of the high intensity bands, which were clearly distinguishable on autoradiographs after a short exposure of several hours, fragments between 2 kbp and 564 bp were considered too difficult to score, and other than in the early screens reported here, were run off RFLP gels. Early screens also investigated the usefulness of *EcoRI* and *HinfI* for generating polymorphisms with the M13 probe, and revealed the presence of similar high-intensity bands within the same size range. *EcoRI* produced clear polymorphisms with the M13 probe, for the same individuals investigated with *HaeIII* (Fig. 4.5 b). The only *HinfI* digests fractionated in this size range were the first to have been prepared, and DNA extracted by the CTAB method used at that stage resulted in poor quality bands, despite which there was a suggestion that MuSh m (R1) and ScCk x1 (R6) could be separated from the others.

Figure 4.5. Polymorphism in high-intensity bands of *H. perforatum* DNA probed with M13, from two independent restriction enzymes a) *Hae*III, and b) *Eco*RI. The size marker was *Hind*III-cut λ DNA, and profiles were generated from RF values relative to the 564 bp band; RF 43 = 4.4 kbp, RF 67 = 2 kbp, and RF 100 = 564 bp. The sample identifier in each lane is followed by the RFLP profile number in brackets.

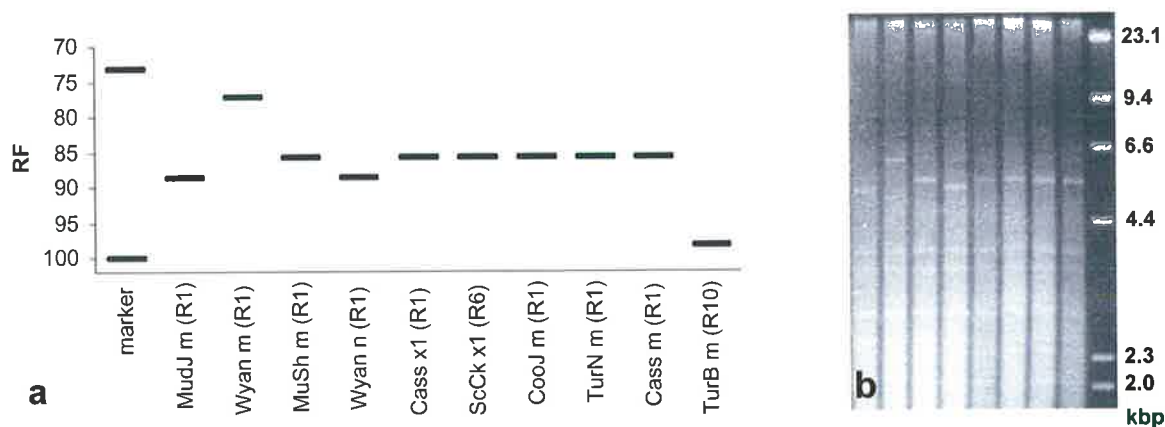


High intensity bands were interesting because they divided the R1 genotype into two groups; a) Wyan m, and b) MudJ m, MuSh m, Wyan n, Cass x1, CooJ m, TurN m and Cass m. This indicated the existence of intra-population variation at the Wyangala Dam site. Of the remaining individuals screened, although TurB m (R10) and ScCk (R6) were distinguished from all the others by RFLP and AFLP multilocus methods, on the basis of high-intensity bands, only the TurB sample displayed a unique pattern, while the ScCk individual could not be distinguished from R1 group b.

4.5.1.4 Ethidium bromide visualization

Ethidium bromide visualization prior to Southern transfer revealed polymorphism in *Hae*III-digested DNA separated in 1% agarose, in one of the early screens described above (Section 4.5.1.3). Polymorphism occurred in high-copy bands between 6.6 and 4.4 kbp, within the range scored for the M13 probe (Fig. 4.6). However, the M13 probe failed to reveal any correlated bands in this region, even over an extended X-ray exposure time, which indicated that the pertinent polymorphic bands did not contain compatible minisatellite sequence. Three groups could be distinguished on the basis of ethidium bromide staining; a) Wyan m, b) MudJ m and Wyan n, and c) MuSh m, Cass x1, CooJ m, TurN m and Cass m. These results support the existence of intra-population variation at the Wyangala Dam site. A second individual with the R1 group b genotype, from Mudgee Common (MudJ m), indicated the presence of this genotype at a minimum of two sites. Of the two individuals distinguishable from all others by RFLP and AFLP multilocus methods, only TurB m (R10) could be differentiated on the basis of high-copy ethidium bromide stained bands, while ScCk x1 (R6) could not be differentiated from R1 group c.

Figure 4.6. Polymorphism in high-copy ethidium bromide-visualized bands of *Hae*III-digested *H. perforatum* DNA. a) profiles generated from RF values between 6.6 kbp (RF = 73) and 4.3 kbp (RF = 100), with *Hind*III-cut λ DNA as the size marker. b) photograph of agarose gel of the first eight individuals in part b, with identical sample order.



4.5.2 AFLP

4.5.2.1 AFLP profiles

Seventy-eight AFLP fragments were scored between 1 and 10 kbp. Among the 298 *H. perforatum* individuals examined, 56 (72%) of the bands were polymorphic, and 22 AFLP profiles could be distinguished. Binary scores for all profiles can be found in Appendix B, and an example of an autoradiograph is given in Fig. 4.7 (page 31).

Twenty of the AFLP profiles were distinguished among 295 of the individuals, which are listed in Table 4.1. Two additional AFLP profiles, belonging to three fine-leaved seedlings that proved to be diploids (Section 4.5.3), are not included in Table 4.1. Two of the fine-leaved individuals (Cass fine-1 and Cass fine-2) that were obtained from Cassilis field-collected seed, were missing four AFLP bands (bands 38, 59, 60 and 74) and had one additional band (51) compared with other samples in the accession (Appendix B). The third fine-leaved specimen (MudJ m F1) was an F1 offspring of a MudJ m ♀ x Wyan m ♂ cross, and showed three similar band absences (bands 38, 60 and 74), compared to its parents.

The 42 individuals for whom an RFLP profile was available were screened for AFLP, with the exception of Ronk 2b, and are identified with an asterisk in Table 4.1. From each individual with a pair of multilocus profiles, a number corresponding to the RFLP profile was assigned to each AFLP profile. Although an AFLP fingerprint was not prepared for Ronk 2b, it shared an R5 profile with six other individuals for which relevant comparisons could be made; Alex 4b, Cast 4b, Chin 2b, Nann 3a, Karr 1o, and SESA 3b (Table 4.1). The latter group shared the A5 profile with all other Ronk samples tested, therefore it is highly likely that Ronk 2b would also demonstrate an A5 profile if re-tested.

Table 4.1. AFLP and/or RFLP profile numbers for 295 *H. perforatum* individuals from 46 populations. RFLP fingerprints were generated for individuals marked with an asterisk. Individuals in underlined type were analyzed cytologically. Individuals in bold type had several PCR-related AFLP band absences, as discussed in Section 4.5.2.2.

Population	State ^a	AFLP number	RFLP number	Identifier ^b	Collection method ^c
Burrendong Dam	NSW	A1	R1	Burr 1, 2, 3, 4, 5, 6, 7*, 8, 9, 10, 11, 12, 13, <u>14</u> , 15, 16, 17, 18, 19, 20	T
Cassilis	NSW	A1	-	Cass a, c, d, e, f, g, h, i, o, p, q, x2, x3	B
		A1	R1c	Cass <u>m</u> *	B
Coolah	NSW	A1	-	CooJ b, c, d, e, o, p	B
		A1	R1c	CooJ <u>m</u> *	B
		A1	R1	Cool o, p* , q	B
Mudgee Common	NSW	A1	R1	MuCo 1, 2, 3, 4, 5, 7*, 8, 9, 11, 12, 13, 14, 15, 17, 18, 19, <u>20</u>	T
		A1	R1	MudJ o* , p, q, r	B
		A1	R1b	MudJ <u>m</u> *	B
Mudgee	NSW	A1	R1	MuSh a, g, h, i, o, p* , q	B
		A1	R1c	MuSh <u>m</u> *	B
		A4	R4	MuTa a, g, h, i, o, p, q* , r	
Turon River	NSW	A1	R1c	TurN <u>m</u> *	B
		A10	R10	TurB b, c, d, e, m*	B
Wyangala Dam	NSW	A1	R1a	Wyan <u>m</u> *	B
		A1	R1b	Wyan <u>n</u> *	
Weddin Forest	NSW	A2	R2	Wedd 1, 3, 4, 5, 6, 7*, 9, 10, 11, 13, <u>14a</u> , 14b, 15, 16, 17, 18, 19, 20a, 20b	T
Adelong	NSW	A3	R3	Adel o, p, q*	B
Avenel	Vic	A4	R4	Aven 1b, <u>2b*</u> , 3a, 4a*	I
Mudgee-L	NSW	A4	R4	MudL f, g, h, i, o, p, q*	B
Violet Town	Vic	A4	-	Viol a, g, h, i	B
Cudgegong Park	NSW	A4	-	Cudg Q6 36/23, Q7 42/38, Q8 44/22, Q9 27/23, Q10 6/25, Q11 25/28 and 8/40, Q12 21/16 and 44/37, Q13 40/15, Q14 10/24 and 25/18, Q15 6/24	Q
Merrendee	NSW	A4	-	Merr Q1 16/4, Q2 10/40, Q3 13/42, Q4 8/4, Q5 47/28, Q6 44/5, Q7 25/37, Q8 43/48, Q9 29/36	Q
Alexandra	Vic	A5	R5	Alex 2a, 3b , 4b*	I
Beechworth	Vic	A5	-	Beec a, g, h, i	B
Benambra	Vic	A5	-	Bena 3f, 4a , 4g, 6f	I
Brockman Hwy	WA	A5	-	Broc 1f, 2f, 3f, 4f	I
Castlemaine	Vic	A5	R5	Cast <u>3f</u> , 3g, 4b* , 5f, 6f	I
		A5	-	CaTa f, g, h, i	I
Chindalup Rd	WA	A5	R5	Chin 1f, <u>2b*</u> , 2f, 4a , 4g	I
Eldorado	Vic	A5	-	Eldo a, g, h, i	B
Karridale	WA	A5	R5	Karr 1f, 1o* , 2a , 4f, 4g	I

Nannup	WA	A5	R5	Nann 1f, 2f, 3a* , 3b , 4f	I
Ronk property	WA	-	R5	Ronk 2b*	I
Ronk property	WA	A5	-	Ronk 1f, 2f, 3f, 4f	I
Bonganditj Forest	SA	A5	R5	SESA 3b* , 5	I
		A7	-	SESA 2	I
		A7	R7	Bong 1b , 2a, 2b*, 3b*	I
Flowerdale	Vic	A5	R14	Flow 1b , 2a, 2b*, 5a	I
Scott Creek	SA	A6	R6	ScCk x1* , x2, x3, o , p, q, r	B
Bemboka	NSW	A7	R7	Bemb a* , g, h, i, o* , p	B
Captains Flat	NSW	A7	R7	CaFl a, o* , p , q	B
Talmalmo	NSW	A7	-	Talm a	
Pierces Creek	ACT	A7	-	Pier 1, 2, 3, 4, 5	F
Smiths Paddock	ACT	A7	-	Smit 1, 2, 3, 4, 5	F
Bundaleer Forest	SA	A8	R8	Bund 1a*, 1b , 2b, 3b*	I
Nelson	Vic	A9	R9	Nels 1b*, 2b, 3a, 3b , 4a	I
Orange-1	NSW	A10	-	Oral a, g, h, j	B
Orange-K	NSW	A10	R10	OraK a*, b , c, d	B
Canada †	-	A11	R11	Cana a*, b , g, h, i	B
Tuena	NSW	A12	R12	Tuen o , p*, q	B
Tuena-J	NSW	A12	R12	TueJ b* , d*, o , p, q, r	B
		A19	-	TueJ a	B
		A20	-	TueJ c	B
Cowra	NSW	A13	R13	Cowr a, b*, d, e, f, g, h, i, o	B
Bridgetown	WA	A14	-	Brid 1f , 2f, 3f, 4f	I
Inverell	NSW	A15	-	Inve a, g, h, i	B
Montpellier†	-	A16	-	Mont a, g, h, i	B
Wonnangatta	Vic	A17	-	Wonn a, g, h, i	B
Crawford Forest	SA	A18	-	Craw a	F

^a State: ACT = Australian Capital Territory, NSW = New South Wales, SA = South Australia, Vic = Victoria, WA = Western Australia.

^b Numerical identifier suffixes indicate seed or fresh material from separate individuals by random, transect or quadrat collections, and alphabetical suffixes indicate seedlings within a single seed lot. Exceptions included quadrat identifiers supplied by Mr. Paul Jupp (Q = quadrat number, followed by co-ordinate), and four bulk-seed-derived individuals used in crosses (Cass x1 and ScCk x1, x2, x3).

^c Collection method: B = bulked seed from multiple plants, I = separate seed lots collected from randomly distributed individuals, T = separate seed lots collected along a transect through the population, Q = separate seed lots collected from individuals defined by quadrat coordinates, F = fresh plant material from discrete individuals.

† Montpellier was the original site of collection of *Aculus hyperici* in Southern France. Canadian seed was obtained from a Perth garden show by John Scott (CSIRO Entomology).

One individual, Flow 2b, was the only exception to the equivalence of RFLP and AFLP profile numbers. When Flow 2b was examined with RFLP, its R14 profile could be distinguished from all other R5 individuals by the presence of an additional faint band (Section 4.5.1.1), but on the basis of AFLP, Flow 2b could not be differentiated from other Flow individuals or other A5 samples.

4.5.2.2 AFLP fingerprint reliability

A subset of individuals was tested for AFLP fingerprint repeatability, and reliability with different extraction methods. There were no differences among fingerprints within each gel, or on different gels, when an identical miniprep extract and AFLP procedure was tested, or when identical restriction-ligation and pre-amplification PCR but separate radiolabeled PCR steps were used (samples tested; Cass x1, x2, x3; ScCk x1, x2, x3). Likewise, the same miniprep extract, but with an entirely separate AFLP procedure (using an identical protocol), resulted in identical fingerprints on different gels (samples Beec i, Bemb g, and Bong 2b). The same conclusion was drawn from samples extracted by the original large-scale DNA method also used to generate RFLP fingerprints (samples Adel q, Cass x1, Cowr b, Wedd 7a), and from a comparison between miniprep and original extraction methods (MudJ m, MuSh m and Wyan n). However, band absence was consistently encountered for a subset of low intensity bands (Bands 2, 5, 6, 7, 9, 10, 12, 23, 24, 25a, 29) in all accessions on the first three AFLP gels (highlighted in bold in Table 4.1). The most likely explanation for band absence in early gels was variation in adapter and/or primer concentration, since the earlier dilutions had come from another laboratory, and the problem was not repeated with subsequent dilutions. Five of the individuals with missing bands on early gels (Alex 2a, Aven 2b, Bund 1b, ScCk x1 and Cass x1) exhibited fingerprints identical to other samples in their accessions when replicated on later gels, and none of the samples within those accessions exhibited any comparable band absences on

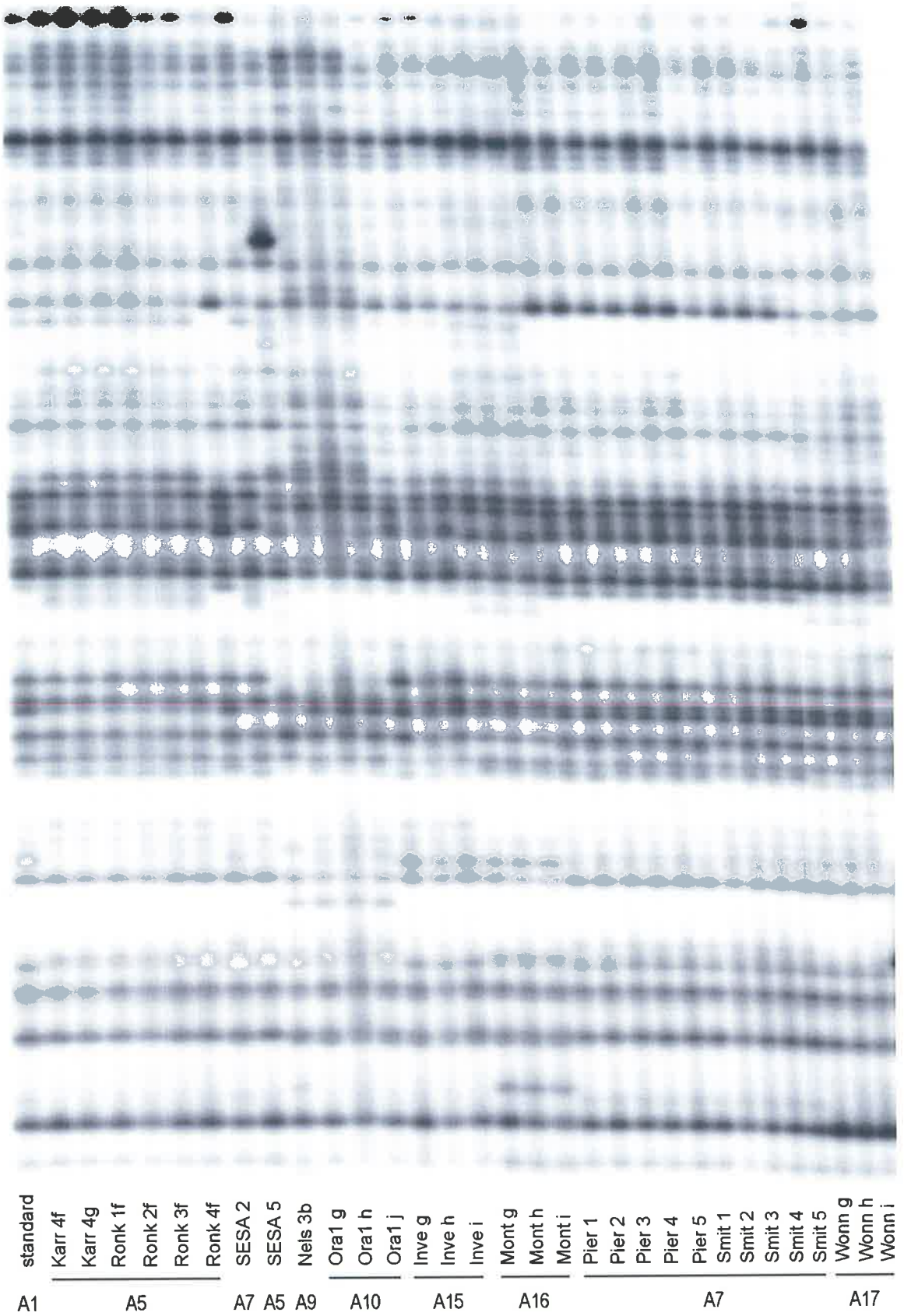
any other gel. Therefore, genetic variation was unlikely to be responsible for the band absences described above. Similarly, in remaining un-replicated samples on early gels affected by missing bands, all positive bands in accessions also appeared in other individuals from the same accessions in later gels, and no positive bands were seen when other samples in the accession were negative. Again, this indicated a low likelihood that genetic variation was responsible for band absence in the early gels.

It was concluded that while care should be taken to reproduce PCR conditions, a high level of confidence could be placed in the fingerprints generated by this method, because replicated samples within and among the majority of gels exhibited identical fingerprints, regardless of DNA isolation method or extraction repeat. Unless otherwise stated, the following results refer to samples from gels unaffected by band absence due to PCR conditions. Adelong samples only appeared on two early gels and the A3 fingerprint presented for this accession should therefore be regarded with caution.

4.5.2.3 *Intra- and inter-population variation*

AFLP profiles were polymorphic among populations (Fig. 4.7). While band intensity varied noticeably among the different profiles, it was consistent among individuals and populations with shared profiles. Populations were monomorphic within 42 of the 46 sites examined with AFLP (for example, Fig. 4.7), including the Wyangala Dam site at which variation was identified with RFLP (Sections 4.5.1.3 and 4.5.1.4). Of the remaining four sites, AFLP detected the same three heterogeneous populations identified by RFLP, at Mudgee, Turon River and Bonganditj Forest (Section 4.5.1.1), and in addition identified variation within the TueJ accession.

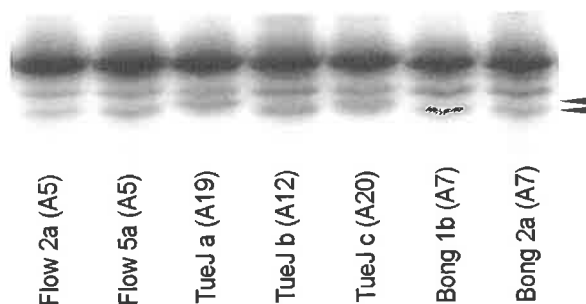
Figure 4.7. AFLP profiles of *H. perforatum* individuals from eleven populations. Samples grouped above each bar share the AFLP profile number below. Cass x1 was used as a standard.



From the Mudgee site, all eight of the MuSh samples screened had an A1 profile, and all eight MuTa were A4. At the Turon River site, the only narrow-leaved TurN sample tested was A1, and five broad-leaved TurB samples were A10. The Bonganditj Forest site revealed two A5 individuals in the SESA accession, and five R7 individuals, one from the SESA and all four from the Bong accession (for example, SESA 2 and 5, Fig 4.7). The SESA accession was collected from randomly selected individuals throughout the population, at a site where *Aculus* mites were released in August 1996, and the Bong accession was a second collection made from the same site and in the same manner, but in the following year.

Minor AFLP fingerprint variation was seen in two samples from the TueJ accession, TueJ a and c (A19 and A20 profiles, respectively), compared with other samples from the population. Band 13 was present in TueJ a and c, and band 14 was absent in TueJ a and possibly also from TueJ c (Fig. 4.8), otherwise fingerprints of both individuals matched the A12 profile (Appendix B). The TueJ accession was a bulk seed collection made from a demography and mite-effect study site (Jupp & Cullen, 1996), approximately 10 km from where the Tuen accession originated.

Figure 4.8. Polymorphism in AFLP fingerprint bands (arrowed) in two individuals from the TueJ accession, TueJ a and c.



A genetic basis for variable morphology at the Castlemaine field site was not supported by the results for the CaTa and Cast accessions. Four seedlings from what was considered to

be a particularly tall plant growing in the field (CaTa) were analyzed, but no difference was noted in morphology in the glasshouse, or in AFLP profile, when compared to five other samples collected at random from the population (Cast 3f, 3g, 4b, 5f, 6f). In particular, the lack of morphological variation within and between the two accessions (N = 15 per accession) under glasshouse conditions suggested that environmental effects were responsible for height variation in the field.

No AFLP variation was detected within transect and quadrat field sites, where the largest numbers of individuals were tested within populations (N = 20, 17 and 19 for transects; 9 and 13 for quadrats), despite screening with two different AFLP primer combinations. Transect samples were made for three populations, by collection of separate seed lots from 20 individual plants, each approximately 10m apart, along a transect through each population. All individuals within the Burr or MuCo transects had A1 profiles, and all individuals within the Wedd transect were A2. Quadrat samples originated from two CSIRO field sites, Cudgegong and Merrendee, at which the impact of *A. hyperici* had been assessed for several years (Jupp & Cullen, 1996), and consisted of separate seed lots from individuals defined by quadrat co-ordinates. All individuals at both quadrat sites had A4 profiles.

Collection methods varied as a consequence of multiple collectors and the use of pre-existing accessions, and it is worth considering the relative value of different methods. Obviously, the most informative samples would be expected to be separate seed lots from individuals widely spaced in the field, for example from transects, quadrats and randomly selected individuals, and as often as was possible in the study, such samples were used. Replication within seed lots from individual plants would be expected to yield very little additional information even with a higher resolution marker system, because of the high degree of apomixis in this species. Collectors in all prior studies on this plant employed

bulk seed collection, largely it seems based on the assumption that *H. perforatum* reproduces clonally. Bulk seed may be less informative, because a separate maternal origin could not be substantiated for each seedling. It was recognized that at several sites where only one accession of bulk seed was available, for example Cassilis and Adelong (Table 4.1), the likelihood of detecting variation in the population might have been reduced. However, this study was not designed to rigorously investigate the level of within-population variation, but to assess the level of variation within the species in Australia. In any case, if the assumption is made that the high degree of apomixis found in the A1 and A6 genotypes (Chapter 3) was comparable for other genotypes, even large sample sizes may not have been sufficient to detect recombination.

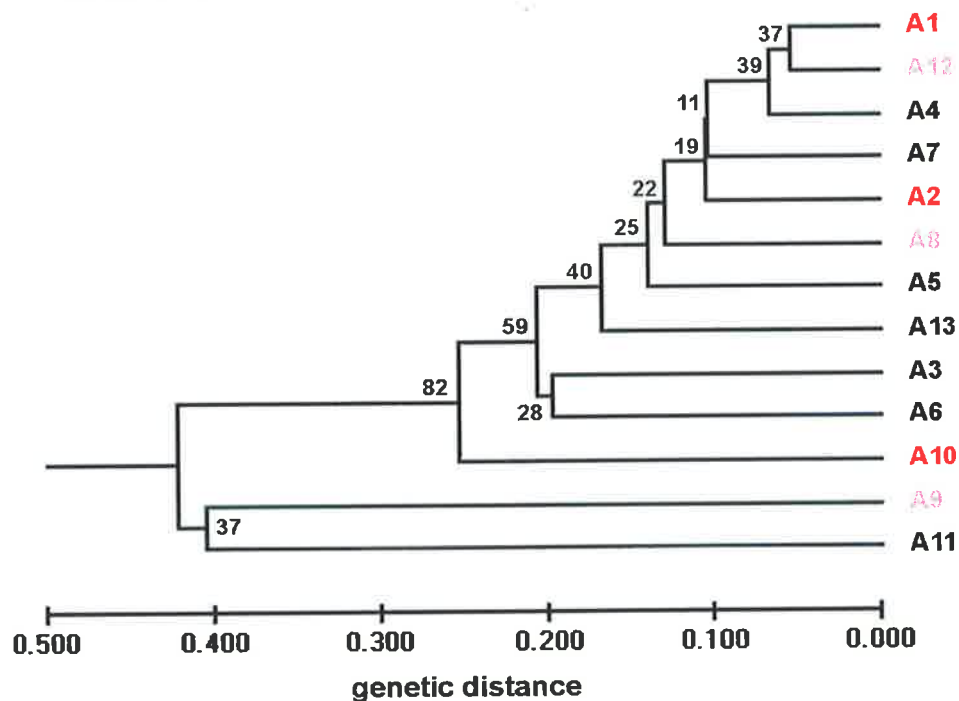
4.5.2.4 Genetic relationship based on AFLP

In order to compare genetic relationships based on AFLP and/or RFLP analysis, a subset of AFLP profiles was analyzed and a pairwise genetic distance matrix (Fig. 4.9) and dendrogram (Fig. 4.10) were constructed as previously described (Section 4.5.1.2). The subset consisted of 13 AFLP profiles corresponding to the same individuals and with the same profile number as the RFLP profiles formerly analyzed, with the exception that Flowerdale could be distinguished from other samples by RFLP but not AFLP profile (profile R14/A13, see Section 4.5.2.1). No tied trees existed, but 78% of bootstrap replicates produced trees containing ties.

Figure 4.9. Genetic distance matrix for 13 AFLP profiles, based on Nei's (1978) unbiased distance.

AFLP profile	1	2	3	4	5	6	7	8	9	10	11	12	13
1	0.000												
2	0.108	0.000											
3	0.198	0.167	0.000										
4	0.066	0.123	0.214	0.000									
5	0.137	0.167	0.262	0.123	0.000								
6	0.167	0.198	0.198	0.182	0.198	0.000							
7	0.108	0.108	0.198	0.094	0.137	0.167	0.000						
8	0.137	0.167	0.230	0.094	0.167	0.262	0.137	0.000					
9	0.386	0.386	0.507	0.445	0.425	0.386	0.425	0.465	0.000				
10	0.246	0.279	0.314	0.230	0.246	0.246	0.214	0.214	0.406	0.000			
11	0.386	0.425	0.425	0.406	0.465	0.386	0.465	0.425	0.406	0.331	0.000		
12	0.053	0.080	0.198	0.066	0.108	0.198	0.108	0.108	0.465	0.279	0.425	0.000	
13	0.152	0.152	0.214	0.167	0.182	0.246	0.214	0.152	0.486	0.262	0.368	0.152	0.000

Figure 4.10. Dendrogram constructed from the distance matrix presented in Fig. 4.9, for the AFLP data sub-set of 13 profiles. The proportion of similar replicates obtained from bootstrapping is given above nodes. Genotypes with low mite-susceptibility are indicated in red (A1, A2 and A10), medium mite-susceptibility in pink (A8, A9 and A12) and all others were highly mite-susceptible (see Chapter 5).

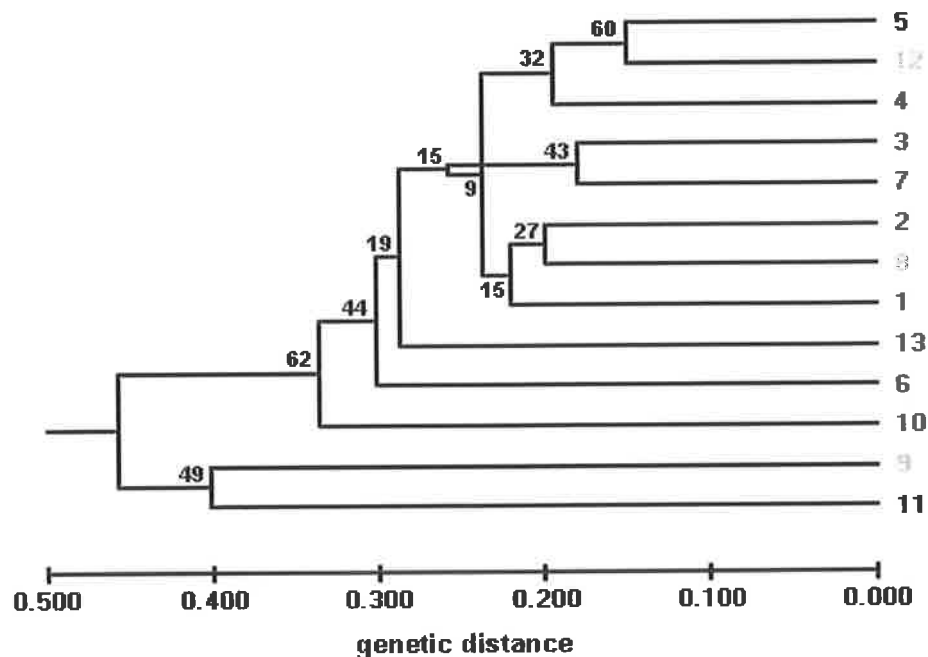


In order to increase resolution, data sets for the 13 corresponding RFLP and AFLP profiles were combined and analyzed as before (Figs. 4.11 and 4.12). Three tied trees existed, and 78% of bootstrap replicates contained tied trees.

Figure 4.11. Genetic distance matrix constructed from combined AFLP and RFLP data from 13 profiles, based on Nei's (1978) unbiased distance.

profile	1	2	3	4	5	6	7	8	9	10	11	12	13
1	0.000												
2	0.219	0.000											
3	0.327	0.261	0.000										
4	0.240	0.240	0.261	0.000									
5	0.240	0.282	0.282	0.219	0.000								
6	0.307	0.253	0.341	0.296	0.285	0.000							
7	0.261	0.199	0.179	0.199	0.219	0.253	0.000						
8	0.219	0.199	0.304	0.219	0.261	0.296	0.199	0.000					
9	0.422	0.489	0.448	0.489	0.462	0.480	0.360	0.435	0.000				
10	0.385	0.361	0.293	0.293	0.315	0.377	0.229	0.293	0.360	0.000			
11	0.431	0.553	0.483	0.457	0.457	0.462	0.525	0.444	0.401	0.381	0.000		
12	0.189	0.250	0.315	0.169	0.150	0.319	0.250	0.209	0.503	0.422	0.418	0.000	
13	0.261	0.282	0.327	0.261	0.261	0.353	0.327	0.304	0.560	0.385	0.444	0.271	0.000

Figure 4.12. Dendrogram constructed from the distance matrix presented in Fig. 4.11, for combined AFLP and RFLP data sets for 13 profiles. The proportion of similar replicates obtained from bootstrapping is given above nodes. Genotypes with low mite-susceptibility are indicated in red (A1, A2 and A10), medium mite-susceptibility in pink (A8, A9 and A12) and all others were highly mite-susceptible (see Chapter 5).

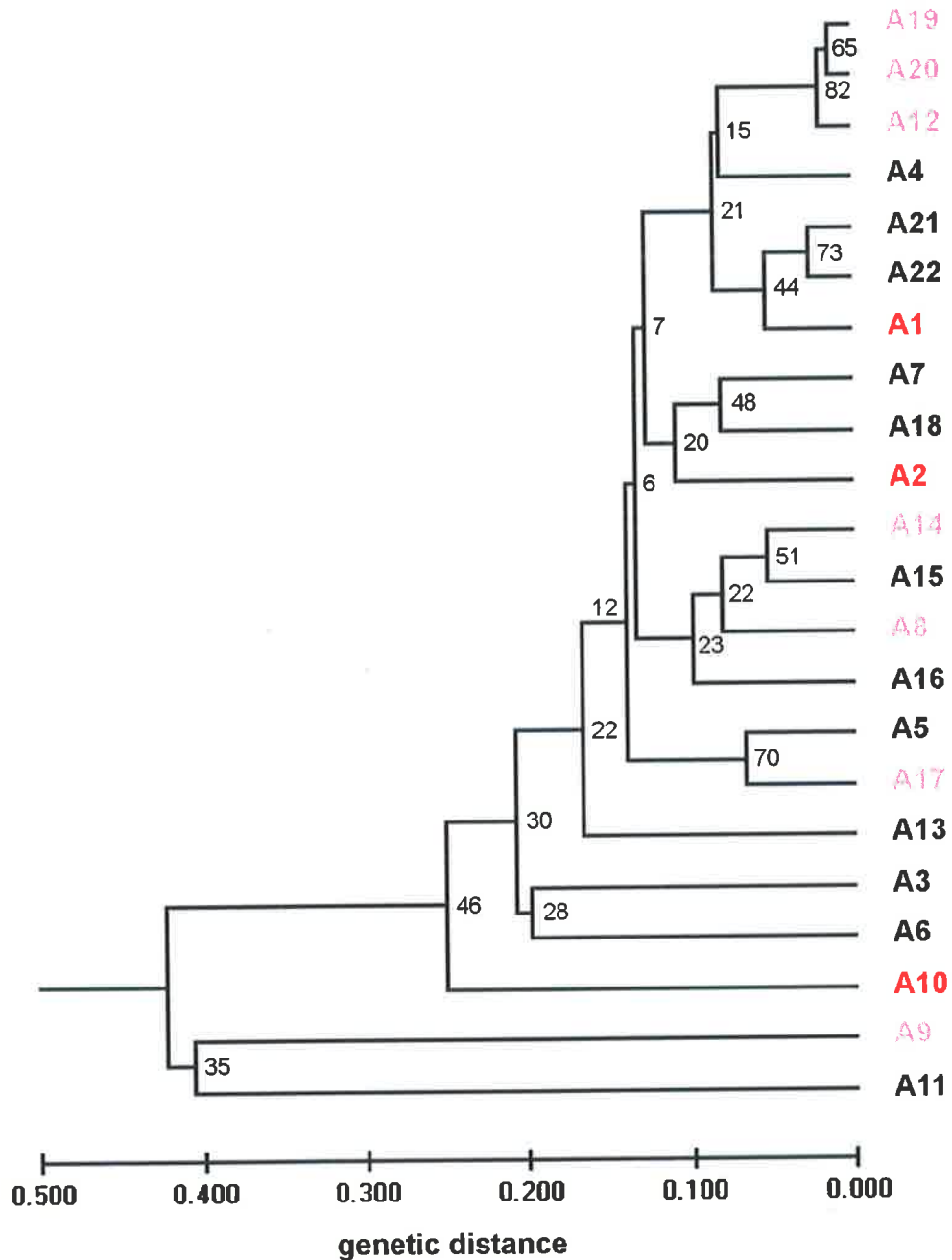


Analysis of the full AFLP data set, including diploids (profiles 21 and 22), was performed as previously (Section 4.5.1.2), and a pairwise genetic distance matrix (Fig. 4.13) and dendrogram (Fig. 4.14) were constructed. Four tied trees existed, and 100% of bootstrap replicates contained tied trees.

Figure 4.13. Genetic distance matrix constructed from full AFLP data set of 22 profiles, based on Nei's (1978) unbiased distance.

AFLP profile	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
1	0.000																					
2	0.108	0.000																				
3	0.198	0.167	0.000																			
4	0.066	0.123	0.214	0.000																		
5	0.137	0.167	0.262	0.123	0.000																	
6	0.167	0.198	0.198	0.182	0.198	0.000																
7	0.108	0.108	0.198	0.094	0.137	0.167	0.000															
8	0.137	0.167	0.230	0.094	0.167	0.262	0.137	0.000														
9	0.386	0.386	0.507	0.445	0.425	0.386	0.425	0.465	0.000													
10	0.246	0.279	0.314	0.230	0.246	0.246	0.214	0.214	0.406	0.000												
11	0.386	0.425	0.425	0.406	0.465	0.386	0.465	0.425	0.406	0.331	0.000											
12	0.053	0.080	0.198	0.066	0.108	0.198	0.108	0.108	0.465	0.279	0.425	0.000										
13	0.152	0.152	0.214	0.167	0.182	0.246	0.214	0.152	0.486	0.262	0.368	0.152	0.000									
14	0.152	0.123	0.182	0.108	0.152	0.182	0.123	0.066	0.406	0.198	0.445	0.123	0.137	0.000								
15	0.094	0.123	0.182	0.108	0.123	0.18	0.123	0.094	0.368	0.198	0.406	0.094	0.137	0.053	0.000							
16	0.167	0.198	0.230	0.123	0.137	0.230	0.167	0.108	0.425	0.182	0.349	0.137	0.182	0.094	0.094	0.000						
17	0.123	0.152	0.214	0.108	0.066	0.214	0.123	0.152	0.445	0.230	0.445	0.123	0.137	0.137	0.108	0.123	0.000					
18	0.108	0.108	0.262	0.123	0.167	0.230	0.080	0.137	0.349	0.246	0.507	0.137	0.246	0.123	0.123	0.198	0.152	0.000				
19	0.080	0.108	0.230	0.094	0.137	0.230	0.137	0.137	0.507	0.314	0.386	0.026	0.182	0.152	0.123	0.167	0.152	0.167	0.000			
20	0.066	0.094	0.214	0.080	0.123	0.214	0.123	0.123	0.486	0.296	0.406	0.013	0.167	0.137	0.108	0.152	0.137	0.152	0.013	0.000		
21	0.039	0.123	0.214	0.080	0.152	0.182	0.152	0.094	0.406	0.262	0.406	0.066	0.137	0.137	0.080	0.152	0.137	0.152	0.094	0.080	0.000	
22	0.066	0.123	0.214	0.108	0.182	0.152	0.182	0.123	0.406	0.296	0.445	0.094	0.167	0.137	0.108	0.182	0.167	0.152	0.123	0.108	0.026	0.000

Figure 4.14. Dendrogram constructed from the distance matrix presented in Fig. 4.13, for the full AFLP data set. The proportion of similar replicates obtained from bootstrapping is given above nodes. Genotypes with low mite-susceptibility are indicated in red (A1, A2 and A10), medium mite-susceptibility in pink (A8, A9, A14, A17, A12, A19 and A20) and all others were highly mite-susceptible (see Chapter 5).

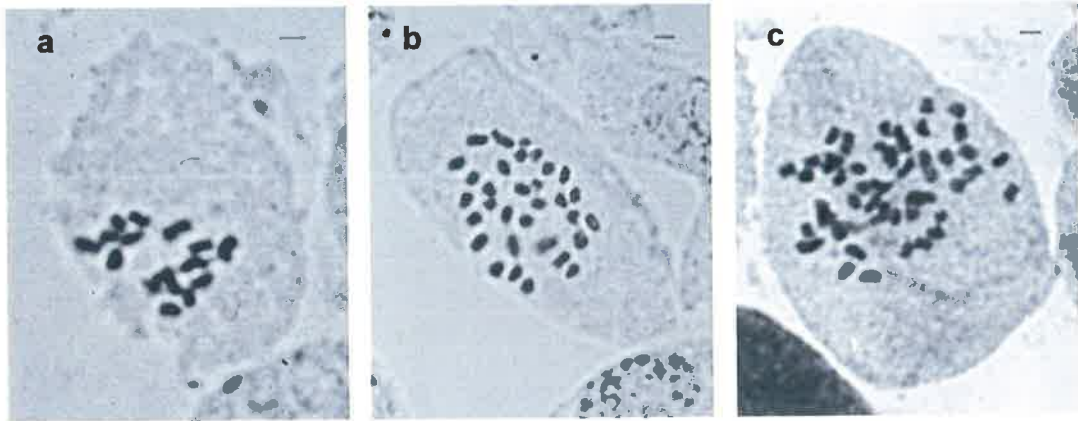


4.5.3 Cytology

Forty-seven individuals were analyzed for chromosome number, identifiers for 43 of which are listed in underlined type in Table 4.1. The latter 43 individuals came from 34 geographically separated populations, and contained all genotypes except A19 and A20 (A1, N=13; A4 and 7, N=3; A5, N=8; A10 and A12, N=2; all others N=1). Additional samples included three fine-leaved specimens (Cass fine-1, Cass fine-2, and MudJ m F1) that had extremely small leaves (Fig.4.16) and atypical AFLP profiles compared to their congeners or maternal parent (Section 4.5.2.1), and one with a larger leaf size compared to congeners (Cass r) for which a multilocus profile was not available.

All individuals examined were tetraploid (for example Fig. 4.15 b) with the exception of Cass r and the three fine-leaved specimens. Of the three fine-leaved specimens, Cass fine-1 (Fig. 4.16) was diploid ($2n = 16$) for three unambiguous cells from each of three meristems (Fig. 4.15 a), and Cass fine-2 appeared to be aneuploid ($2n+1 = 17$) in three unambiguous chromosome counts from two shoots. As a young seedling, the fine-leaved MudJ m F1 also proved to be diploid ($2n = 16$), but as a mature crown in its second year of growth, the latter specimen produced several side shoots with leaf morphology similar to the typical morphology of the R1 maternal parent. Cytological analysis revealed that this plant was mixoploid, the original fine-leaved section being diploid ($2n = 16$) and shoots with maternal leaf morphology being tetraploid ($2n = 32$). The individual with larger leaf size compared to congeners (Cass r) was concluded to be hexaploid ($2n = 48$), on the basis of two unambiguous counts from separate meristems (Fig. 4.15 c). It was difficult to obtain unambiguous results for hexaploids because of the very small size of chromosomes in *H. perforatum*, an experience shared with past authors (Hoar & Haertl, 1932; Robson, 1981).

Figure 4.15. Variation in ploidy of seedlings derived from the Cassilis field-collected accession of *H. perforatum* seed; a) diploid *Cass fine-1* ($2n = 16$), b) tetraploid *Cass m* ($2n = 32$) and c) hexaploid *Cass r* ($2n = 48$). Bar = 1 μm .



4.5.4 Morphology

It was relatively easy to detect diploids by their very small leaf and plant size (Figs. 4.16 and 4.17), and hexaploids by their generally larger size and more vigorous growth, among the large number of seedlings of each accession, and in offspring of artificial crosses that were raised in the glasshouse.

Figure 4.16. Variation in rosette morphology of seedlings from the Cassilis accession; a diploid individual with fine-leaved morphology (*Cass fine-1*) is on the left, and a tetraploid with typical morphology (*Cass m*) is on the right.



Identification of the 20 tetraploid genotypes based on morphology would require a sizeable study, given the large amount of variation observed. Time constraints precluded this, but pictorial records of a selection of genotypes are presented to give an example of the range of variation among genotypes grown under identical conditions in the glasshouse (Figs. 4.17 and 4.18). Reflecting the level of genetic variation detected among and within populations in this study, plant morphology was relatively uniform within genotypes (Fig. 4.17 b and d), but highly variable among genotypes, for example in plant height (Figs. 4.17 and 4.18), number and rigidity of flowering stems, flowers per stem, capsule morphology, and leaf shape. Preserved specimens were made for a number of the genotypes, and are lodged with the Herbarium of the Adelaide Botanic Gardens.

Figure 4.17. Morphology of flowering *H. perforatum*; tetraploids a) *ScCk x1 (A6)*, b) *Cass x1 (A1)*, c) *TurB m (A10)*, d) *Cass m (A1)*, and diploid e) *Cass fine-2*



Figure 4.18. Flowering stage morphology of tetraploid *H. perforatum* individuals a) Cowr a (A13), b) Adel q (A3), c) Bemb o (A7), d) MuTa q (A4), e) Tuen p (A12), and f) Chin 2b (A5).



4.5.5 Distribution

The following results are presented in terms of AFLP profile distribution, with reference to additional variation detected by RFLP where relevant. The A5 profile was the most commonly encountered among populations (N = 11 sites), followed by A1 at seven sites, A4 and A7 at six sites each, A10 at three and A12 at two sites (Table 4.1 and Figs. 4.19-21). Each of the other genotypes was found in only one population. Even from the rudimentary maps, it can be seen that the most widely geographically distributed appears to be the A5 genotype, which was found across southern Australia from the coast of Western Australia to eastern Victoria. Some intra- and/or inter-population variation in the A5 variant was suggested by the existence of an R14 individual at Flowerdale. The A1/R1 genotype seemed to be restricted to northern New South Wales. The three R1 sub-groups detected among a small sub-sample by high intensity M13 band and/or ethidium bromide stain (Sections 4.5.1.3 and 4.5.1.4, and Table 4.1), consisted of one or two individuals from each A1 population (excluding Burrendong Dam); Coolah, Cassilis, Mudgee Short and Turon River contained the group c variant, the Mudgee Common site contained a group b individual, and the Wyangala Dam site further south contained group a and b variants. Sampling more populations, and individuals within populations, may determine whether these distributions overlap. Likewise, more samples would have to be made to determine whether the A4 populations around Mudgee and central Victoria, and the A7 populations around the ACT and at Nelson, represent two isolated and relatively localized distributions or one wide distribution. Future studies may determine more accurately the extent of distributions represented by single or adjacent populations with unique genotypes.

Figure 4.19. Distribution of *H. perforatum* genotypes in southeastern Australia. Triangles and squares indicate single populations of a genotype, and circles represent multiple sites. Genotypes with low mite-susceptibility are indicated by light and dark red symbols (A1, A2 and A10), those with medium mite-susceptibility by pink symbols (A12, A19, A20 and A17), and all other symbols represent highly mite-susceptible genotypes (see Chapter 5, Table 5.10).

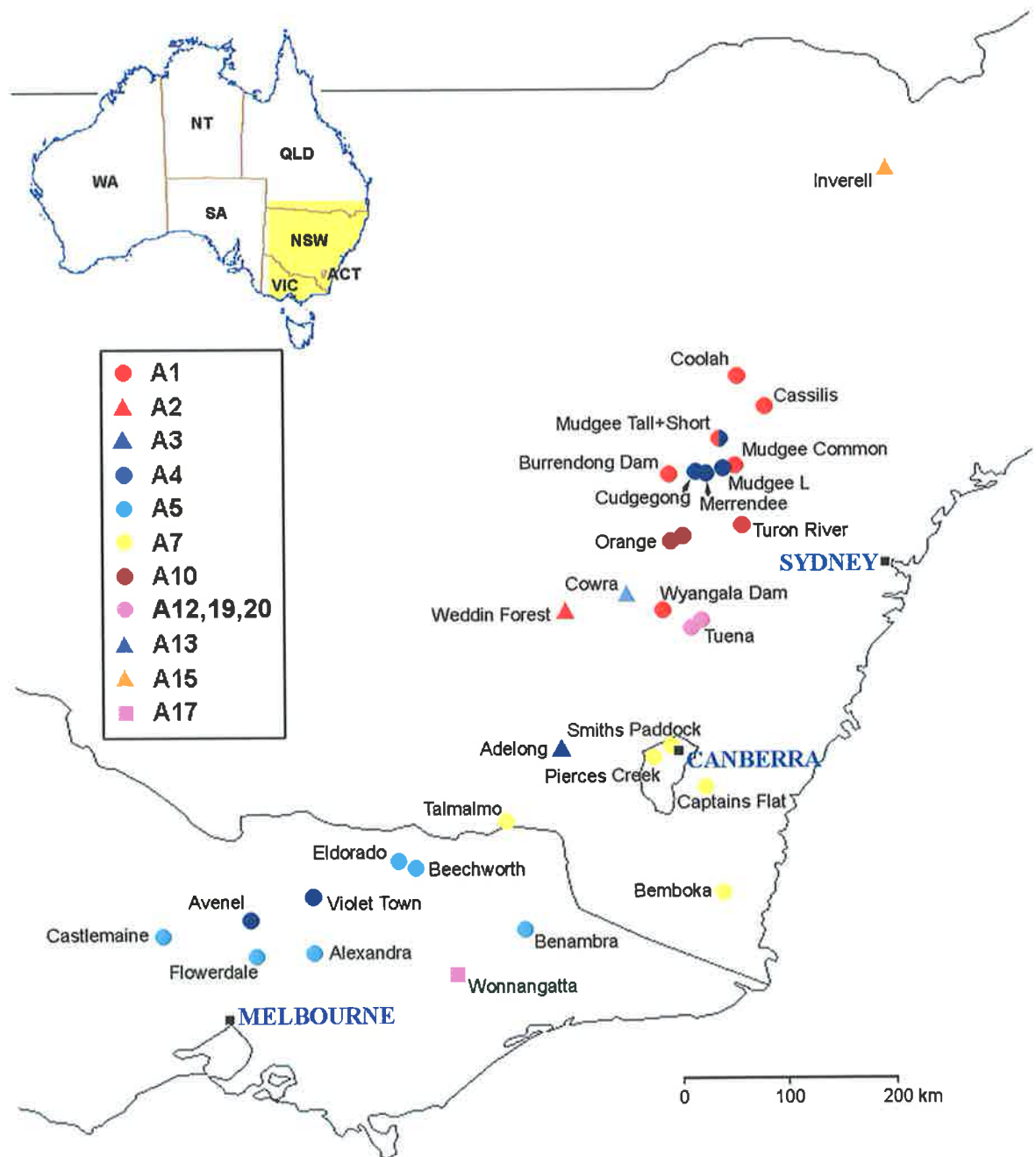


Figure 4.20. Distribution of H. perforatum genotypes in South Australia. Triangles and squares indicate single populations of a genotype, and circles represent multiple sites. Genotypes with medium mite-susceptibility are indicated by pink symbols (A8 and A9) and all other symbols indicate highly mite-susceptible genotypes (see Chapter 5, Table 5.10).

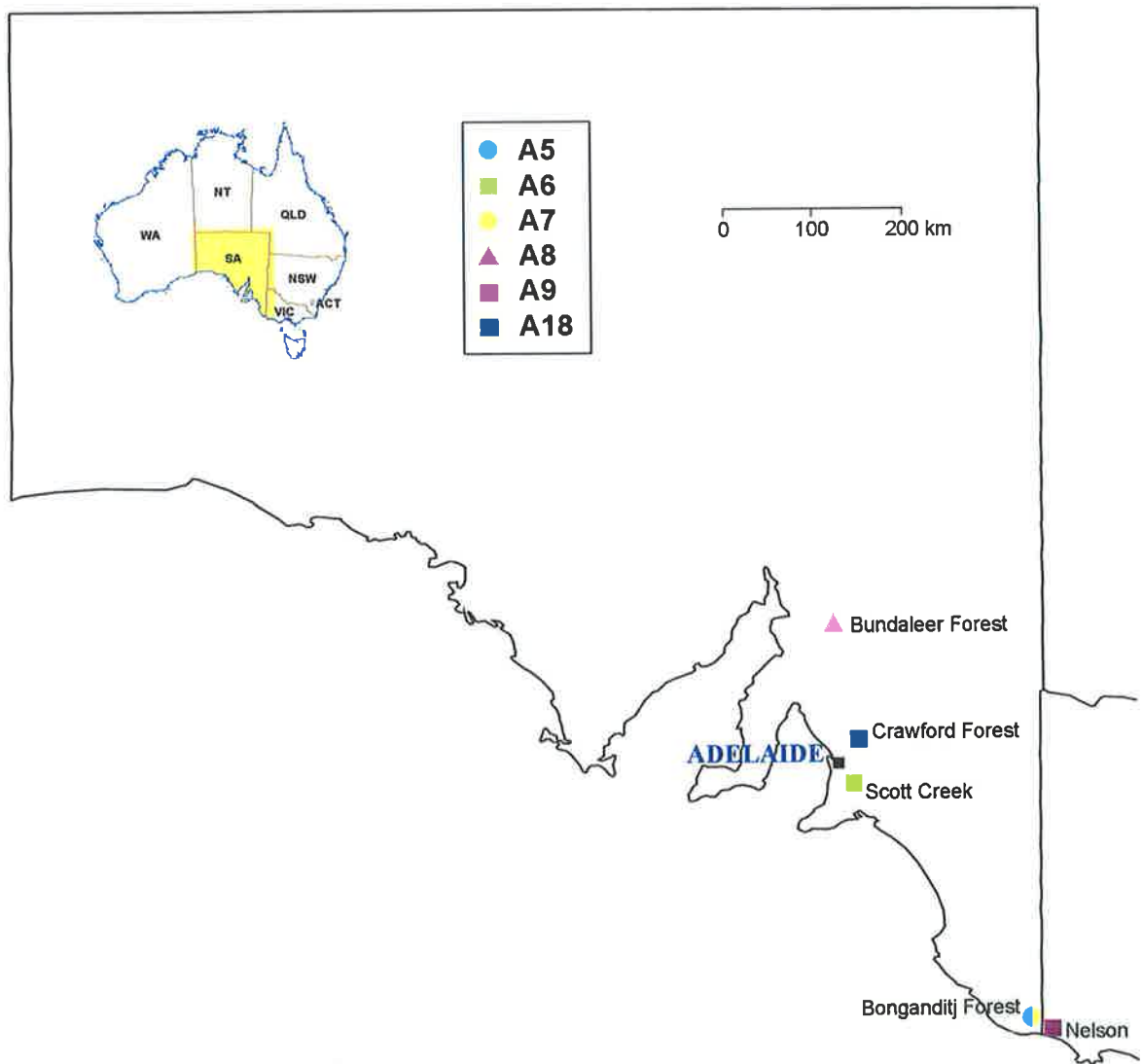
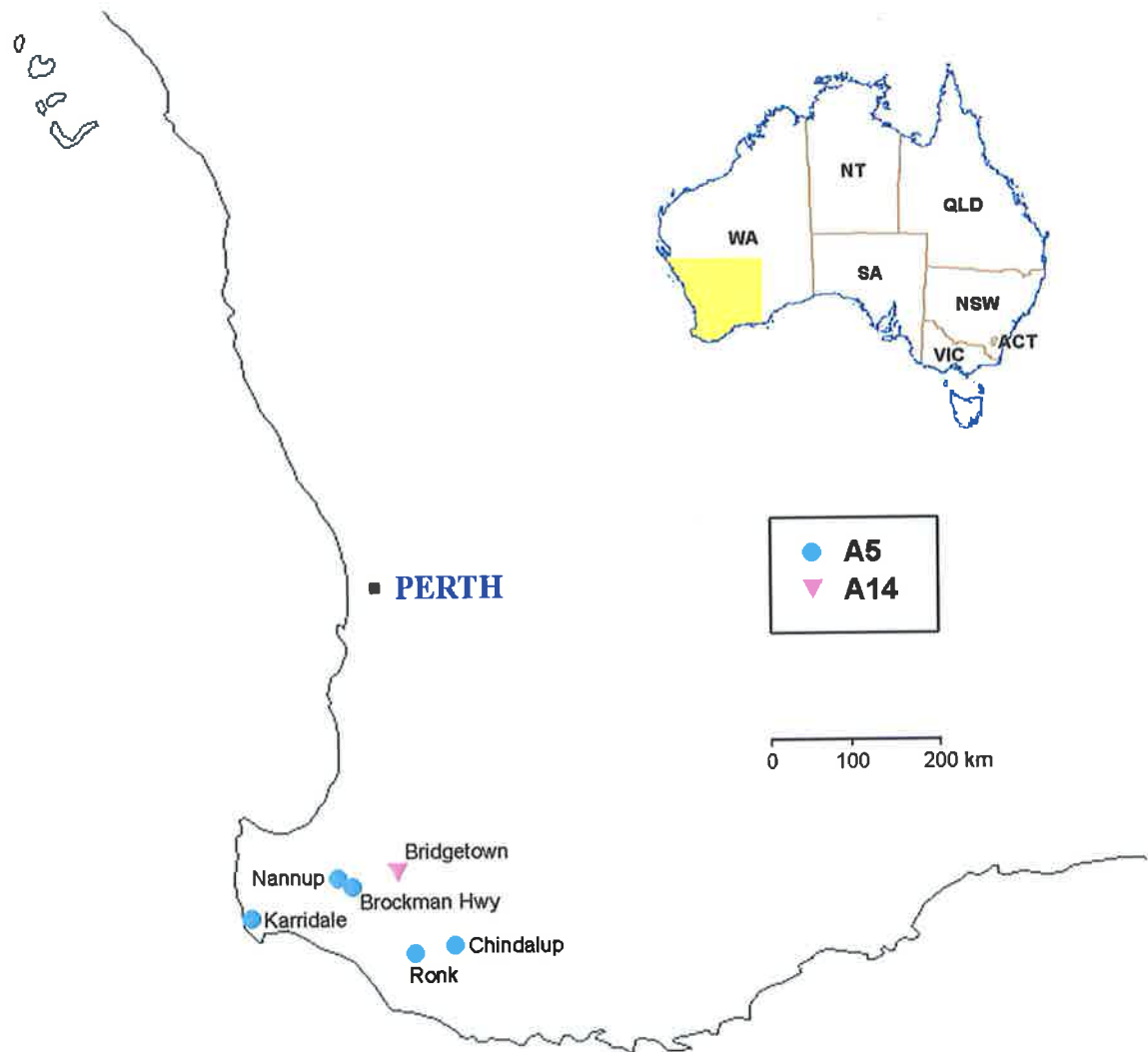


Figure 4.21. Distribution of *H. perforatum* genotypes in Western Australia. Triangles and squares indicate single populations of a genotype, and circles represent multiple sites. Medium mite-susceptibility is indicated by pink, and high mite-susceptibility by blue (see Chapter 5, Table 5.10).



4.6 Discussion

4.6.1 Resolution

Either multilocus method (RFLP with M13, or AFLP) could be used to discriminate among genotypes, with some differences in information content and resolution. AFLP profiles produced with primer pairs *Pst*IAG and *Mse*ICAC contained 56 (72%) polymorphic bands among populations, compared to 44 (100%) with RFLP using the M13 probe. While the percentage of polymorphic bands was lower for AFLP, actual number of polymorphic bands was higher. Among samples for which multilocus profile comparisons could be made, RFLP appeared to be slightly more sensitive; one additional RFLP band in Flow 2b discriminated it from the other R5 samples, but no differences were seen in AFLP profiles for the same individuals. Furthermore, among samples not differentiated by the usual fragment range scored for RFLP or AFLP, the former technique offered slightly higher resolution if high intensity bands or ethidium bromide visualization of digests were considered. *Hae*III restriction digests scored for high-intensity M13 band polymorphism or ethidium bromide visualization revealed two and three sub-groups, respectively, within the R1 genotype. Simply including routine ethidium bromide visualization prior to Southern transfer therefore increase the resolution of the RFLP/M13 technique, and would be less time-consuming than an additional high-intensity band screen. The slightly higher sensitivity and percentage polymorphism of RFLP compared with AFLP may be due to the fact that the M13 probe (used for the former) targets a minisatellite sequence, which is expected to be more variable than random AFLP targets interspersed throughout the genome. It would be interesting to re-examine TueJ a and c with RFLP techniques, since they showed minor band variation in AFLP profiles.

The decision was made on pragmatic grounds to use AFLP markers for the majority of screening. AFLP offers a large number of primers to choose from, resolution can be

controlled by the choice of rare cutter enzyme and number and nature of selective bases, and 50-100 fragments are typically amplified and detected. Because it is PCR-based, AFLP fingerprinting required less DNA, which permitted the development of a faster and higher throughput DNA miniprep extraction method. The AFLP method is inherently a shorter process, with the capacity for a large number of samples, and the added benefit that further markers can be found quickly by the use of additional primer pairs at the selective PCR stage. By comparison, RFLPs are laborious and incompatible with applications that require high throughput, despite their high repeatability (Williams *et al.*, 1993).

Twenty genotypes could be resolved with the AFLP technique. Of these, the A5 group could be split on the basis of the commonly used RFLP technique (*Hae*III digest/M13 probe) into R5 and R14 groups, and the A1/R1 group could be divided into three groups on the basis of *Hae*III restriction followed by ethidium bromide staining. Thus, a total of 23 genotypes could be identified among the forty-six study populations, of which two genotypes were unique to overseas accessions.

4.6.2 Inter-population variation

Twenty-one genotypes were detected among the 44 Australian populations sampled. Populations of *H. perforatum* were polymorphic, which is not surprising given the putative hybrid origin of this facultative apomict (Noack, 1939; Robson, 1981), and evidence for multiple introductions of the plant (Harris & Gill, 1997). Apomixis seems to be associated with hybridization and polyploidy, and high heterozygosity is characteristic of species with this mode of reproduction (Asker & Jerling, 1992; Nogler, 1984; Pamilo, 1987). It is not surprising then, that even a low degree of sexuality in this facultative apomict should produce significant levels of clonal variation, either in the home or introduced range. The high degree of apomixis and relatively short time since establishment of the weed suggests that most of the genotypic variation is a result of multiple introductions of existing

genotypes of the weed. However, we cannot exclude the possibility that some of the genotypes originated within Australia as a result of recombination events or somatic mutation. Somatic variation in either the home or introduced range may be responsible for the presence of minor RFLP band variation among populations, for example between R5 and R14 genotypes (Section 4.5.1.1), and among R1 populations (Sections 4.5.1.3 and 4.5.1.4).

The results substantiated a genetic basis for much of the variation presented by Campbell *et al.* (1992). The “broad-leaved” group, Orange-1 and 2 (A10), was distinctly different morphologically from the samples to which Campbell *et al.* compared it. Among the “narrow-leaved” samples Adelong (A3), Mudgee Tall (A4), Tuena (A12), Captains Flat (A7) and Coolah (A1), Campbell *et al.* (1992) noted differences in stem, bud and/or sepal colour in plants from the first three accessions, which is not surprising given the genetic diversity discovered within this group. However, the present results showed that the “intermediate” Mudgee Short and “narrow-leaved” Coolah accessions supplied by Campbell were the same A1 genotype. When considering RFLP sub-group data, Mudgee Short belonged to R1 group c, and although no R1 subgroup data was available for Campbell’s A1 Coolah accession, another accession from the same Coolah property was also R1 group c. Interestingly, anecdotal evidence was recently provided that mite susceptibility varied at the Coolah property (Richard Arnott pers. comm.) and it may be that one of the “narrow-leaved” genotypes such as A4 comprised Campbell’s study material from Coolah. It is possible that Coolah material supplied for the present study was not the same as that used in Campbell’s study.

4.6.3 Intra-population variation

High diversity among populations contrasted with low variation within populations; only five of the 46 populations tested with a combination of AFLP and RFLP techniques were variable. In particular, no variation was detected within transect (A1 and A2 profiles) and quadrat sites (A4 profile) where sample sizes were largest, which suggested that these populations were monomorphic. Even where sample sizes were small, the lack of variation detected at the majority of sites, together with the probability of a high degree of apomixis, suggested a similarly low level of variation within most Australian populations of *H. perforatum*. Several factors are likely to have contributed to low within-population variation in Australian populations of this weed. Firstly, in the plant's introduced range, many of the populations are likely to have arisen from only a few founders, and secondly, as discussed above, the weed has demonstrated a high degree of apomixis and has been established in this country for a relatively short period of time.

Variation within three of the populations, at Mudgee, Turon River, and Bonganditj Forest, which consisted of a mixture of commonly found genotypes, is likely to indicate immigration through original introductions and/or spread rather than recombination events, given the low level of sexuality in this species and that the genotypes involved were also represented at other sites. A similar scenario may also be responsible for the presence of two R1 subgroups at Wyangala Dam (Sections 4.5.1.3 and 4.5.1.4, and Table 4.1). Somaclonal variation either in the home or introduced range may be responsible for minor band variation within the Tuena-J accession (Section 4.5.2.3).

In the case of most inbred, clonal, or predominantly selfing crop varieties, Morell *et al.* (1995) suggested that the DNA profile of one to several individuals would be representative of the variety, since they exhibit little genetic variation within the taxon. It appears that populations of *H. perforatum* may also be characterized in this manner.

4.6.4 Genetic relationships

The above results emphasized the largely clonal nature of Australian *H. perforatum* populations; therefore, genetic relationships were investigated among profiles rather than populations. Relatedness among genotypes of *H. perforatum* was explored using phylogenetic methods prior to examining susceptibility to *Aculus hyperici* (Chapter 5). Genetic relationships among clones were also examined from the point of view of invasion biology (rather than to reconstruct historical biogeography) since it was clear that most of the variation present in and among the populations of interest had evolved in other regions.

It was quite clear that the two methods produced different genetic distances between clones (Figs. 4.3 and 4.9). This might be explained by differences in the evolutionary history of target sequences. The RFLP targets are minisatellite sequence, which experience low selection pressure and are expected to evolve very rapidly (Sunnucks, 2000), while AFLP targets restriction sites presumably interspersed throughout the genome and with a higher probability of occurring in conserved regions such as functional genes. More rapid evolution of minisatellite DNA probably explains why all RFLP loci were informative among profiles compared with 72% of AFLP loci (Sections 4.5.1.1 and 4.5.2.1). Likewise, somatic variation in an RFLP minisatellite target may have been responsible for the single band difference between R5 and R14, two genotypes that could not be differentiated by AFLP. Differences in selection pressure between targeted regions may account for the fact that genetic relationships based solely on RFLP or AFLP data did not concur. Although poorly resolved and thus speculative, relationships based on AFLP made more biological sense than RFLP when plant morphology was considered.

RFLP analysis indicated that R5 and R14 were very closely related, with strong support from the bootstrap (Fig. 4.4). This result is not surprising given that the two profiles differed by only one band, and as suggested above this may indicate a recent origin via

somatic mutation (Section 4.6.2). Apart from this instance, however, bootstrap analysis of genetic distance using RFLP profiles provided very little statistical support for groupings. In any event, UPGMA analysis revealed three tied trees, and bootstrap values are likely to be misleading (Backeljau *et al.*, 1996).

Even in the absence of tied trees, analysis of the AFLP sub-set did not provide confident resolution, with the exception of a strongly resolved clade excluding A9 and A11 (Fig. 4.10). Although this clade was not resolved by the RFLP dataset, there were no nodes that resolved with confidence that conflict with that resolution. RFLP and AFLP scores would be expected to be completely independent measures of variation, but a combined analysis of 13 profiles made to test relationships more accurately failed to improve the resolution of nodes (Fig. 4.12).

Analysis of the AFLP full data set revealed few groups that resolved with greater than 50% support. In both cases, UPGMA analysis revealed three tied trees, and bootstrap values are again likely to be misleading. Those groups in the full data set that did resolve with confidence included the minor band variants in the Tuena J accession (A12, A19 and A20), diploids with ploidy-related band variation (A21 and A22), and the A5 and A17 profiles. The Tuena J accession profiles differed by only one band (Fig. 4.8), which suggests a recent origin via somatic mutation. The latter two genotypes were clearly closely related (Fig. 4.13 and Appendix B) and may represent recombination events in the home or introduced range.

The results provide some support for the two groups, broad-leaved and narrow-leaved, suggested by Campbell *et al.* (1997) on the basis of morphology (but no support for the intermediate form, as discussed in Section 4.6.2). Despite low bootstrap values, AFLP and combined analyses suggested that the broad-leaved genotype A10 was distantly related to the other forms (A1, A3, A4, A7 and A12) examined by those authors. Moreover, two

other distinctly broad-leaved forms (A9 and A11) were the most distant genetically when compared to all other genotypes in AFLP and combined analyses, and were consistently placed in a separate clade. Although relationships among them were unclear, all the more closely related forms were relatively narrow-leaved, with the exception of A6, a large-leaved tall genotype (Fig. 4.17). From this we might speculate that broad-leaved forms were distantly related to narrow-leaved forms, which would be consistent with a morphological cline between wide-leaved forms across northern Europe and the United Kingdom, and narrow or small-leaved forms in the south (Robson, 1968). The genetic analysis and leaf morphology suggested that genotype A6 was intermediate between morphological groups, which may reflect a geographic origin between the two. The broad-leaved forms were as genetically different from one another as from narrow-leaved forms, which suggests their originating from diverse regions and supports a theory of separate introductions. The narrow-leaved forms appeared to be more closely related and it may be that they originated from a more restricted geographical region in the home range. On the other hand, certain narrow-leaved forms have been recognized to produce higher concentrations of pharmacologically active compounds, and the closer relationships and preponderance of narrow-leaved forms in Australia may reflect selection and breeding for medicinal use in the home range prior to deliberate introduction.

Additional data would be required for ultimate resolution of the relationships among *H. perforatum* genotypes, and should include samples from the centre of origin.

4.6.5 Ploidy

The variation seen in fingerprint pattern at different ploidy levels clearly demonstrates that one should not ignore the ploidy of individuals in molecular analyses of *H. perforatum*. The present study revealed band absences in a diploid progeny compared with the maternal tetraploid parent, and band absences in a diploid and aneuploid ($2n+1 = 17$) compared to

tetraploid congeners. Research presented in Chapter 3 showed that deviations in ploidy level were invariably detected by multilocus methods in progeny of tetraploid crosses, in which cytological examination was made for 121 fingerprinted progeny; hexaploid progeny ($2n = 48$) contained additional donor bands, and an aneuploid ($2n-1 = 31$) lacked bands, compared to the maternal tetraploid. The only exception to the ability of molecular methods to detect ploidy variation was the aneuploid Cass fine-2 ($n = 17$), which was not distinguishable from the euploid Cass fine-1 ($n = 16$). It is therefore likely but not inevitable that individuals sharing a multilocus profile and morphology also share ploidy.

Time constraints precluded examination of all 298 fingerprinted individuals with cytological techniques, but in most cases (excluding A19 and A20) more than one individual with each profile was examined per genotype (Section 4.5.3). Of those examined 43 individuals were tetraploid, and the hexaploid, diploid and aneuploid ($2n = 17$) were included in screening on the basis of unusual morphology. These results suggest that tetraploidy was the most common level in the field, but provided some evidence for the existence of other ploidies. A diploid, aneuploid and hexaploid with unusual morphology were observed in seed progeny from the Cassilis field site, which had otherwise proved to contain the A1 genotype. One can only speculate as to reproductive origin because parentage was unrecorded, and while reduced parthenogenesis or unreduced double-fertilized reproductive pathways may have been responsible, an alternative explanation is that ploidy variants already existed as components of the population. However, the F1 offspring of the A1 genotype cross (MudJ m ♀ x Wyan m) was clearly the product of haploid parthenogenesis, and both pathways have recently been shown to occur commonly in tetraploid *H. perforatum* accessions (Matzk *et al.*, 2001). The hexaploid grew vigorously, and despite the diploids and aneuploid being relatively small plants, they appeared healthy and survived for the duration of the project under glasshouse

conditions; therefore there is no reason to suspect that such seedlings would not germinate and persist under field conditions. Obvious morphological variation was not observed in other genotypes, which could mean that the A1 genotype produces relatively higher proportions of reduced parthenogenetic and unreduced double-fertilized progeny under field conditions, but cytological studies with increased sample size (approximately 20 to 30 seedlings were raised per accession) would be required to address this question.

The results indicated that reduced parthenogenesis in tetraploids could result in naturally occurring diploids within the *H. perforatum* complex. The morphology of the diploids identified in this project raises taxonomic issues. Past authors have indicated the existence of diploid, small-leaved *H. perforatum* in Greece (Papanicolaou, 1984; Strid & Franzén, 1981), Belgium and Italy (Campbell *et al.*, 1997), which were given the varietal epithet *angustifolium*. However, Campbell *et al.* (1997) considered that all small-leaved herbarium specimens examined more closely resembled var. *microphyllum*, and suggested that the latter may all be diploid. Robson (1981) left the question open as to whether varieties should even constitute separate taxa. To complicate the issue, Matzk *et al.* (2001) found that two accessions of diploid obligate sexual *H. perforatum* were not morphologically distinguishable from tetraploids at juvenile or adult stages of development, and reported incorrect determination of three diploid sexual accessions of *H. maculatum* as *H. perforatum*. Confusion over diploid *H. perforatum* is not surprising, since identifications were based on morphology, and one would expect morphological variability among dihaploid progeny of tetraploids as a result of chromosome assortment, the species' hybrid origin, and high heterozygosity. Since all diploid *H. perforatum* reported to date have been obligate sexuals, ongoing hybridization and segregation would also be expected to contribute to variability within diploids.

4.6.6 Morphology

Subjective evaluation revealed plant morphologies characteristic of each genotype, and uniform within seed batches, which reflected low intra- and higher inter-population genetic variation. Plant morphology was complex and the task of identifying genotypes by plant phenotype was not pursued, even though there was some support from phylogenetic analysis for the past groupings into “broad-leaved” and “narrow-leaved” groups within Australia. The current discovery that the majority of genotypes were tetraploid ($2n = 32$), including the three distinctly broad-leaved genotypes (A9, A10 and A11), does not support Robson’s (1968) suggestion that cytological variability may be associated with the morphological cline from broad-leaved, large-flowered forms (var. *perforatum*) in northern Europe to narrow-leaved, smaller-flowered forms (var. *angustifolium*) in the south. However, the results agree with the suggestion (Campbell *et al.*, 1997) that the small-leaved var. *microphyllum* occurring sympatrically with the narrow-leaved var. *angustifolium* in southern Europe may be diploid. Perhaps some narrow-leaved genotypes from the south of the European range are more likely to produce dihaploids than others, since in Australia diploids were only found in progeny of a narrow-leaved A1 maternal parent or seed from an A1 population (Section 4.6.5).

4.6.7 Distribution

The data support existing theories that multiple introductions of the weed have occurred and spread as isolated outbreaks, from which expansion has occurred until populations coalesced (Harris & Gill, 1997). The wide distribution of the A5 genotype across southern Australia from the coast of Western Australia to eastern Victoria (Fig. 4.19) probably reflects length of time since introduction, and suggests that this genotype may have originated from the first outbreak in Victoria. Beechworth and Benambra A5 populations are located either side of Bright, a gold-mining town with the first recorded infestation in

Victoria (French, 1905). The Bright infestation is said to have spread up and down the Ovens Valley, across the state by 1905 via stock feed to the gold mining areas of Clunes, Newstead and Bendigo (near the Castlemaine A5 population), and via railway lines to the north-east of the state by the 1920s (Parsons, 1973). Among the A5 populations RFLP could distinguish two genotypes, R14 at Flowerdale and R5 at all other sites. The close genetic relationship between R5 and R14 profiles suggested a recent origin by somatic mutation (Section 4.6.4), and the isolated occurrence of R14 indicated that it might have arisen within the introduced range of the R5 genotype. However, RFLP samples containing the R5 and R14 profiles were small, consisting of only one individual from each of eight populations, and conclusions about the distribution of R14 are therefore premature.

The pathway of introduction into south-eastern Victoria may have been via Wonnangatta from the Ovens Valley as suspected by Calvert (1932), since the original distribution map of the infestations in the Bright district showed that the weed was also present at Wonnangatta by 1905 (French, 1905). The present study demonstrated that the A5 genotype in the Ovens Valley was not the same but was related to the A17 genotype in the Wonnangatta area (Figs. 4.13 and 4.14). The A17 population may represent spread of a related variant contained in the original introduction, recombination within an Australian population, or a separate introduction. Both genotypes are candidates for spread over the Great Dividing Range and into Gippsland (Parsons, 1973).

The historical pattern of spread westwards across Victoria suggests that the A5 and A7 genotypes at the Bonganditj Forest site in South Australia, near the border of Victoria (Fig. 4.20), and the Western Australian A5 populations (Fig. 4.21), were a result of the spread of existing genotypes. However, the suggested spread from the Bright introduction across the high plains of the Australian Alps via stock routes and into NSW before the end of the last century (Davey, 1919) is not supported by the data. An alternative origin of the first

recorded infestation in the south of NSW in 1899 at Tumbarumba was via importation from England or Germany by a local family (Calvert, 1932). Although population samples were sparse in this area, Tumbarumba is situated centrally between study populations containing the A7 genotype at Talmalmo, Bemboka and Canberra. The A7 genotype did not show close genetic similarity to the A5 genotype from the Ovens Valley (Figs. 4.3, 4.9, 4.11 and 4.13). History, dissimilarity and the geographical extent of the A7 genotype therefore suggest a separate introduction into New South Wales.

The earliest record of the weed in New South Wales is from Mudgee, in 1890 (Harris & Gill, 1997), and may refer to the A1 and/or A4 genotype. In the present study, the A1 genotype was shown to occur only in the Mudgee area, and as such probably represents a separate introduction. However, the A4 genotype occurred in two States, and may either have arrived in Mudgee from central Victoria or the converse may be true, since both were gold mining centres from the early 1850s onwards.

The A6 genotype exclusive to the Scott Creek population is approximately 10km from the site of the earliest recorded introduction in Coromandel Valley, South Australia, in 1881 (Harris & Gill, 1997). While the A6 genotype has probably existed in the country for some time, other genotypes unique to one population may represent more recent introductions. It is important to bear in mind that opportunistic rather than random sampling may have influenced results, and in future field studies it would be of benefit to expand our knowledge of genotype distribution by random sampling within and among many more populations.

4.6.8 Implications for biological control

The results of this study demonstrate that while the assumption of genetic uniformity within asexual weed populations (Burdon & Marshall, 1981) may hold within populations of *H. perforatum*, Australian populations of this facultative apomict are not genetically depauperate. This is not unexpected, because the high heterozygosity of facultative apomictic species creates the potential for production of many new gene combinations that can be stabilized by apomixis.

Given this particular population structure, plant characteristics such as hypericin level and susceptibility to herbivores or pathogens are expected to be relatively uniform within a population, but not necessarily between populations. Failure of *A. hyperici* to establish on some populations of *H. perforatum* in Australia has been linked with variable susceptibility among known forms of the weed in northern New South Wales (Jupp *et al.*, 1997). Similar variation in susceptibility to biological control agents has been experienced with two other apomictic weeds in this country, *Rubus fruticosus* L. aggregate (blackberry) and *Chondrilla juncea* (skeleton weed). The differential susceptibility of genetically distinct forms of blackberry to several forms of the rust fungus *Phragmidium violaceum* has been demonstrated (Evans *et al.*, 1999). Three forms of skeleton weed have been shown to exist in Australia (Burdon *et al.*, 1980; Hull & Groves, 1973), but despite aggressive control of the widespread narrow-leaf form by the agent *Puccinia chondrillina*, success was compromised by a corresponding increase in distribution of two other forms of the weed (Burdon *et al.*, 1981). Despite the expectation of good control within populations of *H. perforatum* susceptible to *A. hyperici*, fears of a similar replacement scenario in the case of this weed are well founded, given resistance in at least one of the genotypes (Jupp *et al.*, 1997), the number of genotypes demonstrated in this study, and the ability of some of them to expand and thrive across a wide range in Australia. Multilocus techniques were

employed in the following chapter, in an examination of the relationship between target weed genotypes and susceptibility to the mite, in order to determine the potential extent of the problem. In the case of resistant genotypes, DNA-based identification and historical evidence of origin would assist in narrowing any future search for more effective natural enemies in the home range.

There is little doubt from the diversity and distribution of genotypes seen in this study, and from historical evidence in the literature, that multiple introductions of the weed have occurred. Evidence was received during this study that introductions were ongoing despite quarantine restrictions; seed originating from Copenhagen was confiscated from a store in Ballarat in 1977, and Canadian material was illegally imported into Perth in 1996. The diversity already present in this country, and the detection of two unique AFLP fingerprints in as many overseas accessions suggests that there is high risk of introduction of new genotypes into Australia. Given the current resurgence of interest in *H. perforatum* as a medicinal herb and on-line access to overseas material via the Internet, illegal importation into this country is likely to be the major cause of future increase in genetic variability in the weed.

4.7 Summary

The multilocus RFLP and AFLP techniques developed in this study provided much-improved methods for the identification of genotypes and estimation of genetic diversity within and among populations of *H. perforatum*. The variation detected in fingerprint patterns at different ploidy levels demonstrated that ploidy should be considered in molecular analyses of this species. Results of cytological and molecular analyses suggested that the majority of populations were tetraploid but a small proportion of other ploidies may also exist. High inter-population variation contrasted with low intra-population variation. With a combination of AFLP and RFLP techniques, 21 genotypes could be identified among the 44 Australian populations, but within-population variation was so low that in most cases populations appeared to be clonal, probably as a result of a high degree of apomixis and introduction via a few founding individuals. Consequently, plant characteristics such as susceptibility to herbivores were expected to be relatively uniform within but not necessarily among populations, and differential control may be experienced. The RFLP and AFLP datasets provided very different genetic distance measures between profiles. Few conclusions could be drawn with respect to relationships because few nodes were resolved with much confidence. Among the well-supported close relationships, it was suggested that somatic variation was responsible for those between R5 and R14, and among Tuena J accession variants, and recombination for that between A5 and A17. There was some evidence from genetic relationships in support of two morphological groups, broad-leaved and narrow-leaved, despite low resolution of nodes. There was little doubt from the diversity and distribution of genotypes included in the study, and from historical evidence in the literature, that multiple introductions of the weed have occurred and are likely to continue in the future. Knowledge of the distribution of genotypes was applied in the following chapter to an examination of the relationship between target weed genotypes and susceptibility to *A. hyperici* in order to determine the geographical extent of resistance.

5 Susceptibility to *Aculus hyperici*

5.1 Introductory review

Variable establishment success of the mite *Aculus hyperici* was experienced at release sites on *Hypericum perforatum* across eastern Australia, and ranged from rapid increases with observable plant damage after two years, to repeated failure to establish (Jupp *et al.*, 1997; Jupp & Cullen, 1996). Variable susceptibility to the mite was demonstrated among four known morphological forms (Jupp *et al.*, 1997). Failure to establish at two sites in northern New South Wales was most likely a result of low host-plant susceptibility, since laboratory experiments showed that mite populations did not increase on plants collected from those sites, compared with significant increases on plants from four other sites with successful establishment (Jupp *et al.*, 1997). A significant negative relationship of mite density with root and shoot growth was also demonstrated, indicating that reduction in plant growth and vigour could be expected in plants from the latter but not the former sites (Jupp *et al.*, 1997). A major concern was that variation in susceptibility to *A. hyperici* would not only reduce the initial impact of this highly promising biological control agent, but as pointed out in Chapter 4, might lead to replacement of susceptible with resistant varieties.

Host-plant susceptibility was investigated among genotypes of *H. perforatum*, for individuals that had been characterized with fingerprinting techniques in Chapter 4. Techniques were developed for inoculation, sampling and assessment of population increase in *A. hyperici*. The correlation of genotype with host-plant susceptibility was examined in the light of field establishment and impact studies, and the extent of resistance to *A. hyperici* predicted from the geographic distribution of genotypes of *H. perforatum*. Factors that might account for variable host-plant susceptibility were examined in Chapters 6 and 7.

5.1.1 Host specificity

Monophagy, in the strict sense, refers to a predator feeding only on one species. However, it is often used more broadly to include host species within a genus or family (Bernays & Chapman, 1994). Host specificity is the most important ecological characteristic in successful biological control of weeds, as it reduces the risk of an agent endangering other plant species (Bernays, 1985; Crawley, 1989; Huffaker, 1978; McLaren, 1993; Wilson, 1964). Mites and plant pathogens often have very narrow host ranges and may be specific to certain forms of a host weed, for example strains of *Puccinia chondrillina* rust fungus and *Aceria chondrillae* gall mite, which are specific to different forms of *Chondrilla juncea* (skeleton weed) (Caresche & Wapshere, 1975; Cullen & Moore, 1983; Wapshere *et al.*, 1989). Unfortunately, such variation in susceptibility within the target weed species may reduce the overall success of a biological control program.

Non-choice tests as sole indicators of host range are considered by some to be inappropriate (Bernays & Graham, 1988; Thompson, 1988), because complex interactions between host and herbivore behaviour, physiology and ecology, ultimately determine host-selection (Willis, 1994). However, such tests at least clearly indicate the taxa on which herbivores are physiologically incapable of surviving (Cullen, 1989).

5.1.2 Coevolution and resistance

Selection acting on genetic variation is the basis of adaptation and coevolution (Futuyma, 1979; Gould, 1983; New, 1988). The ability of organisms to reciprocally evolve defense and counter-defense mechanisms can result in geographical and biological races of both predator and host (Bush & Hoy, 1984). The basis of resistance is essentially biochemical or morphological, and permits a plant to ward off, tolerate or recuperate from the attacks of herbivores which would cause much more damage to other plants of the same species under similar environmental conditions (Kogan, in Jolivet, 1986). Much more quantitative

information on genetic variation in susceptibility to herbivores is available for agricultural crops than for weeds or species of low economic importance. Gould (1983) summarizes studies of variation in response to herbivores in many agriculturally important species, including *Lotus*, ginger, *Passiflora*, alfalfa and wild potato. A genetic basis for variation in weedy populations in susceptibility to herbivore or pathogens has been reported for skeleton weed (Caresche & Wapshere, 1975), cocklebur (Hare & Futuyma, 1978) and blackberry (Evans *et al.*, 1999).

The course of coevolution is most likely thus; plants evolve defenses that are overcome by only a few generalized and specialized enemy species. The specialized herbivore species evolve counter-defenses, this selects for plant species with specific counter-counterdefenses, a subset of specialized herbivores is selected for, and so on. Fortuitous cross-resistance may add new specialists, resulting in new selective pressure, and so new adaptations are selected for. At some point in this specialization, so few herbivore species feed on so few plant species that adaptations no longer result in higher susceptibility to other herbivores (Futuyma, 1979).

It is advantageous to the host plant to evolve a different defense system to coexisting plant species, so that it is less "apparent", *sensu* Feeny (1976), and not subjected to herbivores harboured by others. In turn, it is expected that herbivores become more specialized as plants develop divergent defenses, because it is more difficult to evolve a counter-defense against many different defense systems (Futuyma, 1979). Plant defense chemicals may act as cues for species that specialize on them, and specialist herbivores may become so dependent on these that they do not feed on other potentially suitable plants. However, herbivores can utilize a broader diet if they evolve cross-resistance mechanisms (resistance against one defense is also conferred against another), and host range thus depends on the evolution of cross-resistance (Futuyma, 1979). Cross-resistance in *A. hyperici* may be

responsible for the inclusion of several other *Hypericum* species in the host range of the mite (Section 5.1.4.2). Alternatively, defensive chemicals acting as obligatory feeding stimuli may have been lost in closely related species not included in the mite's host range.

At the subspecific level, evolution of defense systems in plants and herbivores results in genetic polymorphisms among populations of both. Ecological factors such as differences in plant phenology or mobility of insects may reduce interbreeding between herbivore groups to such an extent that "races" with pronounced genetic differences may occur. Intraspecific competition for resources may continue to keep specialized races separate (New, 1988).

5.1.3 Eriophyoid mites and biological control

The Eriophyoidea contains the four-legged phytophagous mites with worm-like shape, known as rust, blister, bud, gall, and erineum mites (Jeppson *et al.*, 1975). Eriophyoids range from 100 to 400 microns in length; hence they are essentially invisible to the naked eye. The Eriophyidae are phytophagous, typically with perennial hosts, and contain the majority of gall and erineum producing species (erinea are an excessive growth of plant hairs) [Cromroy, 1976 #201. Genera in the subfamily Phyllocoptinae, to which *Aculus* belongs, are commonly leaf vagrants rather than gall formers (Oldfield, 1996a; Wapshere, 1984), and include an especially large number of species that cause "russeting" or browning of leaves (Oldfield, 1996b). *Aculus* appear to cause more serious russeting damage than many genera (Jeppson *et al.*, 1975).

Their host specificity, potential to damage plants and ability to disperse readily, make eriophyoids good candidates as both classical and augmentative biological control agents (Boczek & Petanovic, 1996; Cromroy, 1976). Within the group, the Eriophyidae has the greatest potential for weed control, with estimates that 95% of the family is genus specific

(Cromroy, 1976), of which 80% may be restricted to a single plant species (Boczek & Petanovic, 1996). Almost all important weed species in Europe have at least one associated species of eriophyid mite (Boczek & Petanovic, 1996). Eriophyids suppress plant growth and reproduction, and are capable of destroying whole plant populations (Boczek & Petanovic, 1996). Mites in this family usually target specific tissues, therefore they are ideal to include in a suite of herbivores from different feeding guilds, to improve the biological control of a weed (Cromroy, 1976; Willis *et al.*, 1993). *Aceria chondrillae* has been used in the control of *Chondrilla juncea* in Australia (Caresche & Wapshere, 1975), and *A. malherbae* in the control of *Convolvulus arvensis* (field bindweed) in the United States (Boldt & Sobhian, 1993).

5.1.4 *Aculus hyperici*

5.1.4.1 Taxonomy and biology

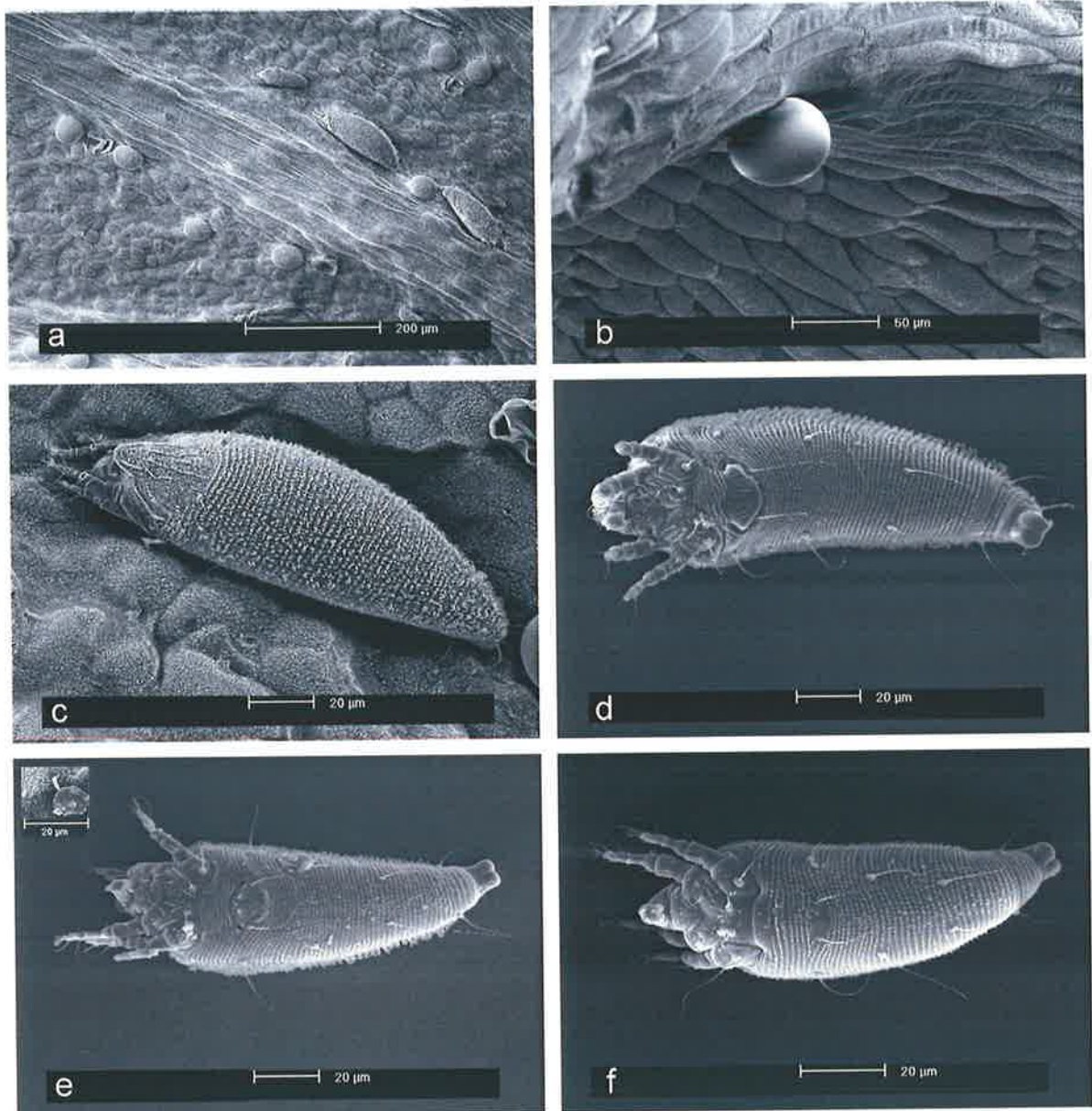
Aculus hyperici (Eriophyidae: Phyllocoptinae) was originally described as *Phyllocoptes hyperici* Liro, before inclusion in the genus *Vasates* (Wapshere, 1984), then *Aculops* (Jeppson *et al.*, 1975). *Aculus hyperici* has become the binomial accepted in biological control, due to synonymy of the latter genus with *Aculus* (Boczek, in CSIRO, 1987). *A. hyperici* has been observed in almost all areas of Europe where *H. perforatum* is found (CSIRO, 1987; Wapshere, 1984). The Australian collection originates from a site at La Salade, 10 kilometres north of Montpellier, France (CSIRO, 1987).

The life cycle of *A. hyperici* in the field is 40 days, with females laying up to 40 eggs (CSIRO, 1995). Mean fecundity at 20°C is 24.6 nymphs per female (CSIRO, 1990). Development time varies with temperature; at 17 and 20°C, respectively, time to adulthood is 8.7 and 7.8 days and adult longevity is 13 and 14 days. Increasing temperature accelerated development but resulted in higher mortality; therefore optimal development

appeared to be at around 15°C. Temperature did not affect percentage eclosion (Valin, 1986). Eggs are approximately 40 µm in diameter, spherical, and glued singly either side of the veins at the base of the leaves in the buds (Valin, 1986), or in dense infestations, on stems and petioles (Willis, 1994). The upper side of the leaf seems to be preferred for oviposition (Valin, 1986; Willis, 1994), but eggs were also commonly observed on the underside of leaves during this project (Fig. 5.1 a and b). Eggs are translucent when laid, becoming opaque prior to eclosion. As is general for eriophyoids, there are two larval and two resting stages (Jeppson *et al.*, 1975). First instar larvae are 75 × 35 µm (Fig. 5.1 a) and second instar larvae are 110 × 45 µm (Fig. 5.1 f).

Resting stages between the first and second stages (nymphochrysalis and imagochrysalis, respectively) are elongated, and last around 24 hours (Valin, 1986). Females (Fig. 5.1 c and d) are generally 150 × 55 µm (Valin, 1986). Unfertilized females produce only males, whereas females hatch from fertilized eggs (Jeppson *et al.*, 1975). Some variation in adult length has been noted, although all stages were found throughout the year (Valin, 1986), in contrast to a report by Wapshere (1984) of a winter resistant deuterozygous stage. Deuterogyny occurs when a secondary female or deuterozygote, quite different in appearance to primary females, carries the species through unfavorable conditions (Jeppson *et al.*, 1975). Males (Fig. 5.1 e) are extremely difficult to distinguish by size from second instar larvae (Valin, 1986). Males deposit spermatophores in areas frequented by females (Fig. 5.1 e, inset).

Figure 5.1. Life stages of *Aculus hyperici*; a) egg, adult, and larval stages on underside of *H. perforatum* leaf midrib; b) egg on underside of leaf; c) adult female, dorsal view; d) adult female, ventral view; e) adult male, ventral view with inset of spermatophore; f) second instar larva, ventral view.



Dispersal is principally by wind, (CSIRO, 1990), but eriophyids may also be transported phoretically (Slykhuis, 1973). In spring, infestations increase and *A. hyperici* disperses to other rosettes by swarming and leaping from the leaf surface of upright summer flowering stems, and in autumn, mites descend the plant and infest basal buds prior to a winter population decline (Wapshere, 1984).

5.1.4.2 Host-specificity of *Aculus hyperici*

Aculus hyperici appears to be restricted to the genus *Hypericum* (CSIRO, 1987; Wapshere, 1984). A mite rating system based on visual assessment of number of dead or live mites per bud or plant was used for host specificity testing (CSIRO, 1987; CSIRO, 1990). Results indicated that the mite could persist for a number of weeks on 17 *Hypericum* species (Table 5.1). However, on all but *H. pulchrum*, *H. tetrapterum* and *H. perforatum*, populations declined and died out after several months (CSIRO, 1987). On *H. pulchrum* and the noxious weed *H. tetrapterum* (St. Peter's wort), symptoms were severe and level of damage was similar to *H. perforatum*. Mild symptoms were noted on *H. canariense*, *H. reptans* and *H. gramineum* only while mite numbers were high. No symptoms of damage were observed on any other species tested (CSIRO, 1987; CSIRO, 1990).

Table 5.1. Persistence of *Aculus hyperici* on species of *Hypericum* tested for host-specificity (adapted from CSIRO, 1990).

species	persistence
<i>H. moseranum</i>	< 4 weeks, with rapid population decline
<i>H. patulum</i>	"
<i>H. canariense</i> *	< 10 weeks, with steady population decline
<i>H. japonicum</i> *	< 12 weeks, few individuals throughout trial
<i>H. beanii</i>	12 weeks, with rapid decline and few individuals after 4 weeks
<i>H. 'Hidcote'</i>	"
<i>H. monogynum</i>	"
<i>H. stellatum</i>	"
<i>H. olympicum</i>	"
<i>H. reptans</i> *	"
<i>H. gramineum</i> *	< 14 weeks, with steady population decline
<i>H. calycinum</i>	16 weeks, with population increase on lower leaves then decrease
<i>H. 'Rowallane'</i> *	"
<i>H. kouytchense</i>	as above, but terminated at 10 weeks
<i>H. pulchrum</i> *	Rapid population increase throughout trial
<i>H. tetrapterum</i> *	Rapid population increase with plant death before end of trial
<i>H. perforatum</i> *	Rapid population increase with some plants dying before end of trial

*excised leaves also used in *A. hyperici* longevity and fecundity trial

Excised leaf trials (marked with an asterisk in Table 5.1) showed significant reduction in longevity and/or fecundity in *A. hyperici* on all *Hypericum* species compared with *H. perforatum*, with the exception of *H. pulchrum*, *H. tetrapterum* and *H. gramineum* (although the non-significance of reduction in the latter may have been due to high variation in the data). Even relatively small reductions in fecundity and longevity in the excised leaf trial seemed to be sufficient to prevent populations persisting in those species (CSIRO, 1990). *A. hyperici* has also been recorded in low numbers on *H. quadrangulum* and *H. hirsutum* outside Australia (CSIRO, 1987).

It was concluded that the Australian endemic *H. japonicum* was not at risk from *A. hyperici*, given results of the specificity tests and lack of contact with *H. perforatum* in the field (CSIRO, 1990). Despite the inability of *A. hyperici* to persist in host-specificity trials on the only other Australian endemic, *H. gramineum*, a concern was expressed that high mite populations on *H. perforatum* in close proximity to the native might act as reservoirs for re-infestation, and that some symptoms of damage may be observed on the native at certain times of the year. However, this risk was expected to reduce as population density of the introduced weed declined (CSIRO, 1990). In a subsequent field study of host selection, *H. gramineum* was significantly less likely to become infested with the mite than was *H. perforatum*, but still had a 31% chance of becoming infested (Willis, 1994). *A. hyperici* was not found on four other taxa outside the genus, which supports the hypothesis that host-selection behaviour is specific to *Hypericum*.

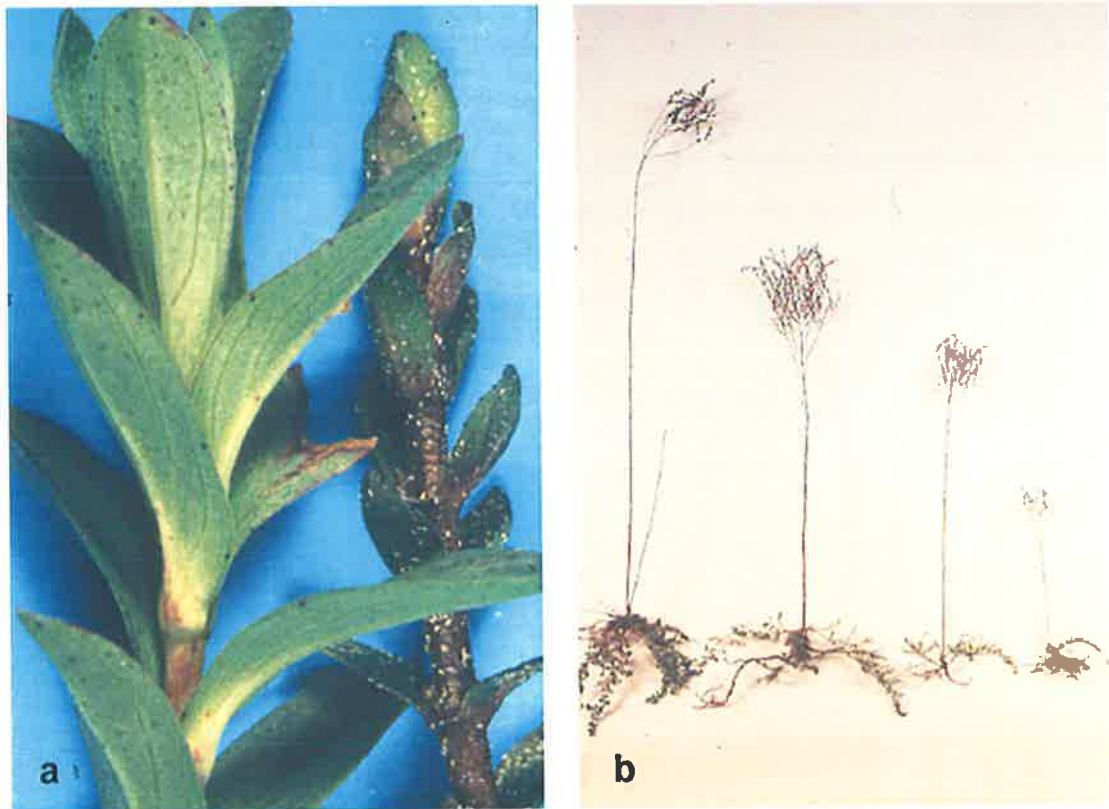
Re-analysis of larval development and reproductive maturation data collected during the host-specificity trials of *A. hyperici*, by a nested analysis of variance structured to reflect the inferred phylogeny of *Hypericum* species tested, supported the original conclusion that *H. perforatum* was the most suitable species for development of the mite (Willis, 1994). Phylogenetic patterns were not detected, suggesting that while there is a clear range in

suitability of different species as hosts, those closely related to *H. perforatum* do not necessarily serve as better hosts than more distantly related species. Thus it appeared that *A. hyperici* responded to the particular traits of individual species. Clear species differences were seen in oviposition and larval development times, but adult longevity was not significantly different among *Hypericum* species, so host-specialization may be of primary importance during oviposition and larval growth (Willis, 1994). Oviposition on *H. perforatum* was significantly higher than on any other species, for the proportion of leaves that received eggs (with the exception of *H. gramineum*), the total number of eggs laid, and the number of eggs hatching. *H. perforatum*, *H. gramineum* and *H. pulchrum* supported the shortest larval development times, which implied that the physiology and chemistry of these species did not retard maturation (Willis, 1994).

5.1.4.3 Impact assessment

Host specificity screening, while it may confirm suitability for establishment and population increase, rarely examines the impact that agents may have on plant growth (Willis, 1994). *A. hyperici* concentrate in the axillary, terminal and flower buds, and on flowers and fruit of *H. perforatum*. Infested leaves become dwarfed and thickened (Fig. 5.2 a), later becoming chlorotic and/or mottled orange-brown and falling from the plant (CSIRO, 1990). Internode length is reduced, and in extreme cases the plant resembles a cushion with short internodes and scale-like leaves (Wapshere, 1984). Flowering summer stems become shortened and deformed (Fig. 5.2 b), flower size is reduced, and fruit aborted. Infested plants show significant decreases in root and shoot growth; hence the mite exerts a continuous stress on the plant that leads to slow decline, which may result in death (CSIRO, 1987; CSIRO, 1990). In a natural population of *H. perforatum*, *A. hyperici* has been credited with 100% and 72% mortality of mature and young plants, respectively (Cullen and Valin unpublished data, in CSIRO, 1987).

Figure 5.2. The effect of the eriophyid mite *A. hyperici* on growth of *H. perforatum*; a) uninfested shoot tip on the left and mite-infested tip on the right; b) impact of mite infestation on shoot growth. Photographs courtesy of CSIRO.



Willis (1994) examined indices of plant growth of four species, *H. perforatum*, *H. tetrapterum*, *H. gramineum* and *H. japonicum*, in the presence and absence of *A. hyperici*. The presence of mites caused reductions in most indices of plant growth, with roots the most adversely affected; on average, root mass, shoot mass, root relative growth rate and shoot relative growth rate were reduced by 25%, 21%, 13% and 8%, respectively. There was no significant interaction between species and mites, which suggested that reduction in growth was similar for each of the species, but problems with mite establishment may have affected these results (Willis, 1994). Results from two further comparative glasshouse studies demonstrated that although there was a significant impact of *A. hyperici* on *H. gramineum*, the indigenous species was less severely affected than *H. perforatum* (Willis *et al.*, 1993; Willis *et al.*, 1995). One of the latter studies with *H. gramineum* and *H. perforatum* demonstrated a three-factor interaction between plant species, the mite *A.*

hyperici and an aphid, *Aphis chloris*. Plant growth decreased by slightly more than the product of their separate negative effects, suggesting a weak positive interaction of *A. hyperici* with the other factors (Willis *et al.*, 1993). However, under more natural conditions in the field, *A. hyperici* had no significant effect on *H. gramineum* shoot growth, and marginal effects on *H. perforatum* (Willis, 1994).

At two CSIRO field sites near Mudgee (Cudgegong and Merrendee) data from individual plants was collected in the presence and absence of *A. hyperici*, from 1994 to 1997 (Jupp & Cullen, 1996). No effect on plant density was found at either site, but significantly lower mean root and shoot dry-weights, root diameter, and number of flowering stems were demonstrated in the presence of mites at Cudgegong (Jupp & Cullen, 1996). At Merrendee, mites had established a year later than at Cudgegong, and results showed similar trends, but only a significant difference for shoot weight (Jupp & Cullen, 1996). Impact assessment was also carried out at two field sites at Castlemaine over an 18-month period, and showed a significant reduction in plant density and plant condition at both sites in the presence of mites, compared to control plots treated with miticide (Mahr *et al.*, 1999).

Host-specificity and impact of *A. hyperici* does not, however, appear to be uniform within *H. perforatum* populations. Susceptibility, as defined by establishment and population increase of *Aculus hyperici*, was extremely low in the intermediate morphotype "Mudgee Short" compared to the broad- and narrow-leaved morphotypes characterized by Campbell *et al.* (Jupp *et al.*, 1997). Negligible population increase was also demonstrated in seedlings from two sites at which the mite failed to establish, compared to seedlings from four sites with medium and high increases corresponding to successful establishment (Jupp *et al.*, 1997). A negative correlation was demonstrated between susceptibility of plants as indicated by accumulated mite rating (overall mite density and feeding pressure over time), and impact on root and shoot biomass (Jupp *et al.*, 1997).

5.2 Aims

A simple and accurate method for estimation of population size of *A. hyperici* was required for comparison of variation in host-suitability among *H. perforatum* genotypes. Accordingly, the method development section includes several investigations of techniques for inoculation and sampling of *A. hyperici*, followed by final protocols.

In order to determine the extent of variation in susceptibility and degree to which it correlated with variation in plant genotype, individuals for whom a DNA fingerprint had been ascertained (Chapter 4) were subjected to bioassay with *A. hyperici*, and mite population size was compared. The correlation of mite-response to genotype was examined, and related back to records of mite establishment in the field, where known. Variable susceptibility was then discussed in relation to genotype distribution and the successful control of *H. perforatum*.

5.3 Plant bioassay method

5.3.1 Mite culture

Mites obtained from susceptible plants near Mudgee, NSW, were raised on potted susceptible plants from Cowra, Scott Creek or Nelson, depending on plant availability. Difficulties with cultures and assays arose early in the project as a consequence of open-plan glasshouse space, with severe attack by two-spotted mite and white-fly, and because sulphur-vapour applied without consultation destroyed mite populations. Subsequently, self-contained growth rooms were used to maintain cultures and conduct assays. Inoculated culture and assay plants were maintained at rosette stage at 20°C: 16°C, 10hr light: 14hr dark, in a growth room equipped with ten 800 µMol metal halide lamps. Cultures were inspected periodically and discarded if contaminated by other mite species, fungal pathogens or insect pests.

5.3.2 Plant preparation for bioassay

Previous trials had utilized seedlings with a maximum age of 6 weeks, consisting of 5-10 shoots (CSIRO, 1987; CSIRO, 1990; Jupp *et al.*, 1997; Willis, 1994). However, a common experience in those trials was that many susceptible plants had died by the end of the experiment. Since the aims of the current study were to assess suitability of the host for establishment and population growth, rather than plant damage, older and larger plants were used in the following experiments to ensure that food was not limiting. Experiments had previously been conducted at 18-25°C (CSIRO, 1987; CSIRO, 1990; Jupp *et al.*, 1997), possibly because plant growth was slow at the optimal temperatures for mites of around 15°C.

Plants were germinated from seed and raised in a glasshouse as described in Chapter 4 (Section 4.4.2). Clonal material was taken from naturally layered stem pieces, or cuttings treated with indolebutyric acid at 2 g/L. At least eight weeks prior to bioassay, fungicide and insecticide were withheld and plants were transferred to a controlled environment room (specifications as for mite culture). Pruning was used to standardize plant architecture, in order to reduce micro-environmental effects as far as possible. Three month-old plants at rosette stage in 4" pots, with shoots 3-10 nodes in length, produced a reasonably standardized quantity of plant material of sufficient size for inoculation with mites, and were used unless otherwise specified.

5.3.3 Inoculation method development

Inoculation levels in previous trials were either 20 or 100 mites, approximately, per plant (CSIRO, 1987; CSIRO, 1990; Jupp *et al.*, 1997; Willis, 1994). In preliminary trials, inocula of around 100 mites were counted *in situ* on excised plant tips, under a binocular microscope. A selection of accessions, prepared as described above (Section 5.3.2), received one inoculum in the centre of each plant by interweaving excised infested tips

among the stems. Harvest was as described in the final harvest and assessment protocol (Section 5.3.4.3). However, slow population increases of three months or more were experienced under these conditions, so the following experiment was conducted to test the effect on population growth of initial release number and spatial arrangement of inocula over the host plant.

5.3.3.1 *Inoculum level and spatial arrangement*

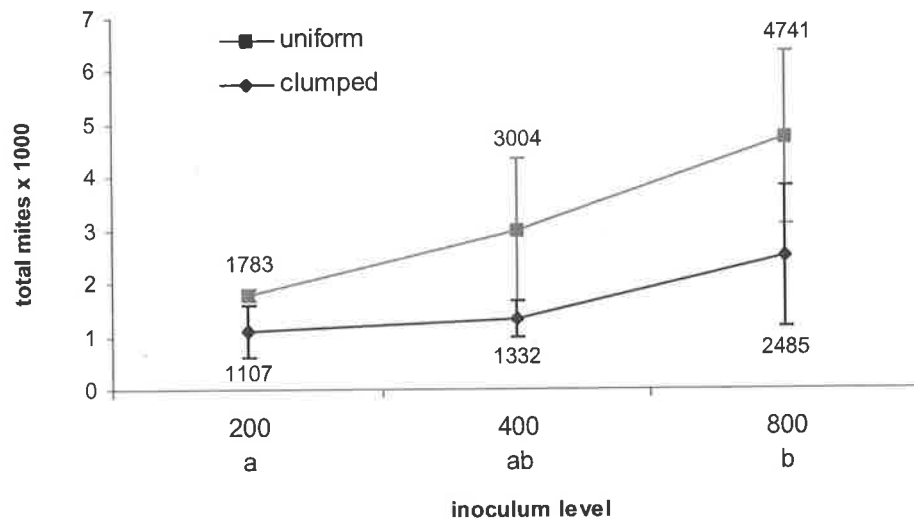
Method

Total number of mites per plant was compared in a replicated 2 x 3 factorial design with three inoculum levels (200, 400 and 800 mites per plant) and two spatial arrangements of inocula on the plant host (“clumped” and “uniform”). Eighteen clones of Nels 3a were prepared as described above (Section 5.3.2), and groups of six were allocated to three blocks depending on minor variations in plant size (for example, largest plants in block 1). Each of the six treatments appeared once per block, and pots were arranged at random within each block. Mites were counted *in situ* on excised leaf tips, using a binocular microscope, and released by interweaving excised infested tips among the stems either in the center of the pot (“clumped”) or dispersed equidistantly over the host plant (“uniform”). To exclude the possibility of re-infestation by dispersal, it was ensured that inoculated plants did not touch, and one additional mite-susceptible replicate with no inoculum was included per block, as a control for cross-contamination. Inoculated plants were maintained in a growth room under the conditions described in Section 5.3.1, harvested 30 days after inoculation as described in Section 5.3.4.3, and total mites per plant were estimated as an indicator of population growth.

Results and discussion

No mites were detected on any of the controls for cross-contamination. The risk of cross-contamination due to migration of mites was therefore assumed to be very low. Data were approximately normally distributed and a two-way ANOVA was performed on total mites recovered (Genstat 5 release 4.2, Lawes Agricultural Trust). Significant differences in final population size were detected among inoculum levels ($Pr = 0.016$), and between spatial arrangements ($Pr = 0.013$), but there was no significant interaction between the two main effects (Fig. 5.3.). Inoculum level means that differed significantly ($\alpha = 0.05$) for the Tukey test (Zar, 1984) are indicated in Fig. 5.3. It was concluded that increasing inoculum level and spacing of inoculum on the host plant resulted in greater population increase.

Figure 5.3. Mean total mites \pm SD, 30 days after inoculation, for three inoculum levels (200, 400 and 800 mites) and two spatial arrangements of inocula (clumped and uniform). Inoculum levels followed by different letters had significantly different means. Numbers indicate estimated treatment means.



The effect of increasing inoculum level on final mite number was as expected; higher initial inoculum resulted in larger populations of mites. The effect of spatial arrangement, with closely spaced inocula resulting in smaller populations, suggested that competition for

food could be limiting to population growth. While that may have been the case at inocula of 200 mites and higher, the situation at lower levels is probably more complex. At very low levels, for example an inoculum of 50 mites, competition for food is less likely to have an effect (note that the interaction was insignificant but there was a trend towards less effect of spacing at low inoculum level) and low density of males may contribute to slow population increases. When the density of spermatophores is very low, one would expect that un-inseminated females would produce mostly male progeny. In that case, with a larval duration of seven days for males and a further seven days for subsequent-generation females, it may take at least 14 days from inoculation before mature females are produced.

5.3.3.2 *Inoculum estimation*

Counting mites on each inoculum was extremely tedious, and a more efficient method was desired. Therefore, fifteen replicate plant tips of approximately 40 mm length, from healthy, actively growing mite cultures showing moderate signs of russeting damage, were placed into 90% alcohol (Section 5.3.4.3), and total mites counted per replicate. Mean number of mites and standard deviation (SD) was found to be 114 ± 41 . The experiment was repeated with five tips per replicate (number = 15) and three tips per replicate (number = 10), and mean number of mites and SD was estimated to be 493 ± 196 and 276 ± 123 , respectively (mean = 99 and 92 mites per tip, respectively).

5.3.3.3 *Inoculation protocol*

Plant tips of approximately 40 mm length from healthy mite culture plants were visually inspected under a binocular microscope for actively growing mite populations and freedom from pests. Depending on the assay, five plant tips (estimated from the study above to average approximately 500 mites) or three tips (300 mites on average) of approximately 40 mm length were selected and broken into several smaller pieces, and applied uniformly across each experimental pot. Only tips from a single culture pot were applied to pots

within an experimental block, and preferably within a single assay. To exclude the possibility of re-infestation by dispersal it was ensured that inoculated plants did not touch.

5.3.4 Sampling method development

Eriophyoids have relatively poorly understood ecologies, primarily because few methods exist for rapidly and accurately estimating population size (Perring *et al.*, 1996). Generally, eriophyoids are unevenly distributed over the host plant; therefore sampling methods must be chosen carefully. Population parameters for *A. hyperici* had been estimated by non-destructive methods in the past; assessing mites per bud according to a visual rating scale, either by examining whole plants after 15 days (CSIRO, 1987), five buds per plant on a fortnightly basis (CSIRO, 1990), or five buds per plant on a fortnightly basis for a period of 14 weeks (Jupp *et al.*, 1997). A rating scale, for example, 0 = no mites, 1 = <10 mites, 2 = 10-25 mites per bud, and so on (Jupp *et al.*, 1997), was used because where live mites were present, their dispersal over the whole plant made it impossible to count them accurately (CSIRO, 1987). However, a rating scale was not considered sufficiently accurate for estimation of *A. hyperici* populations, for comparison of variation in host-suitability among *H. perforatum* genotypes. Time-efficiency was also an important consideration for estimation of population size in the present study, and destructive sampling of whole plants was preferred because large differences in mite numbers were observed among buds of individual plants.

Several efficient, destructive sampling methods using a range of solvents (for example, ethanol, propanol or acetone) have been described for eriophyoids (Elliot *et al.*, 1987; Gibson, 1975; Perring *et al.*, 1996; Zacharda *et al.*, 1988). Alcohol-based techniques were found to be 20% more efficient than direct counting (Zacharda *et al.*, 1988). Ethanol extraction with the addition of ultrasound to dislodge mites from crevices in plant surfaces was used to estimate population size in *Abacarus hystrix* and *Aculodes dubius* on perennial

ryegrass (Gibson, 1975). Almost the same number of mites was removed by 50% or 75% ethanol, as by absolute ethanol (97% of mites), all of which were more suitable than water or water plus wetter (Gibson, 1975). After extraction, mites were easily counted under a dissecting microscope, in a Doncaster nematode counting dish (Doncaster, 1962; Gibson, 1975).

To provide confidence in the accuracy of the sampling scheme being developed, two experiments were performed. The first investigated solvents for recovery of mites from plant material, and the second examined solvent sample volume. Methods and results for both experiments precede a final protocol.

5.3.4.1 Mite recovery experiment

Ultrasonic radiation was tested briefly on *A. hyperici* but not pursued, since it increased processing time, and in 90% ethanol resulted in no significant improvement in recovery compared with shaking. Sonication of a single sample in 90% ethanol recovered 93% of mites compared to $94.1 \pm 2.4\%$ when shaken in 90% ethanol (Table 5.2; $z = -0.46$, $\Pr(|z| \geq 0.46) = 0.65$). The solvents water, water plus detergent, and ethanol (50%, 70% and 90%) were examined. The former two were found to be impractical since mites are waxy and consequently floated and adhered in clumps to containers and pipettes. However, mites were removed efficiently and sank in ethanol, with the added benefit of preservation for assessment at a later date. Three concentrations of ethanol were examined further.

Method

Per replicate, one mite-infested leaf tip was contained in a test tube, covered with ethanol and shaken for 5-10 s, allowed to soak for 10 min, then shaken again. Leaf material was removed and the number of mites remaining on plant material was determined. The number of mites recovered into the solvent was recorded and expressed as a percentage of the combined total for each replicate.

Results and conclusion

Data were approximately normally distributed, and a one-way ANOVA on percentage mites recovered indicated a significant difference ($Pr < 0.001$, power = 0.99) among solvent concentrations (Genstat 5 release 4.2, Lawes Agricultural Trust). Multiple comparisons using the Tukey test ($\alpha = 0.05$) with modifications for unequal n (Zar, 1984) indicated which solvent means differed significantly (Table 5.2). From these results, it was concluded that there was a significantly higher recovery of mites with 90% ethanol as the solvent.

Table 5.2. Mean percentage recovery of mites at three ethanol concentrations. Different superscripts indicate significant differences among solvent means.

ethanol concentration	50%	70%	90%
sample size	6	10	9
mean % mites recovered	83.6 ^a	84.5 ^a	94.1 ^b
SD	4.2	5.1	2.4

5.3.4.2 *Sample volume experiment*

A method of estimating population totals was required because large mite populations were often extracted from experimental plants. Three sample volumes were compared for accuracy of estimation of total mite number, from a standard solution of known mite concentration.

Method

A standard solution was prepared from pooled samples in which mite number had been ascertained by direct counting, and volume adjusted to a concentration of 25 mites/ml. Ten replicate samples for each of three volumes (1, 2 and 5 ml) were made from the standard solution, which was kept agitated. The pipette tip was rinsed with water before and between each replicate.

Results and conclusion

Data were approximately normally distributed, and a one-way ANOVA on mite number indicated a significant difference ($Pr < 0.021$, power = 0.72) among sample volumes (Genstat 5 release 4.2, Lawes Agricultural Trust). Multiple comparisons with the Tukey test ($\alpha = 0.05$) indicated which sample volumes differed significantly (Table 5.3). From these results, it was concluded that 2 and 5 ml sample volumes gave more accurate estimations of the population mean than did 1 ml samples (25 mites/ml). For practical purposes, the 2 ml sample volume gave the best compromise between precision and processing time.

Table 5.3. Mean number of mites for three sample volumes, from a standard solution of 25 mites/ml. N = 10 for each volume. Superscripts indicate significant differences among means.

Sample volume (ml)	1	2	5
Mean mites	26.7 ^b	24.1 ^a	24.5 ^a
SD	2.6	0.8	2.5
Coefficient of variation (V) as %	9.7	3.3	10.2

The coefficient of variation ($V = SD/\text{mean}$) presented as a percentage was used as a measure of relative variability, and thus confidence in the sampling method (Table 5.3). The coefficient of variation for 2 ml samples was within the range of comparable 2 ml sample data from Bioassays 1 and 3 (mean $V = 10.7\%$, range 3.2% to 18.8%, $N = 30$ individuals).

5.3.4.3 Sampling protocol

Plant material from each pot was harvested, lightly packed, into separate 500 ml tissue culture tubs, covered with 90% ethanol and shaken for 5-10 s, allowed to soak for 10 min, then shaken again. The extract was strained through a wide mesh screen, and the tub and retained debris rinsed with a little more ethanol. The total extract was agitated prior to pipetting each of four 2 ml samples into Doncaster nematode counting dishes (Doncaster, 1962). The volume of remaining extract was measured and total volume of solvent calculated. Number of mites per ml was averaged over the four samples, and multiplied by total volume to give total mites per plant.

5.3.5 Plant Bioassay 1

Six replicates of each of ten individual *H. perforatum* plants (Cass a, m, x1, Wyan m and n, MuSh m, MudJ m, TurB m, ScCk x1, Cowr a) were cloned, prepared for assay and inoculated with mites as described in the methods (Sections 5.3.2 and 5.3.3.3). Initial inoculum was approximately 500 mites per plant. One clone of each plant was allocated to each of six blocks, according to minor variations in plant size (for example, largest plants were used in block 1). Pots were arranged at random within each of the six blocks in a growth room, under the conditions described in Section 5.3.1. An additional mite-susceptible replicate (Cowr a) with no inoculum was included per block as a control for cross-contamination. Whole plants were harvested after eight weeks, and total mites per plant were calculated as described (Section 5.3.4.3). Transformation of data for total mites ($\log_{10} x+1$) was performed to correct for unequal variances and satisfy assumptions for analysis of variance (ANOVA) (Genstat 5 release 6.1, Lawes Agricultural Trust).

5.3.6 Plant Bioassay 2

Eleven blocks, each containing clones of eight plants (Cass a, x1, x2, x3, Cowr a, ScCk x1, x2, x3), were prepared, inoculated and set up in a growth room as described for Bioassay 1, with the exception that whole plants were harvested after four weeks. Although each block was of four weeks duration, groups of blocks (1-3, 4-7 and 8-11) were assayed at different time periods. Total mites per plant at four weeks were calculated, and data were analyzed as described in Bioassay 1.

5.3.7 Plant Bioassay 3

Four plants from each of 23 accessions (listed in Table 5.6) were prepared for assay and inoculated with mites as described in the methods (Sections 5.3.2, 5.3.3.3). Initial inoculum was approximately 500 mites per plant. In this assay, individual seedlings were used instead of clones, because their AFLP fingerprints were identical within each

accession (Chapter 4, Table 4.1). One individual of each accession was allocated to each of four blocks according to minor variations in plant size. Pots were arranged at random within each of the four blocks in a growth room with specifications as per Section 5.3.1. Five individuals from four additional accessions were included in block 1 only, because all other seedlings prepared from those accessions had died (SESA 2 and 5, CaFl a, Talm a, Tuen o). Whole plants were harvested after four weeks and total mites per plant were calculated as before (Section 5.3.4.3). For the 23 accessions that were present in all four blocks, analysis was as for Bioassay 1.

5.3.8 Plant Bioassay 4

Two clones of each of 22 plants (listed in Fig. 5.7) were prepared from the Cudgegong and Merrendee quadrat sites (thirteen and nine individuals, respectively, from each site). Clones were inoculated and allocated to two blocks as described for Bioassay 1, with the exception that whole plants were harvested after four weeks. Total mites per plant at four weeks were calculated as described and analysis was as for Bioassay 1.

5.3.9 Plant Bioassay 5

Four plants from each of 14 accessions (listed in Table 5.7) were prepared for assay and inoculated with mites as described in the methods (Sections 5.3.2, 5.3.3.3). Initial inoculum was approximately 300 mites per plant. Individual seedlings were used because sufficient clonal material was not available at the stage this screen was conducted. One individual of each accession was arranged at random within each of four blocks in a glasshouse with specifications as per Section 5.3.1. Whole plants were harvested after four weeks, and total mites per plant were calculated and analysis was as for as for Bioassay 1.

Difficulties arose as a consequence of severe pest contamination. Where obviously reduced plant quality existed, an individual datum was treated as missing (a total of four plants).

AFLP fingerprints were available for most of the individuals, and were identical within each accession. However, for some individuals no AFLP fingerprint was available, because plants died shortly after assay, no clonal material survived, and DNA extractions from preserved material were not successful.

5.3.10 Plant Bioassay 6

Four individuals from each of 15 accessions (listed in Table 5.8) were prepared for assay and inoculated with 500 mites per plant as described in Bioassay 3. Within accessions, all individual seedlings had identical AFLP fingerprints, with the exception of TueJ. Only individuals 1, 7, 14 and 20 from Burr, MuCo and Wedd transects were assayed, because no variation in A1 genotype had been shown within populations (Chapter 4) and susceptibility among plants with the same genotype was consistent in other assays. One individual from each accession was allocated to each of four blocks according to minor variations in plant size. Pots were arranged at random within each of the four blocks in a growth room with specifications as per Section 5.3.1. Whole plants were harvested after six weeks and total mites per plant were calculated and analyzed as described for Bioassay 1.

5.3.11 Plant Bioassay 7

Three clones of each of the tetraploid individuals Cass m, MudJ m and Cowr a (all $2n = 32$), and of the diploids Cass fine-1 and MudJ m F1 (both $2n = 16$), and aneuploid Cass fine-2 ($2n = 17$) were prepared, inoculated and allocated to three blocks as previously described for Bioassay 1. However, initial inoculum was lower, approximately 300 mites per plant, because the diploids and aneuploid were very small. Total mites per plant at 4.5 weeks were calculated as described, and analysis was as for Bioassay 1.

5.4 Results

Bioassay results consistently demonstrated uniform mite-susceptibility within each genotype, but a range of susceptibilities among genotypes. Very low susceptibility was found in the three genotypes with A1, A2 and A10 fingerprints, and medium susceptibility in seven others, in contrast to the high susceptibility found in the majority of genotypes. Detailed results of each assay are presented below and collectively discussed in Section 5.5. Fingerprint numbers referring to RFLP and AFLP profiles were presented in Chapter 4 (summarized in Table 4.1). The terms “log mites” and “mite number” are used with the understanding that statistical inferences were made from \log_{10} total mites.

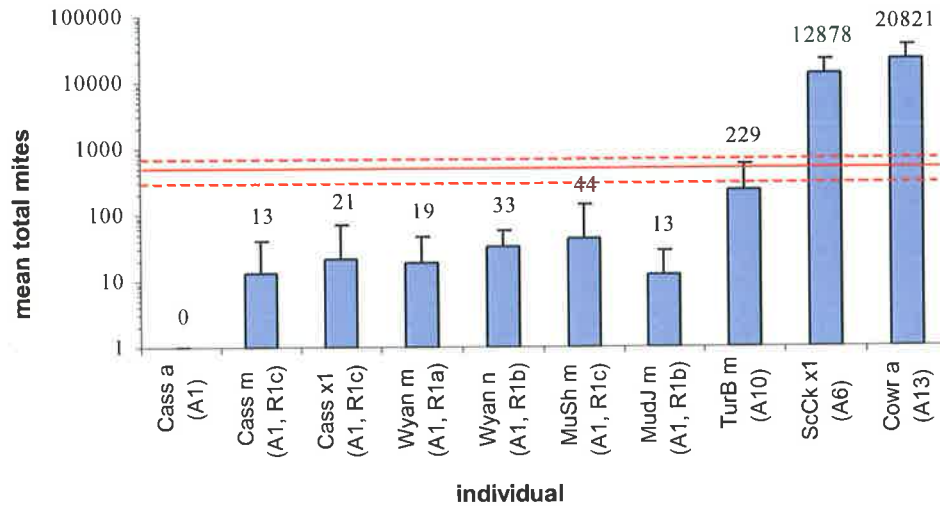
5.4.1 Plant Bioassay 1

No mites were detected on any of the mite-susceptible negative controls for cross-contamination (Covr a). Since no mites had been detected on any of the Nels 3a mite-susceptible negative controls either (Section 5.3.3.1), the risk of cross-contamination due to migration of mites was assumed to be very low and negative controls were not included in any of the subsequent assays.

A highly significant difference among individuals ($Pr < 0.001$) was shown by ANOVA with blocking, on log mites. For descriptive purposes, untransformed means and standard deviations were graphed (Fig. 5.4). Only those groups of interest were discussed in relations to LSD (Table 5.4), because it is not valid to make all pairwise comparisons in multiple comparison testing (Sokal & Rohlf, 1995).

When the initial inoculum level of approximately 500 mites per plant was considered, it could clearly be seen that mite populations increased on A6 and A13 genotypes (Fig 5.4). By comparison, populations underwent a severe decline on the A1/R1 genotypes, and decreased slightly on the A10 individual.

Figure 5.4. Mean total mites at eight weeks, for 10 *H. perforatum* individuals in Bioassay 1. Genotypes are given in brackets, and means for each individual are presented above bars. Solid and dashed lines indicate mean inoculum \pm standard deviation.



There was no significant difference among means for individuals belonging to the R1 subgroups a, b and c (Table 5.4), with the exception of a significant difference between Cass a and Wyan n (due to collapse of mite populations on all replicates of the former), indicating that host-plant suitability was relatively uniform within the A1/R1 genotype. Similar mite response among subgroups suggested that polymorphic markers defining those groups were not associated with susceptibility to *A. hyperici*.

Table 5.4. Susceptibility of 10 *H. perforatum* individuals in Bioassay 1, in terms of mean log mites. Genotype is indicated by AFLP and/or RFLP profile number. Number of clonal replicates = 6. Means are arranged in increasing order, LSD = 0.835.

AFLP/RFLP number	Accession	Identifier	Mean log mites
A1	Cassilis	Cass a	0
A1/R1c		Cass m	0.465
A1/R1c		Cass x1	0.512
A1/R1c	Mudgee	MuSh m	0.633
A1/R1b	Mudgee Common	MudJ m	0.673
A1/R1a	Wyangala Dam	Wyan m	0.752
A1/R1b		Wyan n	1.288
A10	Turon River	TurB m	1.568
A6	Scott Creek	ScCk x1	3.947
A13	Cowra	Cowr a	4.200

Mite numbers on A6 and A13 plants did not differ significantly from each other, and were significantly greater than on all other genotypes (Table 5.4). Significantly more mites were found on the A10 individual than for the A1 group, but significantly less than the A6 and A13 individuals (Table 5.4).

5.4.2 Plant Bioassay 2

As previously, ANOVA on log mites showed a highly significant difference among individuals ($Pr < 0.001$). There was no significant difference among individual plants with the A1 genotype, or with the A6 genotype (Fig 5.5 and Table 5.5), which suggested that mite response was consistent within each of those genotypes. Mite numbers on A1 individuals were significantly lower than on A6 individuals, and Cowr a (A13) supported significantly larger mite numbers than any ScCk (A6) plant (Table 5.5).

Figure 5.5. Mean total mites at four weeks, for eight *H. perforatum* individuals in Bioassay 2. Genotypes are given in brackets, and means for each individual are presented above bars. Solid and dashed lines indicate mean inoculum \pm standard deviation.

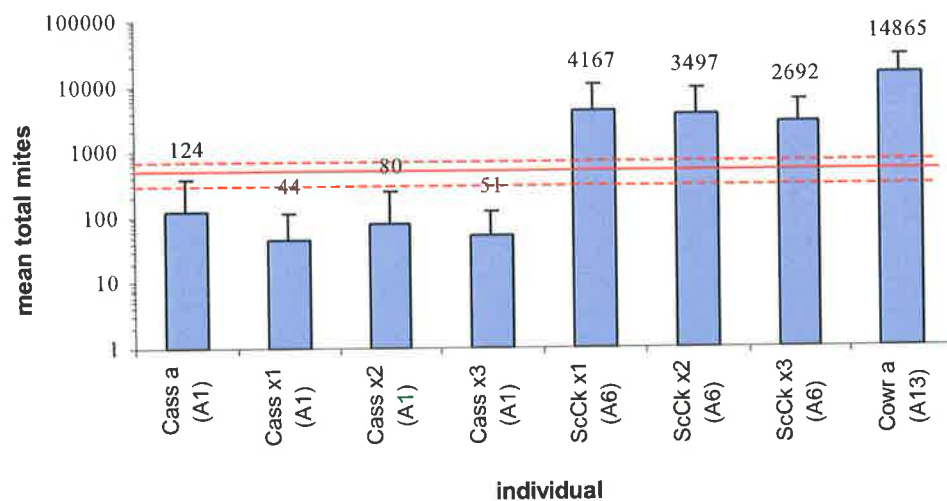


Table 5.5. Susceptibility of eight *H. perforatum* individuals in Bioassay 2, in terms of mean log mites. Genotype is indicated by AFLP profile number. Number of clonal replicates = 11. Means are arranged in increasing order, LSD = 0.431.

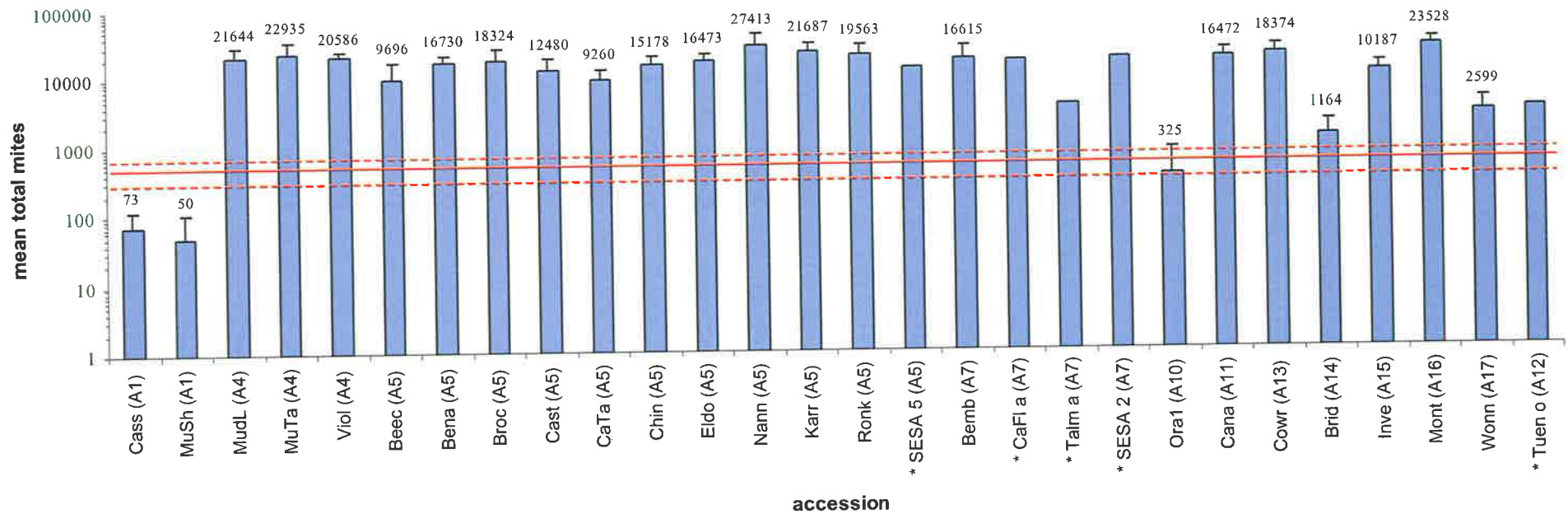
AFLP number	Accession	Identifier	Mean log mites
A1	Cassilis	Cass x2	0.853
		Cass x1	0.930
		Cass x3	1.020
		Cass a	1.108
A6	Scott Creek	ScCk x3	3.077
		ScCk x2	3.092
		ScCk x1	3.096
A13	Cowra	Cowr a	3.976

In contrast to Bioassay 1, statistically significant differences in susceptibility were seen between Cowr a and ScCk x1 individuals (Table 5.5). These results indicate that among assays that differ in length, conclusions drawn from significant differences in relative susceptibility should be made with caution. However, the trend of relative susceptibility between A6 and A13 individuals in both Bioassay 1 and 2 was similar. Most importantly, in both assays populations increased significantly from initial inoculum levels, and the A6 and A13 individuals tested were clearly suitable hosts. In contrast, individuals with the A1 genotype supported lower-than-inoculum levels of mites, and some populations had died out by the end of four weeks.

5.4.3 Plant Bioassay 3

ANOVA showed a highly significant difference in log mites among accessions ($Pr < 0.001$). In contrast, there was no significant difference among accessions with the same genotype, for the three genotypes (A1, A4 and A5) represented by more than one accession (Fig. 5.6 and Table 5.6).

Figure 5.6. Mean total mites at four weeks, for 23 *H. perforatum* accessions in Bioassay 3. Genotypes are given in brackets, and means for each accession are presented above bars. Solid and dashed lines indicate mean inoculum \pm standard deviation. Un-replicated accessions are indicated with an asterisk.



The results for genotype A1 confirmed previous conclusions of uniform mite susceptibility within that genotype, and consistent mite response to genotypes A4 and A5 demonstrated that susceptibility was relatively uniform within those genotypes. Together with the consistent response to genotype A6 (Bioassay 2), these results suggested that host-suitability might be consistent within any given genotype.

Among genotypes A1, A10 and A13, a similar pattern of response was observed relative to the initial inoculum and to each other (compare Bioassays 1 and 3, Figs. 5.6 and 5.4). Again, the A1 individuals appeared to be poor hosts, individuals with the A10 genotype sustained mite populations at around the original inoculum level, and the A13 genotype supported large increases in mite populations. The accessions with A4, A5, A7, A11, A15 and A16 genotypes supported mite numbers similar to the A13 genotype, and thus represented equally suitable hosts. Two other accessions with populations higher than inoculum levels, Brid and Wonn (A14 and A17 genotypes, respectively), supported mite numbers significantly higher than the A1 and A10 genotypes but significantly lower than all of the most suitable genotypes, except for two A5 accessions (Table 5.6).

For the four un-replicated accessions included in block 1 only (asterisked in Fig. 5.6), comparisons were made to replicated A5 (SESA 5) or A7 (CaFl a, SESA 2 and Talm a) accessions. Total mites for SESA 5 (12,639 mites) fell within one standard deviation of the mean calculated over blocks for all A5 accessions ($16,232 \pm 8,672$). Compared to the mean for the Bemb (A7) accession ($16,615 \pm 9,244$), total mites for CaFl a (15,546 mites) and SESA 2 (17,120 mites) fell within one standard deviation, and for Talm a (3,586 mites) within two standard deviations. There was no representative of the A12 genotype in this assay with which to compare Tuen o (2,794 mites), but mean total mites for the same accession in Bioassay 5 appeared very similar (mean = 2,315 mites; Fig. 5.9). Formal

comparison was not made for A12 samples between assays because inoculum levels and assay conditions were different.

Table 5.6. Susceptibility of 23 *H. perforatum* accessions in Bioassay 3, in terms of mean log mites. Genotype is indicated by AFLP profile number. Number of replicate individuals = 4. Means are arranged in increasing order, LSD = 0.574

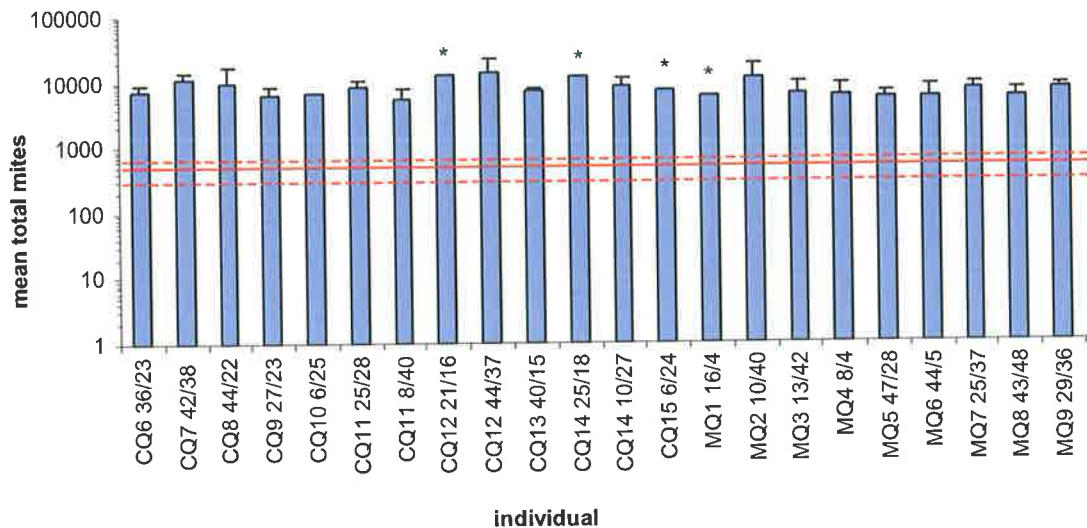
AFLP number	Accession	Identifier	Mean log mites
A1	Mudgee	MuSh a, g, h, i	1.000
A1	Cassilis	Cass f, g, h, i	1.493
A10	Orange	Oral a, g, h, j	2.153
A14	Bridgetown	Brid 1f, 2f, 3f, 4f	3.003
A17	Wonnangatta	Wonn a, g, h, i	3.378
A5	Beechworth	Beec a, g, h, i	3.870
A5	Castlemaine	CaTa f, g, h, i	3.948
A15	Inverell	Inve a, g, h, i	3.990
A5	Castlemaine	Cast 3f, 3g, 5f, 6f	4.055
A5	Chindalup Rd	Chin 1f, 2f, 4a, 4g	4.147
A7	Bemboka	Bemb a, g, h, i	4.153
A11	Canada	Cana a, g, h, i	4.195
A5	Eldorado	Eldo a, g, h, i	4.205
A5	Benambra	Bena 3f, 4a, 4g, 6f	4.213
A5	Brockman Hwy	Broc 1f, 2f, 3f, 4f	4.232
A13	Cowra	Cowr f, g, h, i	4.243
A5	Ronk property	Ronk1f, 2f, <u>3f</u> , 4f	4.257
A4	Violet Town	Viol a, g, h, i	4.305
A5	Karridale	Karr 1f, 2a, 4f, 4g	4.310
A4	Mudgee	MuTa a, g, h, i	4.313
A4	Mudgee-L	MudL f, g, h, i	4.315
A16	Montpellier	Mont a, g, h, i	4.360
A5	Nannup	Nann 1f, 2f, 3a, 4f	4.400

underlined = missing datum

5.4.4 Plant Bioassay 4

No variation in AFLP fingerprint was found within or between Cudgegong and Merrendee quadrats; despite screening with two different primer combinations, all had the A4 fingerprint (Chapter 4, Section 4.5.2.3). There was no significant difference in log mites ($Pr = 0.38$) among any of the plants from either quadrat. These results add further support to the conclusion that host-suitability was relatively uniform within a genotype (Fig. 5.7).

Figure 5.7. Mean total mites at four weeks, for 22 *H. perforatum* individuals with the A4 genotype from Cudgegong (CQ) and Merrendee (MQ) quadrats, in Bioassay 4. Solid and dashed lines indicate mean inoculum \pm standard deviation.



Plants were assayed under identical conditions and at the same time as Bioassay 3, so mean log mites for all individuals was compared to the mean of all plants with the A4 genotype in Bioassay 3. A two-sample *t*-test showed no difference in mean log mites among A4 individuals from the two assays, with $t_{0.05(2), 23} = -0.18$, and $\Pr(|t| \geq 0.18) > 0.50$.

Quadrat samples originated from two CSIRO field sites near Mudgee, Cudgegong and Merrendee, where impact assessment on individual plants over a three-year period demonstrated that the presence of mites caused significant reduction in indices of growth (Section 5.1.4.3). There was no recorded plant damage in either of the two years, 1996 and 1997, for four of the Cudgegong individuals for whom data was collected (CQ11 8/40, CQ12 44/37, CQ13 40/15 and CQ14 25/18); however, the similar response to other A4 individuals (Fig. 5.7) suggested that initial inoculum levels or environmental factors were more likely to have been responsible than variation in plant susceptibility.

5.4.5 Plant Bioassay 5

Difficulties with pest contamination were experienced with this assay (see method) therefore conclusions are presented cautiously. Genotype was undetermined for 11 individuals (asterisked, Fig. 5.8) and an assumption of genetic uniformity within those accessions could therefore not be made. However, a comparison of total mites suggested similar mite responses and thus shared genotype within those accessions (Fig. 5.8). In the ScCk accession, one datum was missing (ScCk r), and total mite number was very low on another (ScCk o) compared with results for other individuals (see also Bioassays 1 and 2). Despite this, the fingerprint and morphology of both individuals matched others within the accession. While variation in susceptibility within this accession cannot be excluded, samples with low mite numbers may be a consequence of difficulties with this assay and lack of clonal replication, rather than low susceptibility.

Given the generally similar mite response within accessions, and very low intra-population genetic variation (Chapter 4), there was a reasonable likelihood that within each accession, un-fingerprinted plants shared the same genotype as fingerprinted individuals. Therefore, results were combined in order to look at overall pattern of response among genotypes (Fig. 5.9).

Figure 5.8. Total mites for 14 H. perforatum accessions in Bioassay 5, from an initial inoculum of 300 mites. Genotypes with brackets indicate accessions with all individuals fingerprinted. Genotypes without brackets indicate accessions in which at least one individual (asterisked) had not been fingerprinted. Exclamation marks indicate missing data.

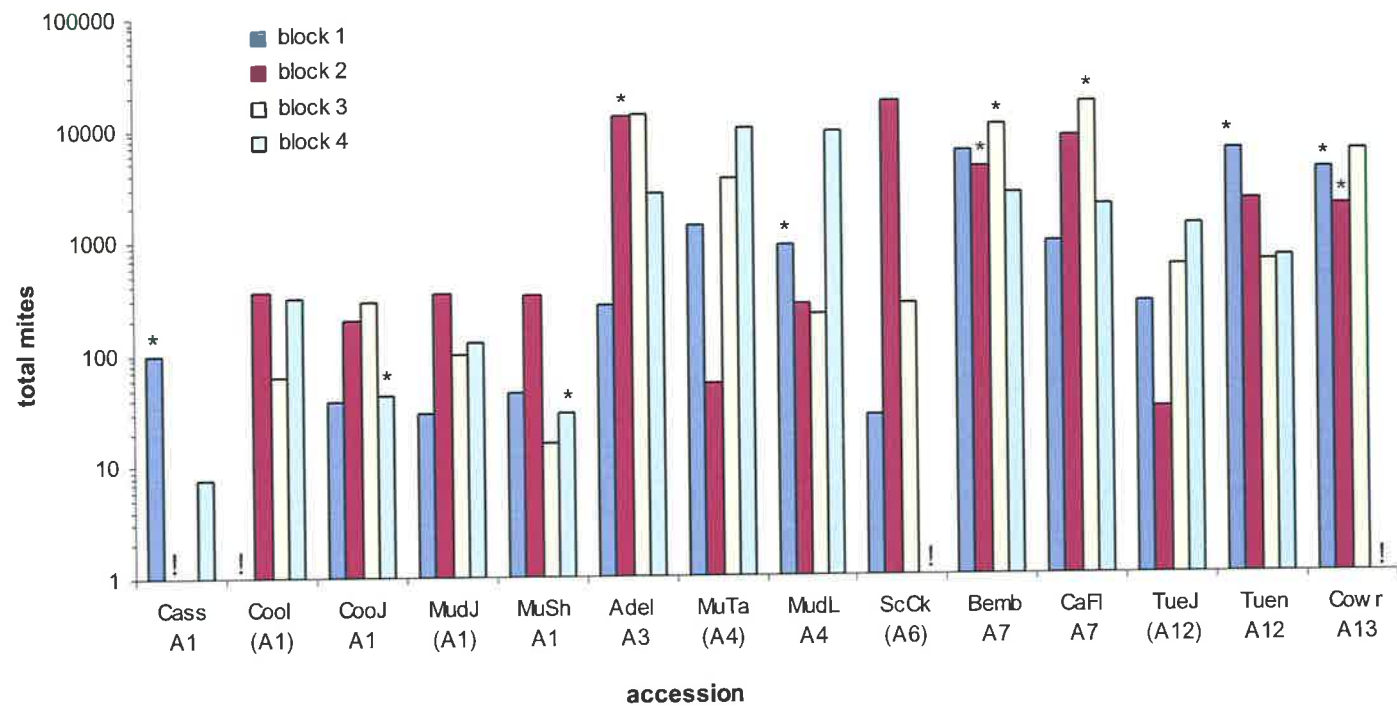
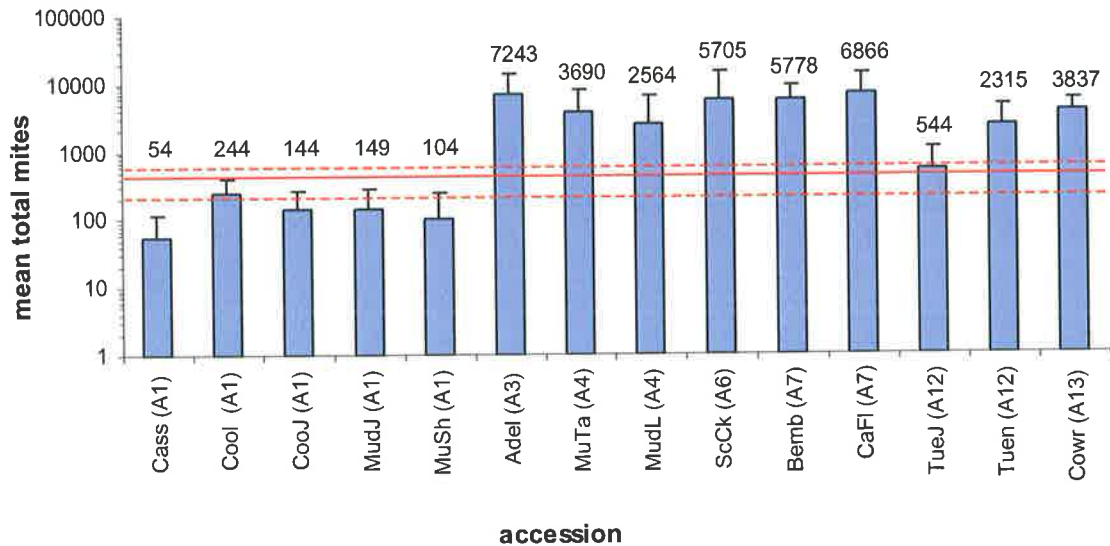


Figure 5.9. Mean total mites at four weeks, for 14 *H. perforatum* accessions in Bioassay 5. Means for each accession are presented above bars, and genotypes for the majority of individuals (see Fig. 5.8 and text for explanation) are given in brackets. Solid and dashed lines indicate mean inoculum \pm standard deviation.



Highly significant differences were found in log mites among accessions ($P < 0.001$). Again, host-plant suitability was relatively uniform within genotypes; there were no significant differences among accessions within a particular genotype where more than one accession was tested (A1, A4, A7 and A12), with one exception within the A1 genotype (Table 5.7). A significant difference between the Cass and both MudJ and Cool accessions (all A1 genotype) was probably due to the fact that mites had completely died out on one of the Cassilis individuals (Fig. 5.8). Among genotypes, it was clear that A3, A7 and A13 differed significantly from A1. There was a suggestion that the A12 genotype was a less suitable host than the A3, A7 and A13 genotypes: TueJ (A12) supported significantly smaller populations, and there appeared to be fewer mites on Tuen (A12) but the difference was not significant (Table 5.7). Non-significant differences in the latter case and among the other genotypes were likely to have been a consequence of high variation and large LSD (Table 5.7).

Importantly, A1 was again demonstrated to be a poor host, with final mite populations below the inoculum level, and all other genotypes were clearly suitable hosts (Fig. 5.9). All genotypes represented in other assays (A1, A4, A6, A7, A12 and A13), showed similar levels of mite response to those seen previously (compare Fig. 5.9 with Figs. 5.6 and 5.4). The A3 genotype (Adel) was not represented in any other assay; it was not significantly different from, and was thus concluded to support mite populations comparable to, all except A1 and TueJ (A12) accessions.

Table 5.7. Susceptibility of 14 *H. perforatum* accessions in Bioassay 5, in terms of mean log mites. Genotype is indicated by AFLP profile number. Number of replicate individuals = 4. Means are arranged in increasing order, LSD = 1.014.

AFLP number	Accession	Identifier	Mean log mites
A1	Cassilis	Cass o, p, q, s*	0.998
A1	Mudgee	MuSh o, p, q, r*	1.725
A1	Coolah	CooJ o, p, m, r*	2.000
A1	Mudgee Common	MudJ o, p, q, r	2.025
A1	Coolah	Cool o, p, q, r	2.245
A12	Tuena-J	TueJ o, p, q, r	2.425
A6	Scott Creek	ScCk o, p, q, r	2.732
A4	Mudgee-L	MudL o, p, q, r*	2.875
A4	Mudgee	MuTa o, p, q, r	3.075
A12	Tuena	Tuen o, p, q, r*	3.175
A3	Adelong	Adel o, p, q, r*	3.500
A13	Cowra	Cowr o, p*, q*, r	3.599
A7	Captains Flat	CaFl o, p, q, r*	3.600
A7	Bemboka	Bemb o, p, q*, r*	3.700

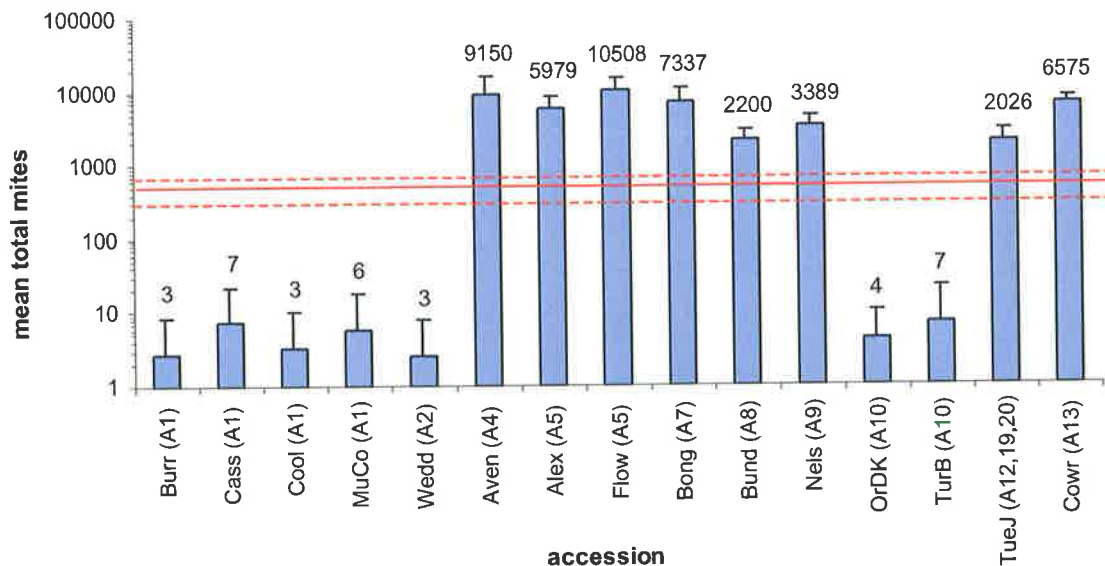
* = no fingerprint data, bold = missing data

5.4.6 Plant Bioassay 6

Data from TueJ individuals were combined for comparison with other accessions, even though TueJ a and c varied from the A12 fingerprint at two band locations (Chapter 4 Section 4.5.2.3, and Appendix B), because the standard deviation for total mites was relatively small and within the range for other accessions (Fig. 5.10).

All genotypes represented in other assays (A1, A4, A5, A7, A10, A12 and A13) showed similar relative patterns of mite response to those seen previously (compare Fig. 5.10 with Figs. 5.6 and 5.4). Again, genotypes A1 and A10 were shown to be poor hosts, on which mite populations had virtually collapsed by six weeks (Fig. 5.10). In contrast, all other genotypes produced significantly higher mite numbers and were clearly suitable hosts (Fig. 5.10 and Table 5.8).

Figure 5.10. Mean total mites at six weeks, for 15 *H. perforatum* accessions in Bioassay 6. Genotypes are given in brackets, and means for each individual are presented above bars. Solid and dashed lines indicate mean inoculum \pm standard deviation.



Mite numbers on TueJ were significantly higher than on the A1, A2 and A10 genotypes, but not significantly lower than other accessions, with the exception of Flowerdale (Table 5.8). However, as in the case of Bioassay 5, there was a suggestion that the A12 genotype was a slightly less susceptible host, compared with some of the most suitable genotypes.

Table 5.8 Susceptibility of 15 *H. perforatum* accessions in Bioassay 6, in terms of mean log mites. Genotype is indicated by AFLP profile number. Number of replicate individuals = 4. Means are arranged in increasing order, LSD = 0.665.

AFLP number	Accession	Identifier	Mean log mites
A2	Weddin Forest	Wedd 1, 7, 14a, 20a	0.264
A1	Burrendong Dam	Burr 1, 7, 14, 20	0.270
A1	Coolah	Cool b, c, d, e	0.289
A1	Mudgee Common	MuCo 1, 7, 14, 20	0.348
A10	Turon River	TurB b, c, d, e	0.369
A1	Cassilis	Cass a, c, d, e	0.370
A10	Orange-K	OraK a, b, c, d	0.478
A12,19,20	Tuena-J	TueJ a, b, c, d	3.276
A8	Bundaleer Forest	Bund 1a, 1b, 2b, 3b	3.324
A9	Nelson	Nels 1b, 2b, 3b, 4a	3.511
A5	Alexandra	Alex 2a, 3b, 4b	3.771
A13	Cowra	Cowr a, b, d, e	3.809
A7	Bonganditj	Bong 1b, 2a, 2b, 3b	3.836
A4	Avenel	Aven 1b, 2b, 3a, 4a	3.877
A5	Flowerdale	Flow 1b, 2a, 2b, 5a	3.986

Genotypes A2, A8 and A9 were not tested in any other formal assay. Susceptibility in the A2 accession (Wedd) was similar to the A1 and A10 genotypes; mite populations on these three genotypes were significantly lower than on any other accession but not significantly different from each other (Table 5.8). The A8 genotype from Bundaleer Forest supported mite populations comparable with all other suitable hosts, including TueJ. Mite number for the A9 genotype (Nels) was within the range predicted from Nels 3a in the inoculation method experiment (Section 5.3.3.1); mean total mites would be expected to fall between 3,000 and 4,000 from an initial inoculum of 500 mites (Fig. 5.3).

5.4.7 Plant Bioassay 7

ANOVA on log mites showed a highly significant difference among individuals ($P < 0.001$). Relative mite number on the tetraploid Cass m, MudJ m (both A1) and Cowra (A13) individuals included as controls was similar to that seen for those individuals in Bioassay 1 (Fig. 5.4). There was no significant difference in mite number between the MudJ m tetraploid and its A21 diploid progeny, but a significant difference between the Cass tetraploid, and its A22 diploid and aneuploid congeners (Table 5.9).

Figure 5.11. Total mites for diploid, aneuploid and tetraploid *H. perforatum* at 4.5 weeks, from an initial inoculum of 300 mites, Bioassay 7. Mean total mites for each individual is presented above bars. Solid and dashed lines indicate mean inoculum \pm standard deviation.

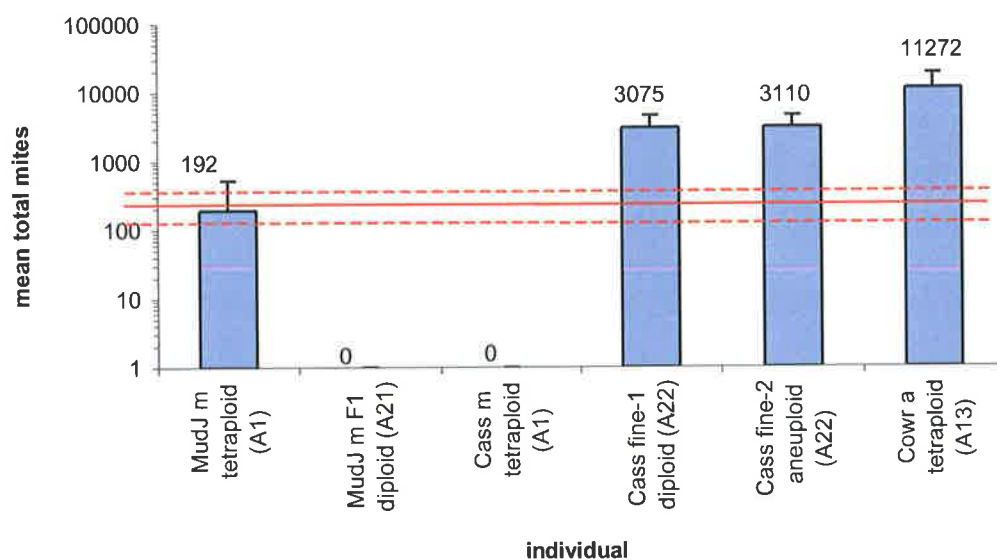


Table 5.9 Susceptibility of diploid, aneuploid and tetraploid *H. perforatum* individuals in Bioassay 7, in terms of mean log mites. Genotype is indicated by AFLP profile number. Number of replicate individuals = 3. Means are arranged in increasing order, $LSD = 1.272$.

AFLP number	Accession	Identifier	Mean log mites
A1	Cassilis	Cass m	0
A21	progeny of MudJ m	MudJ F1 (diploid)	0
A1	Mudgee Common	MudJ m	0.920
A22	Cassilis	Cass fine-1 (diploid)	3.451
A22	Cassilis	Cass fine-2 (aneuploid)	3.459
A13	Cowra	Cowra a	3.977

The diploids ($2n = 16$) and aneuploid ($2n = 17$) all had fingerprints with band absences compared with the maternal parent or congeners (Chapter 4, Section 4.5.2.1). The diploid MudJ m F1 was an offspring of a MudJ m ♀ x Wyan m ♂ cross, and its A21 fingerprint showed three band absences (bands 38, 60 and 74) compared to its A1 parents (Appendix B). The A1 tetraploid Cass m, A21 diploid Cass fine-1 and aneuploid Cass fine-2 were congeners from Cassilis field-collected seed; the A22 fingerprint shared by the diploid and aneuploid was missing four AFLP bands (bands 38, 59, 60 and 74) and had one additional band (51) compared with the A1 fingerprint (Appendix B). While the presence of the Cass diploid and aneuploid may reflect pre-existing clonal variants in the field, it is more likely that they resulted from haploid parthenogenesis in a Cassilis tetraploid with A1 fingerprint, given that morphological variation was not noted in the field (Paul Lutschini, pers. com.), and that low intra-population variation existed at most sites.

The Cass diploid and aneuploid supported significantly higher mite populations than the tetraploid congener, but not significantly different to the Cowr a control (Table 5.4). One may postulate that during reduction of the tetraploid chromosome set, a gene or group of genes conferring resistance was lost from the Cass diploid and aneuploid, but not the MudJ diploid. The absence of bands 38, 59, 60 and 74 implies that those loci were in the heterozygous state in the tetraploid parent. However, the common absence of bands 38, 60 and 74 *per se* does not appear to be associated with the loss of a resistance factor, since only the Cass diploid and aneuploid showed increased mite-susceptibility. Band 59, which was only absent in the latter individuals, was not likely to be linked to a resistance gene, because it was present in so many AFLP fingerprints of highly susceptible genotypes (Appendix B), as well as low susceptibility genotypes A1 and A10. So it seems that while the loss of a resistance gene(s) in the Cass diploid and aneuploid has occurred, none of the AFLP markers that were absent were associated with it.

5.5 Discussion

Bioassays were used to examine 230 fingerprinted individuals from 41 of the 44 populations sampled, which included 20 of the 21 genotypes characterized with RFLP and AFLP techniques (Table 4.1, Chapter 4). Eleven additional individuals from nine accessions, that were not fingerprinted, were also included in Bioassay 5. Pier, Smit and Crow accessions were not formally assayed because samples were obtained too late in the project. However, both Pier and Smit accessions had the A7 fingerprint, and mites had been shown to establish in the field at both Pierces Creek (CSIRO, 1995; Jupp, 1993b; Jupp & Cullen, 1996) and Smiths Paddock (Willis, 1994; Willis *et al.*, 1993; Willis *et al.*, 1995). The Crow accession (A18) consisted of fresh material from one plant in the field, on which mites were present.

Bioassays using clonal plant material showed similar mite numbers between replicates (Bioassays 1, 2, 4 and 7), which indicated that the method was reproducible. The bioassay protocol was found to be adequate for statistical investigation of relative susceptibility among genotypes, despite occasional large standard deviations.

5.5.1 Susceptibility within genotypes

Results indicated that susceptibility was correlated to genotype. The consistent level of susceptibility within a genotype mirrored the low intra-population variation discussed in Chapter 4. Where more than one individual or accession with the same genotype was tested in a given assay, no significant difference was found in mite number. For example, 49 individuals with the A1 genotype consistently demonstrated low susceptibility to mites, with non-significant differences among individuals or accessions in each of the six bioassays in which that genotype was tested; two apparent exceptions of statistically significant differences resulted from mites dying out completely on Cass individuals (Bioassays 1 and 5), from which one would not infer biological significance. Six other

genotypes consistently demonstrated non-significant differences in mite number where multiple individuals or accessions with the same genotype were tested, within a given assay (genotypes A6 in Bioassay 2; A4, A5 and A7 in Bioassay 3; A4 in Bioassay 4; A4, A7 and A12 in Bioassay 5; A5 and A10 in Bioassay 6).

5.5.2 Susceptibility among genotypes

Consistent susceptibility within a genotype contrasted with variable susceptibility between genotypes. Susceptibility ranged from extremely low mite numbers or total population collapse in genotypes A1, A2 and A10, to very high populations in, for example, genotype A13 (Bioassay 6, Fig. 5.10).

Genotypes were broadly categorized into “low”, “medium” and “high” susceptibility classes (Table 5.10). Assignment of genotypes to susceptibility classes is discussed for each category below, and was based on statistical significance within an assay, mite number as a percentage of the highest mean for that assay (particularly where a genotype was represented by only one accession in one assay), and consistent trends for a given genotype relative to others. Where class distinctions were difficult to make at above inoculum levels, differences were inferred only where biological significance appeared striking or was linked to field establishment history.

Relative increases above inoculum levels were not comparable between assays, because assay lengths varied, and conditions could not always be standardized among assays conducted at different points in time. However, increases relative to the inoculum level are provided as a rough guide for medium and high susceptibility classes, using assays of four-week duration.

Table 5.10. Susceptibility class for 19 genotypes of *H. perforatum*, summarized from bioassays. Class colour codes are those used in distribution maps in Chapter 4 (Figs. 4.11, 12 and 13).

Class	Genotype	Accession/individual	Bioassay
Low	A1	Cassilis, Wyangala Dam, Mudgee Short, Mudgee-J, Coolah, Coolah-J, Burrendong Dam, Mudgee Common	1, 2, 3, 5, 6, 7
	A2	Weddin Forest	6
	A10	Orange-1, Orange-K, Turon Broad	1, 3, 6
	A21	MudJ m F1 progeny (diploid)	7
Medium	A8	Bundaleer Forest	6
	A9	Nelson	6
	A12, 19, 20	Tuena, Tuena-J, Tuen o	3, 5, 6
	A14	Bridgetown	3
	A17	Wonnangatta	3
	A22	Cass fine-1 (diploid), Cass fine-2 (aneuploid 2n = 17)	7
	High	A3	Adelong
A4		Mudgee Tall, Mudgee-L, Violet Town, Avenel, Cudgegong Park, Merrendee	3, 4, 5, 6
A5		Alexandra, Beechworth, Benambra, Brockman Hwy, Castlemaine, Castlemaine Tall, Chindalup, Eldorado, Flowerdale, Nannup, Ronk, SESA 5	3, 6
A6		Scott Creek	1, 2, 5
A7		Bemboka, Bonganditj, Captains Flat, Talm a, SESA 2	3, 5, 6
A11		Canada	3
A13		Cowra	1, 2, 3, 5, 6, 7
A15		Inverell	3
A16		Montpellier	3

“Low” susceptibility indicated a decline in mite populations to below inoculum levels, and in no case was mite number above 3.3% of the highest mean for a given assay (Table 5.10, genotypes A1, A2 A10 and A21). This group of low-susceptibility genotypes was significantly different from all other genotypes in every assay in which they appeared, with the exception of Bioassay 5 (due to poor experimental conditions and large variances). In all instances where field data were also available for sites with the A1 genotype, mites had failed to establish; at the Coolah and Cassilis field sites in the Liverpool Ranges, NSW (Jupp *et al.*, 1997; Jupp & Cullen, 1996) and Mudgee Common and Burrendong (Paul Jupp and Paul Lutschini, pers. com.). The present results for Coolah, Mudgee-J and Mudgee

Short agree with the conclusion of low susceptibility, when seedlings from the same seed batches were previously tested in the laboratory using a mite rating system (Jupp *et al.*, 1997). Low mite-susceptibility in the A2 accession from Weddin Forest supports the observation in April 1997 (G. Mayo) that mites had not established after release in 1991. However, the evidence presented here for very low susceptibility in the A10 genotype conflicts with anecdotal evidence of establishment on this broad-leaved form at the Turon River site (Paul Lutschini, pers. com), and with the conclusion of susceptibility in the Orange-1 accession (Jupp *et al.*, 1997). On the basis of the latter two reports, the Orange-1 accession had been used initially for mite cultures, but lack of mite establishment led to its discontinued use. It may be that this accession contained a mixture of genotypes, or was not the same accession obtained by Jupp *et al.* (1997).

“Medium” susceptibility genotypes (Table 5.10, genotypes A8, A9, A12, A14, A17, A19, A20 and A22) showed only relatively small to moderate increases of between 1.1 and 5.2 times the initial inoculum levels over a four-week period. For all genotypes included in this group, mite number for all samples was less than 33% of the highest mean for a given assay. For A14 and A17 genotypes, mite number differed significantly from both low and highly susceptible genotypes, and was only 4% and 10% of the highest mean, respectively (Bioassay 3). However, in genotypes A8, A9 and A12, an apparent biological difference was not clearly supported by statistical significance. A subjective decision was made to include the A12 genotype in the “medium” susceptibility class, based on consistently lower mite numbers across assays. Mean mites for genotype A12 were 8% and 32% of the highest mean for Bioassay 5, and 19% of the highest mean for Bioassay 6. Establishment and increase at the Tuena-J site had required three release attempts (Jupp *et al.*, 1997), build-up was slow and mites more difficult to find than at other release sites (Paul Jupp pers. com.), and previous laboratory experiments using a mite rating system had concluded

that the Tuena and Tuena-J accessions were of intermediate susceptibility (Jupp *et al.*, 1997). Genotype A8 from Bundaleer Forest was only included in one assay, and mean total mites was 21% of the highest mean (Bioassay 6). According to the collector, establishment at Bundaleer was slow but some effect has been noted in the field (Mary Simon, pers. com.). The A9 genotype from Nelson supported populations with a mean total of 32% of the highest mean for Bioassay 6, and was also included in this group because, when it was used for mite cultures, population increase was noticeably slower than on the Cowra genotype.

The majority of genotypes fell into the category of "high" susceptibility (Table 5.10, genotypes A3, A4, A5, A6, A7, A11, A13, A15 and A16), and supported large mite population increases of up to 55 times the initial inoculum level over a four-week period. These genotypes were not significantly different from each other in mite number in any assay in which they appeared, with the exception of A6 and A13 in Bioassay 2. Highly susceptible genotypes were always significantly different from low, and in some cases from medium susceptibility genotypes, with some exceptions in Bioassay 5 due to high variances. The high susceptibility of plants from Cudgegong and Merrendee quadrats (A4) and the A5 accession from Castlemaine (A5) corresponded well to the significant reductions in plant growth in A4 accessions, and plant growth and density in the A5 accession in the field (Section 5.1.4.3). The results for the Talmalmo accession (A7) agreed with the conclusion of high susceptibility when seedlings from the same seed batch were previously tested in the laboratory using a mite rating system (Jupp *et al.*, 1997). However, although mites had established at Talmalmo, no plant mortality or decline in plant density was seen over a four-year period (Jupp & Cullen, 1996). The same A7 genotype was also present at Pierces Creek, where mites showed rapid establishment, dramatic increases, deformation of rosettes and reduced seeding capacity (Jupp, 1993a).

However, the latter field data could not be directly compared to bioassay results because the Pierces Creek accession was omitted due to time constraints. Marginal decreases in growth of *H. perforatum* were demonstrated in a field impact study of transplanted seedlings raised from “locally collected” seed from the vicinity of Canberra (Willis, 1994), which were likely to have the A7 genotype. The Beechworth collection tested by Jupp *et al.* (1997) also showed high susceptibility and establishment in the field, but was not the same accession characterized in the present study. However, the A5 genotype appeared widespread in Victoria, and together with low intra-population variation, suggests that the Beechworth collection in the former study was most likely to be the A5 genotype. At the Bonganditj site, with a mixture of A5 and A7 genotypes, establishment was confirmed during the present study, only 8 months after release of mites. At that time, plant damage was seen in the form of a distinctive circle of russetting extending out to approximately 10 metres from the release point. Seven years after release, impact on the population appeared to have been considerable (Fig. 5.12). The A16 genotype was collected from the vicinity of Montpellier, near the original site of impact assessment and host-specificity testing (Wapshere, 1984) and collection of *A. hyperici* in southern France (CSIRO, 1987), from which one would anticipate high mite-susceptibility.

Figure 5.12. H. perforatum at Bonganditj Forest near Donovans in the south-east of South Australia, in a) summer just prior to and b) winter at release in 1996 of Aculus hyperici (dried flower heads are reddish brown), and c) summer 2004. Arrowhead indicates release site.



5.5.3 Distribution and control

Bioassay-based estimates of susceptibility to *A. hyperici* corresponded well to previous establishment and impact assessment records, for the six genotypes for which data was available (low susceptibility genotypes A1 and A2, medium susceptibility A12, and high susceptibility A4, A5 and A7). Susceptibility classes were therefore used to estimate potential control by *A. hyperici*, given the distribution of genotypes across the weed's Australian range. Referring back to the preliminary distribution maps in Chapter 4 (Figs. 4.11, 12 and 13), it can be seen that the three low susceptibility genotypes (indicated by light and dark red symbols) are located in central New South Wales, and on the basis of above results one would not expect *A. hyperici* to contribute to their control. The five medium susceptibility genotypes (indicated by light and dark pink symbols, Figs. 4.11, 12 and 13) are located across the states; Tuena in New South Wales, Wonnangatta and Nelson in Victoria, Bundaleer Forest in South Australia, and Bridgetown in Western Australia. On the basis of this study, one would expect a lower impact or a longer time to achieve similar control in weed populations consisting of genotypes with medium susceptibility, in comparison to high-susceptibility genotypes. Highly susceptible genotypes are widely distributed (all other colours and symbols, Figs. 4.11, 12 and 13) and one would expect the best control at these sites given the results of this study, and field establishment and impact assessment studies by other workers (Jupp & Cullen, 1996; Mahr *et al.*, 1999).

Given the history and mode of spread of the weed in this country (Harris & Gill, 1997), it is probable that low and medium mite-susceptible forms of *H. perforatum* will spread and replace highly susceptible forms as the mite controls them. However, without improved maps of the distribution of *H. perforatum* genotypes, it will not be possible to determine whether replacement of one form by another is occurring.

5.5.4 Host specificity and genetic relatedness

The situation with *A. hyperici* and *H. perforatum* seems to be similar to that of host-specificity in *Aceria chondrillae*, a gall-forming eriophyid released in Australia for the control of the triploid apomict *Chondrilla juncea*, or skeleton weed (Caresche & Wapshere, 1975). Strains of the mite *A. chondrillae* from France, Italy and Greece were specialized to geographic forms of skeleton weed, with particular suitability of the Greek mite strain for control of the narrow leaf form common in Australia. From the present study, there is sufficient evidence of such low susceptibility to *A. hyperici* in particular genotypes of *H. perforatum*, to justify collection of strains of the mite suitable for control of those forms.

No close genetic relationship of resistant forms of the weed could be seen from the results of any of the phylogenetic analyses presented in Chapter 4. However, close relationships cannot be discounted because few of the nodes in any of those analyses were resolved with confidence. Within susceptibility classes, neither low susceptibility genotypes A1, A2 and A10 (highlighted in red) nor medium susceptibility genotypes (highlighted in pink) appeared to be closely related (Figs. 4.4, 4.10, 4.12 and 4.14). In particular, it was interesting to note that genotypes with very low or medium susceptibility to *A. hyperici* were to be found in both groups based on leaf morphology, the “broad-leaved” (A9, A10 and A11 genotypes) and “narrow-leaved” (most other genotypes) forms.

It seems peculiar that within the species *H. perforatum*, there exist genotypes with extremely low susceptibility, while several other species in the genus are apparently suitable hosts. In the home range across Eurasia, host specialization is likely to have been a complex evolutionary process fueled by hybridization and polyploidization, and driven by density-dependent selection. Cross-resistance to defensive chemicals in *A. hyperici* may be responsible for a similar level of susceptibility in *H. tetrapterum* and *H. pulchrum* as was

shown for *H. perforatum* (Table 5.1). Alternatively, chemicals acting as obligatory feeding stimuli may have been lost in those closely related species not included in the mite's host range. The corollary in the plant, resistance to a range of herbivores, has not been reported in biological control agents of *H. perforatum*. In the case of *Aphis chloris*, observations of several aphid-infested plants during the present study showed that aphid populations could increase and cause severe plant damage on both the *Aculus*-resistant A1 genotype (MudJ, CooJ, Cass accessions), and the highly *Aculus*-susceptible A5 genotype (Karr accession).

5.5.5 Genetic control of resistance

The *H. perforatum* – *A. hyperici* interaction may consist of a gene-for-gene system, similar to resistance in wheat to Hessian fly (at least five loci in wheat confer resistance to the pest, which have corresponding counter-resistance loci, and 12 fly biotypes can be distinguished) (Bernays & Chapman, 1994). The range of mite-susceptibility seen in this study and the evidence from loss of chromosomes in ploidy variants (Bioassay 7) is not inconsistent with an hypothesis of single gene control, since the plant is a tetraploid. Heterozygotes would be expected to fall into three classes, with one, two or three copies of the resistance gene or linkage group, and if there were incomplete dominance for this resistance factor, dosage effect could result in a range of susceptibilities. The loss of one set of chromosomes appears in some cases to have resulted in a loss of resistance, which seems inconsistent with involvement of multiple genes across chromosomes. The complex genome and reproductive biology of tetraploids (Chapter 3), precluded the use of classical crosses to explore the genetic basis of resistance in the present study, since very large numbers of progeny must be screened with molecular and cytological tools in order to identify recombinant progeny, and the interpretation of segregation ratios becomes complex. On the other hand, diploids with high and low susceptibility present an ideal opportunity to pursue the question in the future, due to their simpler genome and

reproduction by primarily sexual means (Chapter 3). In the event of attempting such crosses among diploids, it is worth noting that seed set in all diploids was extremely poor when inflorescences were bagged and self-pollinated, which suggested that a compatibility system may be operating in this species. Compatibility aside, if resistant and susceptible diploid parents were used and the F_1 backcrossed to the phenotypically dissimilar parent, resistant and susceptible progeny would be expected in a ratio of 1:1 in the case of a single gene or tightly linked group. Molecular markers associated with the trait would provide reasonable evidence of single gene control. In the absence of such a cross, RFLP and AFLP fingerprints for field-collected material were examined, but no markers were associated with susceptibility to *A. hyperici*. However, further screening with other AFLP primer pairs would be relatively simple, and may locate markers associated with resistance.

5.6 Summary

The results strongly suggested that susceptibility to *A. hyperici* was correlated to *H. perforatum* genotype. Consistent susceptibility within a genotype mirrored the pattern of low intra-population genetic variation detected in Chapter 4, and contrasted with variable susceptibility among genotypes. Plant susceptibility ranged from “low”, where mite populations consistently declined from initial inoculum levels, sometimes to the point of complete collapse, through “medium” susceptibility, with relatively slow population increases ranging from 4% to 33% of the most highly susceptible genotype, to “high”, where mite populations could increase as much as 55 times the initial inoculum level in only four weeks. Comparison of genotypes of known susceptibility with field establishment history and impact studies (low susceptibility A1 and A2, medium susceptibility A12, and high susceptibility A4, A5 and A7 genotypes) led to the prediction that *A. hyperici* would fail to establish at all populations consisting of the three low-susceptibility genotypes in New South Wales (A1, A2 and A10), and establish but provide

relatively slow or sub-optimal control on the seven medium-susceptibility genotypes dotted across the continent (A8, A9, A14, A17, A12, A19 and A20), compared to the nine widespread highly susceptible genotypes (A3, A4, A5, A6, A7, A11, A13, A15 and A16). It is quite plausible that those with lower susceptibility will replace highly susceptible genotypes over time. The range of mite-susceptibility seen in this study and the evidence from ploidy variants is not inconsistent with an hypothesis of single gene control, which could be tested by marker segregation in progeny of classical crosses. No RFLP or AFLP markers were associated with susceptibility to *A. hyperici* for the field-collected material examined.

6 Mechanisms of resistance to *Aculus hyperici*

6.1 Introduction

Several physical factors were investigated for potential contribution to resistance mechanisms to *Aculus hyperici* in *Hypericum perforatum*. During the investigation of susceptibility to *A. hyperici*, leaf-colour variation was noted among genotypes of *H. perforatum*. One of the low susceptibility genotypes (A1 fingerprint) consistently displayed a more blue-green leaf colour by comparison to more susceptible genotypes, which was suggestive of leaf wax variation. Therefore, the leaf surfaces of several genotypes with variable mite-susceptibility were examined for wax density, wax depth and cell morphology. In addition, evidence of physical impediment to feeding and oviposition was sought in mites fed on low and highly susceptible genotypes of the weed.

6.2 Review of herbivore-plant interactions

Our understanding of the complex interplay between herbivore, plant host and environment has in large part been gleaned from studies on insects, which can be loosely extrapolated to include other arthropods. Arthropod-plant interactions are influenced in the host by plant chemicals, physical structures and abiotic factors such as stress, and in the herbivore by sensory systems, developmental or physiological factors, host selection behaviour, experience, and ecological factors such as interactions with other organisms (Bernays & Chapman, 1994). Factors with a genetic basis are considered in the following review.

The aerial parts of *H. perforatum* contain numerous polyphenolic constituents belonging to the bioflavonoid and anthraquinone classes, diterpenoids, and n-alkanes (Bombardelli &

Morazzoni, 1995; Southwell & Bourke, 2001), which may act as feeding deterrents or stimulants (Repcák & Mártonfi, 1997).

6.2.1 Plant defenses

Physical features such as surface texture or exudate, and leaf shape, size and colour, may affect choice by visual cues, or by influencing an insect's ability to walk or feed on tissue (Bernays & Chapman, 1994; New, 1988). Small insects in particular are often confined to certain plant parts, of which associated structures and chemistry influence feeding (Bernays & Chapman, 1994).

Although nutrient concentrations influence arthropod-plant interactions, the narrow host ranges of phytophagous insects depend mostly on the presence of secondary plant metabolites only found in one or a small number of taxa (Bernays & Chapman, 1994). Defensive chemicals are often located in surface tissues or waxes, and are thus encountered as the herbivore starts to feed (New, 1988). Toxins may be activated on breakdown of tissue, metabolized into active form by the consumer, or rapidly induced by herbivory.

6.2.2 Herbivore response

Discrimination of host from non-host, and host plant quality is made either by the female parent or the neonate larva. Since *A. hyperici* is largely wind-dispersed, pre-alighting discrimination of host plant is less likely to be important than post-alighting discrimination through chemical and tactile stimuli (Willis, 1994). Principal phagostimulants are nutrients (usually sugars), and some common secondary chemicals. Phagodeterrents may cause feeding to cease, but do not cause an insect to move away from the plant. A balance between phagostimulant and deterrent components and concentrations is probably

responsible for feeding choice (Bernays & Chapman, 1994). A chemical that stimulates one species may deter others, and vice versa.

Insects adapted to specific plant species are generally relatively insensitive to the secondary compounds that characterize their host plants (Bernays & Chapman, 1994; New, 1988). Herbivores may detoxify defensive chemicals by conjugation, reduction, hydrolysis or oxidation by enzymes such as mixed function oxidases (New, 1988). There are many examples of sequestration of defensive chemicals and use by herbivores as defense against predators. Behavioural adaptations may result in the ability to feed on plants containing deterrents, for example by feeding on older leaves in which concentrations are lower, or by avoiding resin canals (Bernays & Chapman, 1994). However, it has been argued that the role of plant chemistry has been overemphasized in host-selection by arthropods (Bernays & Graham, 1988; Thompson, 1988), and that complex interactions between host and herbivore behaviour, physiology and ecology, ultimately determine host-selection (Willis, 1994).

6.3 Aims

Several physical factors in the plant, leaf wax density, wax depth, and cell morphology, were investigated to see if any correlation existed with low and high mite-susceptibility. Evidence was sought for physical impediment to feeding and oviposition in the mite, by comparison of mites fed on low and highly susceptible genotypes.

6.4 Method

Plants at rosette stage, with known DNA profile (Chapter 4) and shown to have either low or high susceptibility to *A. hyperici* (Chapter 5), were inoculated with mites by intertwining branches with highly-infested living branches of mite cultures for 24 hours. Three days later, scanning electron microscopy (SEM) was used to examine infested leaves

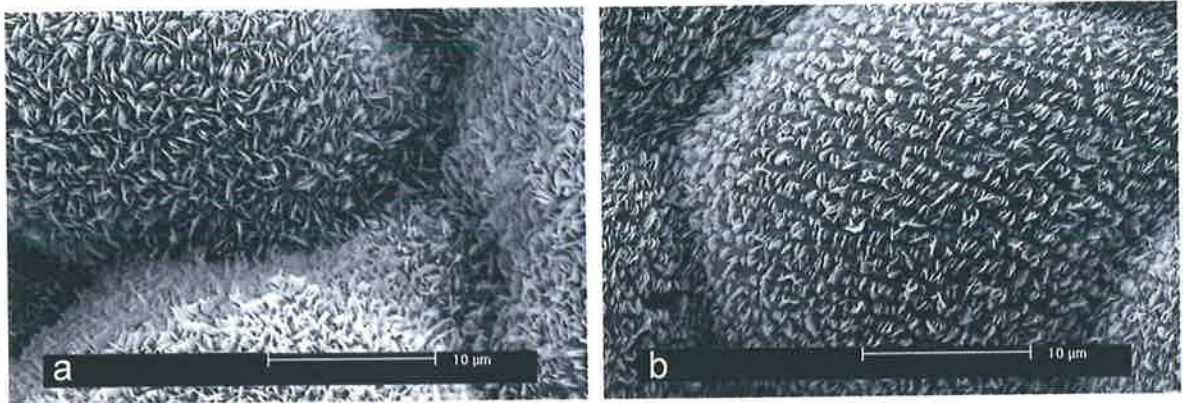
and mites on inoculated plants. Experimental plants included low susceptibility genotypes Cass a and CooJ b (both A1) and OraK b (A10), and highly susceptible genotypes Aven 1b (A4), Flow 5a (A5), Bong 2a (A7) and Cowr a (A13).

Examination was made with a Philips XL30 field emission (FEG) scanning electron microscope, with an Oxford CT 1500 cryostage. The method chosen to prevent collapse of soft structures involved snap freezing in liquid nitrogen, followed by a sublimation step to remove ice crystals. Leaves were either held in a clamp for freeze fracturing, or attached to carbon-based adhesive tape.

6.5 Results and discussion

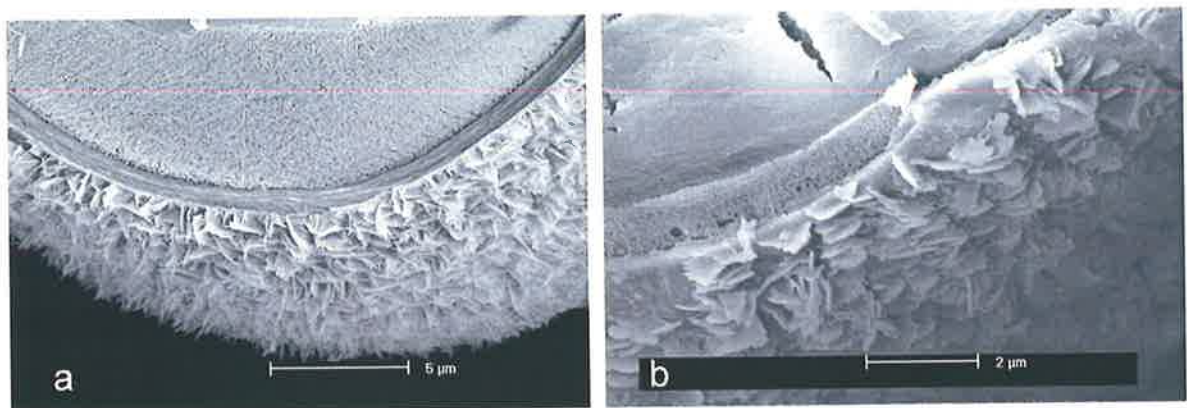
Scanning electron microscopy (SEM) revealed that the leaf cell surface of all *H. perforatum* genotypes tested was covered with wax plates extruded at approximately 90° to the epidermis (for example Fig. 6.2). Evidence of feeding damage, in the form of depressions caused by punctured cells (for example Fig. 6.3a), was seen the upper leaf surface as well as the lower. At first it was thought that variation in wax plate structure might directly prevent feeding by reducing the ability of mite mouthparts to access or penetrate the epidermis, either via increased wax density or depth. Although it seemed at first that wax plates in the low susceptibility A1 individual Cass a (Fig. 6.1a) were denser than in the highly susceptible A13 individual Cowr a (Fig. 6.1b), too much variation existed over the leaf surface and among leaves of different ages for any firm conclusions to be drawn.

Figure 6.1. Wax density on upper epidermis of *H. perforatum* individuals a) Cass a, low susceptibility genotype A1; b) Cowr a, high susceptibility genotype A13.



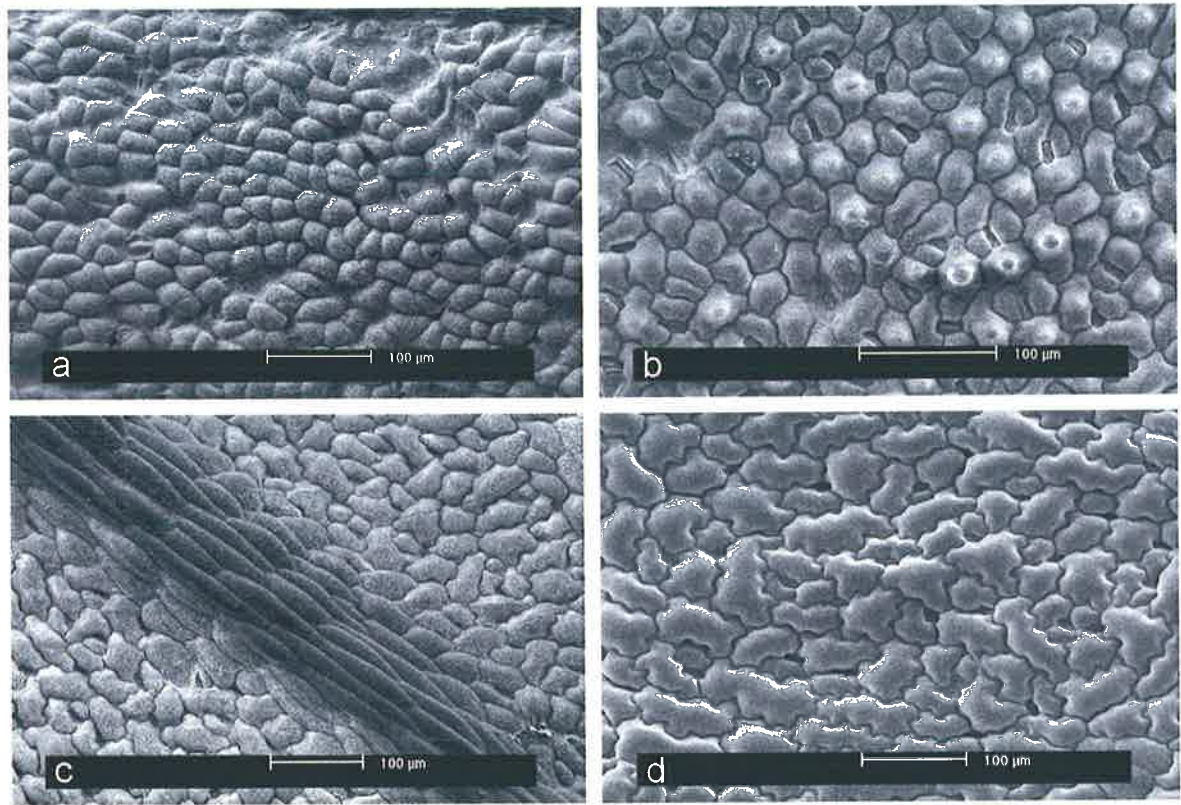
Similarly, despite an initial hint of differences in wax depth (Fig. 6.2), variation over the leaf surface and among leaves of different ages was too high for freeze fracturing of leaves to reveal any consistent differences among genotypes.

Fig 6.2. Wax depth on lower epidermis of *H. perforatum* individuals a) Cass a, low susceptibility genotype A1; b) Cowr a, high susceptibility genotype A13.



The investigation of wax structure was not pursued further, because the amount of variation was large and the method was slow and expensive. However, during the course of the investigation a record of variation in leaf cell pattern was made for several of the genotypes (Fig. 6.3 a-d).

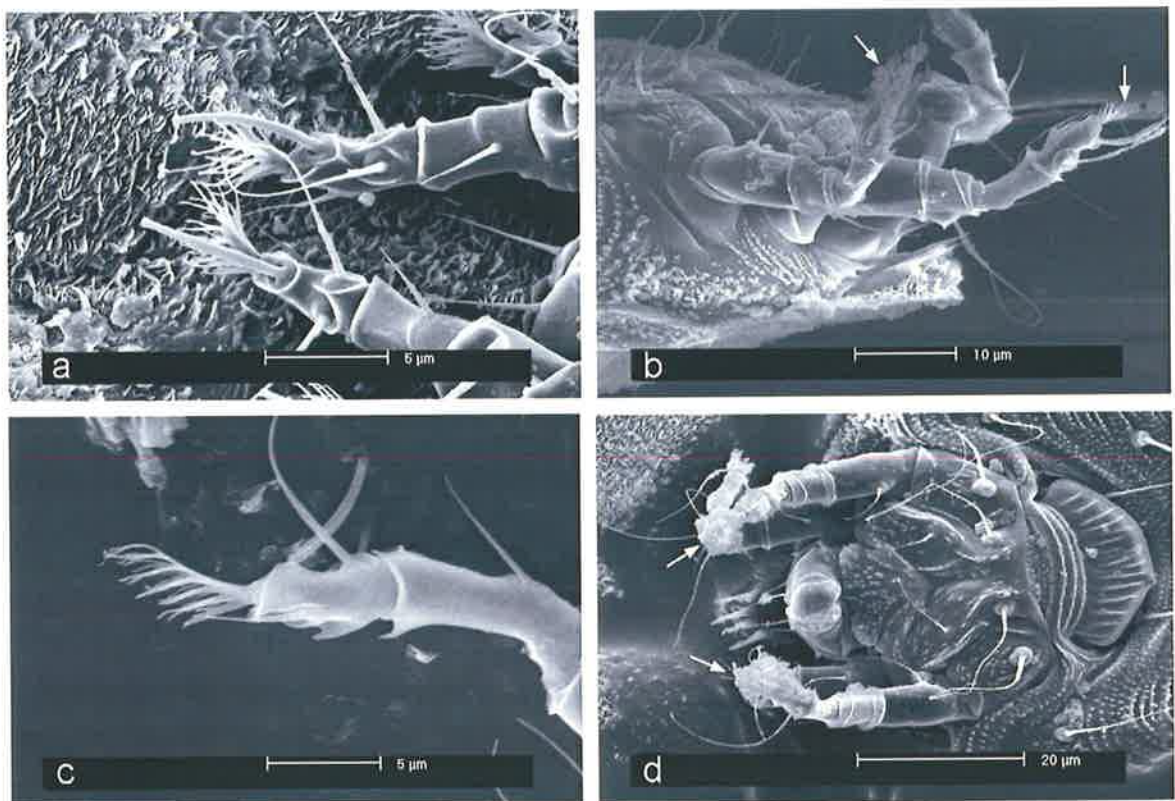
Fig. 6.3. Leaf cell shape of *H. perforatum* lower epidermis (except where noted); highly susceptible individuals a) *Cowr a* (upper epidermis) and b) *Flow 5b*; low susceptibility individuals c) *Cass a* and d) *OraK b*.



It may be that a physical factor related to cell shape or size influences mite behaviour, for example, by acting as a stimulus to feed or oviposit. Oviposition appeared to occur mainly in the shallow depressions left by punctured cells, and it may be that the availability of such oviposition sites had some influence on fecundity. A great deal of variation was seen in cell shape, but this character was unlikely to have influenced mite behaviour, given that no striking difference was seen between low susceptibility individual *Cass a* and highly susceptible *Flow 5b* (Fig. 6.3). Cell size appeared to be lower in the two susceptible genotypes examined (compare Fig. 6.3 a and b, with c and d). However, before reaching any conclusion, far more extensive testing would be required in order to determine whether cell size was consistent across and among leaves of various ages, and for all genotypes within susceptibility groups.

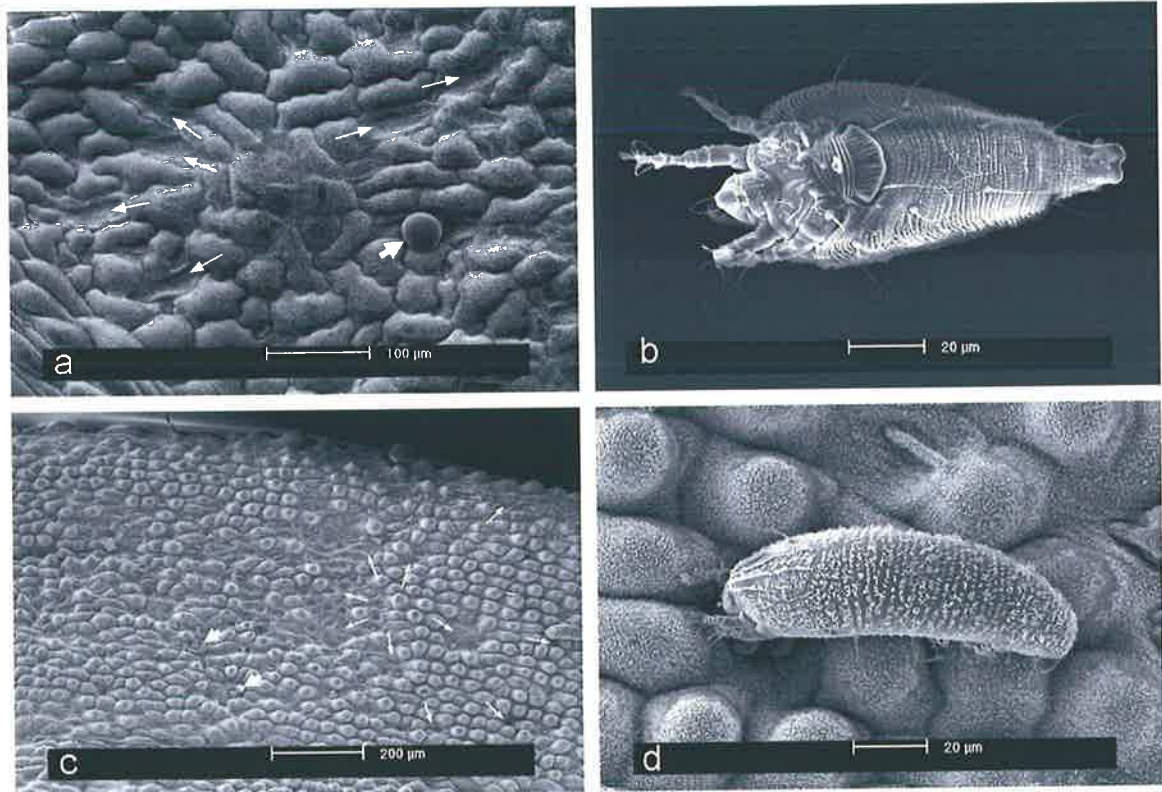
Evidence for physical impediment to feeding was also sought by examining mites that had been feeding for three days on either low or highly susceptible *H. perforatum* leaves. No conclusions could be made regarding reduction of sensory stimuli or feeding by direct obstruction of feather-claws and mouthparts, because wax-like deposits were observed on those structures in individuals on both low and high susceptibility genotypes (Fig. 6.4).

Fig 6.4. Wax-like deposits (arrowed) on feather-claws of mites fed for three days on high (a and b) and low mite-susceptible (c and d) *H. perforatum* individuals; a) *Cowr a*, A13 genotype, dorsal view; b) *Aven 1b*, A4 genotype, ventral view; c) and d) *CooJ d*, A1 genotype, lateral views.



Some evidence of feeding and oviposition was seen on low susceptibility individual *Cass a*, three days after inoculation (Fig. 6.5a), but the majority of mites on those plants were dead or constricted in length (Fig. 6.5b). In contrast, feeding damage on highly susceptible genotypes was more widespread (Fig. 6.5c), and mites appeared to be quite normal (Fig. 6.5d), with no dead mites observed in any of the samples examined.

Fig. 6.5. Leaf surface with evidence of depressions caused by feeding damage (narrow arrows) and oviposition (eggs or exuviae, wide arrows), and mites fed for three days on *H. perforatum* a) and b) low susceptibility individual Cass a; c) and d) high susceptibility Cowr a. The structure located centrally in part a is a black hypericin-containing gland.



6.6 Summary

The results were too inconclusive to exclude hypotheses concerning the influence of physical factors on population dynamics. It remains to be shown what physical or chemical mechanisms are responsible for low *Aculus* mite-susceptibility in *H. perforatum*.

7 Hypericin variation in *Hypericum perforatum*

7.1 Introduction

Grazing has been proposed as an option for the control of mite-resistant *Hypericum perforatum* (St. John's wort) genotypes that are unlikely to be controlled by *Aculus hyperici*. However, *H. perforatum* contains the toxin hypericin, and unless carefully managed, stock grazing the plant may suffer weight loss, reduced wool or milk production, low fecundity and survival rates, or abortion (Bourke, 1997; Campbell & Delfosse, 1984; Parsons, 1957).

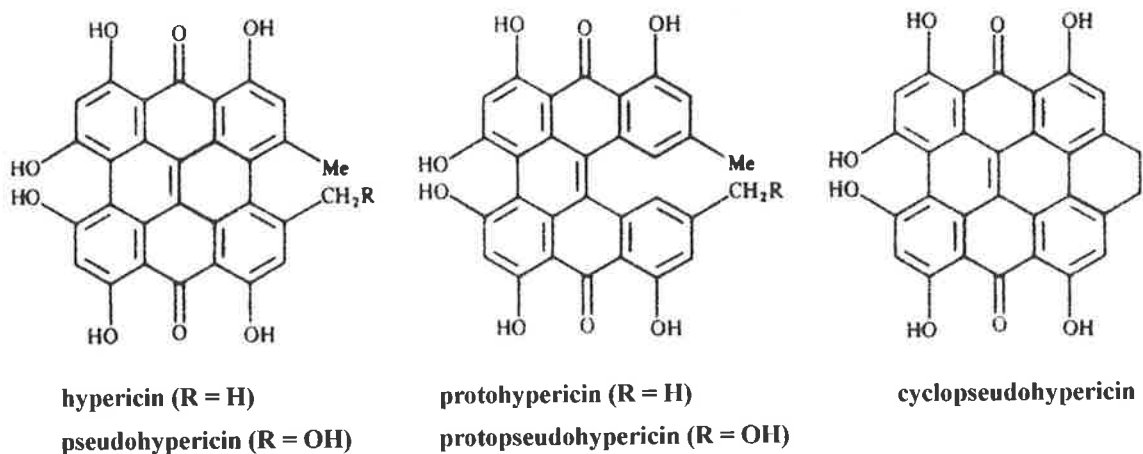
Investigation of *H. perforatum* in Australia indicated higher hypericin levels in a narrow-leaved compared to a broad-leaved "variety" (Southwell & Campbell, 1991). It was considered that grazing management could be improved if information were available on variability in hypericin levels, between plant forms and at different stages of the plant's life cycle (Cullen *et al.*, 1997). A grazing management system subsequently proposed for St. John's wort took into account hypericin variation between the broad- and narrow-leaved morphotypes (Bourke & Southwell, 1999; Southwell & Bourke, 2001). However, the current project examined the identical seed batches used by Southwell and Campbell (1991), and demonstrated that A1, A4 and A12 genotypes existed among the narrow-leaved populations tested (Coolah, Mudgee and Tuena, respectively). A collaborative study was subsequently undertaken with Dr. Chris Bourke (Orange Agricultural Institute, Orange, NSW) and Dr. Ian Southwell (NSW Agriculture, Wollongbar, NSW), in which hypericin variation was examined among ten of the genotypes characterized during the present study. The results are discussed in relation to the role of hypericin in plant susceptibility to *Aculus hyperici*, and to toxicity of the different genotypes and control of *H. perforatum* by grazing management.

7.2 Review

7.2.1 *H. perforatum* toxicity

The first report of *Hypericum* toxicity appeared in the literature in 1787 (Mitich, 1994). The toxic principles are thought to be hypericin and pseudohypericin, which, along with their precursor 'proto' analogues (Fig. 7.1), cause photosensitization, stimulant or depressive effects on behaviour, and altered temperature perception (Bourke, 1997). Horses are most sensitive to hypericin, followed by cattle, sheep and goats, in that order (Southwell & Campbell, 1991). It has been estimated that sheep are affected by 4% of their body weight of St. John's wort, compared to 1% for cattle (Southwell & Campbell, 1991). Skin pigmentation or protection from sunlight reduces the effect (Daley, 1937). However, all stock grazed on St. John's wort-dominant pasture will experience health problems and associated production losses will occur despite variation in susceptibility due to breed or colour.

Figure 7.1. Chemical structure of hypericin-related pigments; hypericin, pseudohypericin, protohypericin, protopseudohypericin, and cyclopseudohypericin (from Southwell, 1991).



7.2.2 Hypericin and hypericin-related pigments

Hypericin is one of a mixture of polyhydroxyphenolic pigments (Fig 7.1) contained in the glands of the stem, leaves, flowers and capsules (Southwell & Campbell, 1991). Hypericin is chemically stable to drying and heat (Bourke, 1997) and persists in hay or old standing plants (Everist, 1974). The hypericin molecule has both hydrophilic and lipophilic properties conferred by hydroxyl and methyl groups, and as a result interacts with cell membranes. The hydrophilic nature of hypericin causes rapid absorption from the gastrointestinal tract and passage of the molecule through the liver without detoxification (Bourke, 1997).

Visible light of wavelengths peaking at 580 nm (range 540-590 nm) energizes the molecule, leading to the production of singlet oxygen and disruption of cell membranes. Photosensitization can result, which is a very disfiguring and painful skin inflammation occurring in unprotected skin exposed to light. The disorder can appear in as little as 24 hours, and will continue as long as ingestion of hypericin and exposure to sunlight occur (Bourke, 1997). Independently of photo-energization, hypericin crosses the blood-brain and blood-mammary gland barriers. Due to the inhibitory effect of hypericin on catechol-o-methyl transferase, adrenalin and noradrenalin accumulate, causing increased heart rate with vasoconstriction, diarrhoea, and an agitated mental state (Bourke, 1997). In the brain, small amounts have an antidepressant or stimulatory effect, however large quantities cause severe depression (Bourke, 1997; Dodd, 1920; Everist, 1974). Hypericin also appears to disturb the temperature control centre, causing an exaggerated perception of hot or cold, or elevation in body temperature.

7.2.3 Hypericin levels in Australian *H. perforatum*

Hypericin levels in Australian *H. perforatum* were compared among three “narrow-leaved” accessions (Coolah, Mudgee and Tuena) and a “broad-leaf” form from Orange that were grown in a common garden (Southwell & Campbell, 1991). Using Soxhlet extraction and spectrophotometry the latter authors found higher hypericin concentrations in the narrow-leaved compared to the broad-leaved accessions (Table 7.1). In all plants hypericin concentrations were higher in the flowers compared to the leaves (Table 7.1) and increasing toxicity during summer was explained by flowering stems containing the highest levels of hypericin (Southwell & Campbell, 1991). The variation found among the parts of a single broad-leaved plant ranged from 40 ppm in stems to 2150 ppm in flowers, thus the variation within a plant was greater than the difference between comparable plant parts of the two varieties. Therefore, the relative toxicities of broad and narrow-leaved varieties were considered less significant than variation among their different plant parts (Southwell & Campbell, 1991).

Table 7.1. Mean hypericin content (\pm standard deviation) of leaves and flowers from different populations of *H. perforatum* (adapted from Southwell and Campbell 1991).

Variety	Population	Plant part	N measurements	Hypericin content (ppm)	
				Mean	SD
Broad-leaved	Orange-1	leaves	3	370	20
		tops*	5	2030	250
	Orange-2	leaves	5	380	100
		flowers	3	2360	170
Narrow-leaved	Mudgee	leaves	5	1040	130
		flowers	1	2980	-
	Coolah	leaves	7	1050	110
		flowers	3	4180	250
	Tuena	leaves	4	1110	100
		flowers	3	5030	180

*the top 8-10 cm of leaf, stem and flower

Hypericin levels in broad-leaved and narrow-leaved varieties growing in the field (at Orange and Tuena, respectively) were assessed over a two-year period (Bourke & Southwell, 1999) and are summarized in Table 7.2. Two- to three-fold greater hypericin levels were frequently recorded for the narrow-leaved compared to the broad-leaved form. In both varieties, the static winter growth phase was far less toxic than the flowering summer stage. In keeping with earlier conclusions (Southwell & Campbell, 1991), the difference in toxicity of broad and narrow-leaved varieties during any period was considerably less than seasonal variation among plant growth stages.

Table 7.2. Hypericin content of broad- and narrow-leaved varieties growing in the field in the central Tablelands of NSW (summarized from Bourke and Southwell, 1999).

Time period	Growth stage	Hypericin content (ppm)	
		Broad leaf (Orange)	Narrow leaf (Tuena)
mid Sept to early Nov	upright flower spikes	100 rising to 500	300 rising to 600
mid Nov to late Dec	full flowering	500 rising to 2500	1000 rising to 5000
early Jan to mid Mar	fruit capsule development	1000 falling to 400	3000 falling to 1500
late Mar to mid Jun	prostrate winter stems	400 falling to 100	1500 falling to 300
late Jun to early Sept	winter stem static growth	between 50 and 100	between 100 and 300

7.2.4 Grazing management

There is anecdotal evidence to suggest that repeated grazing by livestock will eventually suppress St. John's wort growth (Bourke & Southwell, 1999). However, the recommended use of coloured breeds of goat, sheep and cattle, to minimize the photosensitizing effect of hypericin, has not been adopted by producers for a variety of reasons. A grazing management system was therefore proposed for white wool-producing Merino sheep, the most common livestock-producing enterprise in southeastern Australia (Bourke & Southwell, 1999).

The daily intake tolerance level for adult merino sheep carrying four months or more of wool, in the most intense sunlight period of the year, was calculated to be 160 ppm hypericin (Bourke & Southwell, 1999). Using data for daily intake tolerance and hypericin content variation, a grazing management system for the control of the weed was proposed, in which merino sheep are grazed on St. John's wort-infested rangeland when the risk of poisoning is lowest but impact on plant growth is likely to be high (Bourke & Southwell, 1999). Adult white wool merino sheep from a fine or superfine wool producing bloodline and with a fleece of at least four months growth were recommended, to reduce the effect of photosensitization on exposed skin. To suppress plant growth in the least toxic winter growth stage (Table 7.2), a broad-leaved biotype from Orange could be grazed from May 1 to Oct 14 (19 weeks), and narrow-leaved biotype from Tuena from July 1 to Sept 14 (6 weeks). When weed density has decreased the grazing period can be extended, but sheep should always be moved off pasture before shoot growth exceeds 5-10 cm height because poisonous levels of hypericin will rapidly develop.

7.3 *Aims*

The aim was to compare the hypericin content of flowering stems of individual plants of *H. perforatum* for which genotype had been determined in Chapter 4. The relationship between hypericin concentration and mite-susceptibility was examined, and discussed in relation to the role of hypericin in the susceptibility of genotypes to *A. hyperici*, and to toxicity and control of St. John's wort genotypes by grazing management.

7.4 *Materials and methods*

Eighteen individual plants, representing ten different genotypes, were propagated by subdivision of crowns, repotted into 10" pots, randomized across benches and grown in a glasshouse as described in Chapter 4 (Section 4.4.2). Total flowering shoots were

harvested into brown paper bags and dried for 10 h in an oven at 70°C, in the dark. Flowering shoots were harvested approximately two weeks after the opening of the first flower. Due to variation in time to flowering three harvests had to be made, on 10th and 27th December 1999, and 28th January 2000 (Harvests 1, 2 and 3; Table 7.3). Sufficient plant material was available from one of the harvests (27th Dec 1999) to directly compare hypericin content of 17 individuals (nine genotypes) in a one-way ANOVA (Genstat release 6.1).

Hypericin concentrations were determined by Dr. Southwell and Dr. Bourke as follows (Southwell & Bourke, 2001). Dried, ground plant material (2 g), was accurately weighed into a Soxhlet thimble and extracted with 200 ml *t*-butyl methyl ether for 4 h by Soxhlet, to extract chlorophyll. The thimble was dried *in vacuo* for 16 h and re-extracted with absolute 200 ml absolute ethanol for until the red hypericin pigments ceased extracting (8 h). The solution was made up to 500 ml with absolute ethanol, and the visible spectrum measured from λ 500-700 nm. Three separate measurements (replicates) of each individual extraction were made. Hypericin concentrations were determined using the ϵ_{592} 4.16×10^4 value for absorption maximum in ethanol, equivalent to that obtained by using an authentic sample. The λ 592 nm absorptions of the ethanol solution provided combined concentration estimates for hypericin, pseudohypericin, protohypericin and protopseudohypericin, with sufficient distance from co-occurring phloroglucinols and flavonoids to prevent their interference. Where chlorophyll was incompletely removed, an adjustment equivalent to 15% of the λ 660 nm chlorophyll absorption was made to allow for the contribution by the secondary λ 608 nm chlorophyll band. The linearity of hypericin absorption at λ 592 nm was established for absorption values from 0 to 1.8 (i.e. 0 to 5500 ppm).

7.5 Results

7.5.1 Harvest 2

ANOVA on samples from Harvest 2 showed a highly significant difference ($Pr < 0.001$) in hypericin level (Table 7.3). The Tuen p individual (A12) produced significantly more hypericin than all other individuals, with the exception of those with the same genotype. The A10, A7 and one of the A8 individuals (Bund 3b) produced significantly lower levels of hypericin than all other samples except the borderline A1 Wyan n, but were not significantly different from each other. Hypericin values for the latter group were less than half of the top hypericin producers, and all other samples lay between the two extremes.

Table 7.3. Mean hypericin concentration and standard deviation (SD) at three harvest dates. Samples are arranged in increasing order of means for Harvest 2, LSD = 267 ppm. Low mite-susceptibility genotypes are indicated in red (A1/R1, A2 and A10), medium mite-susceptibility in pink (A8, A9 and A12), and all others were highly mite-susceptible (Chapter 5).

AFLP or RFLP number	Identifier	Hypericin concentration (ppm)					
		Harvest 1		Harvest 2		Harvest 3	
		Mean	SD	Mean	SD	Mean	SD
A9	Nels 3b					1076	64
A8	Bund 3b			1109	73		
A7	Bong 2b			1212	85		
A10	TurB m	941	39	1294	111		
A10	OraK a			1373	304*	1539	111
A1/R1b	Wyan n			1640	59		
A8	Bund 1b	1172	206	1792	179		
A6	ScCk x1	1380	71	1818	279		
A4	Aven 2b	973	70	1935	33		
A4	Aven 4a	981	98	1984	205		
A13	Cowr b	1378	83	2131	112		
A2	Wedd 7	1355	133	2139	148		
A2	Wedd 20b	1573	4	2152	171		
A1/R1c	Cass x1	1953	183	2325	163		
A4	MuTa q			2439	133		
A12	TueJ b			2573	222		
A12	TueJ d			2726	120		
A12	Tuen p			2767	169		

* only two replicate measurements of the extract were made

Genotype appeared generally to be associated with hypericin level, with the following exceptions. The Wyan n individual produced significantly less hypericin than the other A1 individual (Cass x1), which might be explained by genetic variation, since the former belonged to R1 subgroup b and the latter to subgroup c (Table 7.3). The significant differences between A4 samples (Aven 2a or 4a, and MuTa q) and between A8 samples (Bund 3b and Bund 1b) cannot be explained by variation detected by either AFLP or RFLP fingerprinting methods. However, RFLP subgroup data was not available for these samples and low levels of variation cannot be excluded. Alternatively, hypericin variation may have been due to slight differences in flowering stage. Additional individuals would have to be examined in order to resolve these issues.

These results are in accord with past conclusions (reviewed in Section 7.2.3) that a broad-leaved form from Orange produced less hypericin than narrow-leaved forms from Mudgee, Coolah and Tuena (Southwell & Bourke, 2001; Southwell & Campbell, 1991). Genotypes of all plants from exactly the same seed batches used in the latter studies were known (Chapter 4). For the present hypericin study, only sufficient plant material from the original Tuena (A12) and Mudgee Tall¹ (A4) seed batches was available, and in lieu of Orange-1 (A10), Coolah (A1) and Mudgee Short (A1), accessions with the same genotype were compared. The Tuen p individual (A12) produced significantly more hypericin than the A1, A4 and A10 genotypes (Table 7.3), as noted earlier. The MuTa q (A4) and Cass x1 (A1/R1c) individuals were not significantly different from each other, but both produced significantly more hypericin than the A10 genotype (OraK a and TurB m). The borderline non-significant difference of the A1/R1c individual (Wyan n) and A10 genotypes was probably due to genetic variation within the A1/R1 genotype, as noted above, so this individual was excluded from the comparison.

¹ It was unclear from Southwell and Campbell's (1991) paper whether "Mudgee" referred to the Mudgee Short (A1/R1c) or Mudgee Tall (A4) accession examined in Chapter 4, so both were considered here.

Hypericin level did not appear to be associated with mite-susceptibility class. For example, genotypes A1, A2 and A10 with low mite-susceptibility (in red, Table 7.3) ranged from low to high hypericin, and a similar situation was observed for medium and highly mite-susceptible classes.

7.5.2 Harvests 1 and 3

Samples from the same plant in Harvests 1 and 2 were consistently higher in hypericin at the second harvest (Table 7.3), as expected due to more advanced flower development (Section 7.2.3). The pattern of hypericin level relative to each other varied between individuals sampled in both Harvests 1 and 2. The discrepancy was probably due to minor variation in maturity of flowering stems, because it was very difficult to collect flowering stems at exactly the same stage.

Two individuals were harvested one month after Harvest 2, due to late flowering. Harvest 3 represented the only sample from the A9 broad-leaved genotype (Nels 3b), which had the lowest hypericin level measured in this study, although it could not be directly compared to other samples in the analysis of variance. The other Harvest 3 sample was a second collection from the OraK a individual, made because insufficient replicate measurements had been available from Harvest 2 (Table 7.3). The hypericin level for OraK a at Harvest 3 was roughly comparable with that seen at Harvest 2.

7.6 Discussion

Genotype appeared generally to be associated with hypericin level, but it was clear that hypericin concentration was not associated with mite-susceptibility, since genotypes within each of the susceptibility classes displayed a wide range of values (Table 7.3). This suggests that hypericin-related pigments are not involved in the mechanism of mite resistance. Importantly, the low mite-susceptible A1 and A2 genotypes and medium

susceptibility A12 genotype were among the highest hypericin producers. The A1 and A2 genotypes, and to some degree the A12 genotype, are not expected to be controlled by *A. hyperici* (Chapter 5) and due to high hypericin are likely be more difficult to control by grazing management, but the A1 genotype in particular may also pose a more extensive threat to livestock production because it appears to be the most widely distributed in northern NSW (Chapter 4). The low susceptibility A10 and medium susceptibility A8 and A9 genotypes may be less problematic because they do not seem to be distributed widely, and despite degrees of resistance to *A. hyperici* may be amenable to control by extended grazing pressure because they were among the lower hypericin producers.

On re-examination of the genetic analysis presented in Chapter 4, neither low nor high hypericin genotypes appeared to cluster together (Figs. 4.4, 4.10, 4.12 and 4.14). Moreover, low hypericin was not exclusively associated with a broad-leaved “biotype”, as has been suggested (Christopher Bourke, pers. com; Southwell and Campbell, 1991); although both the broad-leaved genotypes (A9 and A10) were low hypericin producers, two narrow-leaved genotypes (A7 and A8) were also low in hypericin. However, it would be prudent to increase the number of samples per genotype in order to verify this. It is interesting to note that genotype A6, with somewhat intermediate leaf morphology between the two groups, was intermediate between the lowest and highest hypericin levels.

Genotypes could vary in their potential toxicity to animals but hypericin variation was not likely to have serious implications for grazing management, since levels fell within the range from which recommendations had previously been made. The results demonstrated that the highest and lowest hypericin-producing genotypes examined in past studies (narrow-leaved A12 and broad-leaved A10 genotypes, respectively) were representative of the upper and lower extremes for all the Australian genotypes tested. Many of the narrow-leaved genotypes that were examined produced considerably less hypericin than the

narrow-leaved A12 genotype from which recommendations for grazing management of St. John's wort were made (Bourke & Southwell, 1999). Consequently, the latter control recommendations using merino sheep (summarized in Section 7.2.4) are still acceptable and in the case of some narrow-leaved genotypes may even be conservative. However, the flowering times of different narrow-leaved genotypes were somewhat variable even under controlled conditions, and there is a risk that earlier-flowering genotypes may reach toxic levels during the recommended six-week "safe grazing" phase on winter growth (July 1 to Sept 14), since hypericin levels rise rapidly with the growth of flowering spikes (Bourke & Southwell, 1999). Although we do not have data on seasonal variation in hypericin for all genotypes, the general pattern it is unlikely to differ greatly from the broad- and narrow-leaved forms examined by Bourke and Southwell (1999). Plant growth stage was considered more critical in determining toxicity than variation among narrow- and broad-leaved forms (Southwell & Campbell, 1991), which implies that it would be relatively less important to identify the genotypes of narrow-leaved plants than to monitor onset of flowering and move sheep off pasture before shoot growth exceeds 5-10 cm height, as recommended (Bourke & Southwell, 1999).

The results are also significant to the pharmaceutical production industry and herbal practitioners. Over the past decade St. John's wort has gained in popularity as a medicinal plant for use as an anti-viral and anti-depressant. The bioactive ingredients are not well understood, but possible candidates include hypericin and related bisanthraquinones, as well as flavonoids and phloroglucinols (Southwell & Bourke, 2001). Hypericin yields are likely to vary two- to three-fold among field-collected Australian material, thus from a commercial production or herbalists' perspective collection from previously characterized populations or standardization of extracts may be very important.

7.7 Summary

Hypericin-related pigments do not seem to be involved in the mechanism of mite resistance since genotypes within each susceptibility class displayed a wide range of hypericin values. The low mite-susceptible A1 and A2 genotypes and the medium susceptibility A12 genotype were among the highest hypericin producers. The A1 and A2 genotypes, and to some degree the A12 genotype, are not expected to be controlled by *A. hyperici* and due to high hypericin are likely to be more difficult to control by grazing management, but the A1 genotype in particular poses the greater threat to livestock production because it appears to be the most widely distributed in northern NSW. The low susceptibility A10 and medium susceptibility A8 and A9 genotypes may be less problematic because they do not appear to be distributed widely, and despite resistance to *A. hyperici* may be more easily controlled by grazing because they are lower hypericin producers. Leaf morphology was not a reliable indicator of low hypericin levels, since the low hypericin samples included both narrow- and broad-leaved genotypes. All genotypes produced hypericin levels within the range of high and low hypericin-producing forms examined in past grazing management studies (narrow-leaved A12 and broad-leaved A10 genotypes, respectively). Existing grazing recommendations using merino sheep are consequently still appropriate and in the case of some narrow-leaved genotypes may even be conservative. However, this would apply only as long as sheep are moved off pasture before shoot growth exceeds 5-10 cm height, since plant growth stage has previously been found to be more important than genotype in determining toxicity. An awareness of the two- to three-fold difference in hypericin levels among Australian genotypes may also be very important to the pharmaceutical production industry and herbal practitioners.

8 General discussion

Mismatch between biological control agent and weed genotypes is one of the factors significantly affecting successful agent establishment and efficacy. In order to distinguish between genetic variation and other possible factors responsible for failure of biological control, information is required on variation within and between weed or agent populations. To this end, an examination was made of genetic variation in the invasive weed *Hypericum perforatum*, and the influence of mismatch between agent and weed biotypes on establishment and efficacy of the mite *Aculus hyperici*.

At the inception of the project, three morphological groups (broad-leaved, narrow-leaved and intermediate) were thought to exist within Australian *Hypericum perforatum*, but there was no detailed information on population genetic structure. There was an indication that variability in this phenotypically plastic species had a genetic basis, but the extent and source of genetic variation had not been investigated, primarily because of a lack of reliable characters such as those provided by molecular tools. It had been demonstrated that mite-susceptibility varied among several morphotypes and that this could affect biological control with *Aculus hyperici*, but the biological and geographic extent of such phenotypic diversity was not known.

The conclusions of the present studies on reproductive biology and genetic variation in *H. perforatum* are discussed below, followed by consideration of genetic variation in susceptibility to *A. hyperici* and its influence on establishment and impact. Biological control success is discussed in the context of host range and mismatch of agent and target weed, and hypotheses for success or failure are examined. Future research and the prospects for biological control of this weed are suggested.

8.1 Diversity and biological control of *H. perforatum*.

8.1.1 Reproductive biology, genetic variation and population structure

Even when the degree of apomixis is as high as was found in two Australian genotypes (Chapter 3), the reproductive biology of this facultative apomict provides potential for variation and the development of complex population structures over time. Reproductive pathways appear to depend on ploidy, and include obligate sexuality in diploids, obligate or facultative apomixis in tetraploids, and obligate apomixis in hexaploids, as well as the unusual pathway of diploid doubling. Tetraploids may display a variable degree of sexuality, apomixis, and reduced pseudogamous or unreduced double fertilized egg sacs. Euploid and aneuploid variation is not uncommon in this species and may arise within reproductive or somatic tissue, as noted during this project (Section 4.5.3). These reproductive strategies, together with somatic mutation and the high heterozygosity associated with the presumed hybrid, multisomic origin of *H. perforatum*, have apparently led to a variable species with potential for diversity and evolution in the introduced range.

The results showed that the major determining factors in the genetic diversity and structure of invasive populations of *H. perforatum* were likely to be the number of introductions and the reproductive biology of the plant. The Australian study populations were characterized by relatively high inter-population variation and low intra-population variation (Chapter 4). The conclusion was reached from population structure and historical records that multiple introductions were the most likely source of high inter-population variation (21 genotypes detected among 44 populations), with a minor contribution by somatic variation and recombination in the introduced range. A predominance of tetraploids with a high degree of apomixis (94.2%) was the most probable cause of low intra-population variation, even though major weed incursions had occurred more than a century earlier.

8.1.2 Variation in susceptibility to *A. hyperici*

Strong evidence was found for a genetic basis to variation in mite-susceptibility, and for a clear mismatch of *A. hyperici* with a number of plant genotypes that is likely to result in failure of biological control of *H. perforatum* in some areas (Chapter 5). In plant bioassays designed to exclude the effect of environment as far as possible, mite-susceptibility phenotypes clearly corresponded to particular *H. perforatum* genotypes, irrespective of bioassay or accession. A consistent level of mite-susceptibility within each population sample and a range of susceptibility among samples with different genotypes reflected the low intra- and high inter-population genetic variation. Further, a genetic explanation (loss of a chromosome set) was suggested for the medium mite-susceptibility seen in diploid and aneuploid congeners of a resistant tetraploid (Section 5.4.7). Genotypes were grouped into three classes, depending on whether they sustained decreases (low susceptibility, or “resistant”), relatively small increases (medium susceptibility) or large increases (high susceptibility) in mite numbers compared with initial inoculum levels.

Three resistant genotypes (A1, A2 and A10) were collected from eleven sites in New South Wales, seven medium susceptibility genotypes were collected from five sites scattered across the continent, and the remaining nine genotypes from 25 Australian and two overseas collections were highly susceptible to *A. hyperici* (Table 5.10 and Figs. 5.19-21). While a consistent level of control with *A. hyperici* is expected within most populations as a result of their genetic uniformity, variation in mite-susceptibility is highly likely to influence overall success of biological control with *A. hyperici*. Comparison of six genotypes of known susceptibility for which field establishment history and impact studies were available (Section 5.5.2) suggested that *A. hyperici* will provide optimal control on populations of the highly susceptible genotypes (e.g. Fig. 5.12), establish but provide

relatively slower or sub-optimal control on the medium-susceptibility genotypes, but will fail to establish at all in populations consisting of the three resistant genotypes.

8.1.3 Success of biological control

Two key issues regarding successful biological control of weeds were raised in the introductory chapter. Firstly, more evidence was required to demonstrate that genetic variation in biological control populations has an influence on establishment and control success, in order to distinguish between failure due to genetic variation and demographic or environmental factors. Secondly, it has been argued that weeds with lower genetic diversity are more easily controlled biologically, and that breeding system provides a good first approximation of genetic diversity and population structure for predicting success. A major weakness in tests of this hypothesis was admitted to be the lack of direct data on the genetic population structure of weeds.

8.1.3.1 Genetic variation, host range and control success

The evidence reviewed above clearly showed that genetic variation in *H. perforatum* influenced establishment and growth of *A. hyperici*, which is highly likely to affect the overall success of biological control with this agent. Even so, it is important to point out that genetic diversity *per se* may not influence control success when measures of variation, such as DNA fingerprinting, do not relate to the factor(s) selected for by an agent. Host range for a given agent is dictated by gene(s) for physical or chemical cues and defenses that may vary within species or genera. Thus, biological control success depends on the quality, but not necessarily the quantity, of genetic variation in the weed. Problems arise for the biocontrol practitioner when genetic variation in host suitability is not reflected by taxonomic categories such as “species” or “subspecies”. For example, even the morphologically defined groups within *H. perforatum* were inadequate to describe variation in susceptibility to *A. hyperici*.

The case of *H. perforatum* and *A. hyperici* emphasizes the crucial role that host range plays in successful biological control of weeds. The present results support the suggestion that agent species with typically narrow host ranges such as mites and pathogens have an increased risk of less effective control by comparison with agents with broader host ranges, because it is more likely that resistant genotypes will exist in the host population (Burdon & Marshall, 1981). Mismatch due to sub-specific variation in host range has compromised the success of biological control programmes with several other agents with narrow host ranges: eriophyid mite species *Aceria chondrillae* and *Aceria malherbae* used for biological control of *Chondrilla juncea* and *Convolvulus arvensis* respectively (Rosenthal, 1996), and *Eriophyes cynodoniensis* on Bermudagrass (Cromroy, 1976); and in the rust fungi *Puccinia chondrillina* and *Phragmidium violaceum* used for control of *Chondrilla juncea* (Burdon *et al.*, 1981) and *Rubus fruticosus* (Evans & Gomez, 2004; Evans *et al.*, 1999) respectively. However, despite the risk of reduced effectiveness due to mismatch, agents with narrow host ranges minimize off-target effects and improve the level of safety, an increasingly important criterion in biological control (Hopper, 2001; Willis *et al.*, 2002). Such agents also provide scope for “fine-tuning” to match strains of the agent with the weed, although this might mean that success takes more time.

In host specificity tests, the first step in the sequence of plants to be tested according to the centrifugal phylogenetic method is inclusion of other forms of the same plant species (Wapshere, 1974). The importance of this step for agents with narrow host ranges is reinforced by the results of this study. The logic of the centrifugal phylogenetic method dictates that the full range of plant forms known to exist in the introduced range should be included; testing all the broad- and narrow-leaved *H. perforatum* morphotypes described (Campbell, 1987; Campbell *et al.*, 1989) at around the time of *A. hyperici* host-specificity

screening (CSIRO, 1987; CSIRO, 1990) would have alerted researchers to future difficulties with the resistant A10 and A1 genotypes.

The second level of plants in host-specificity tests includes other species within the genus. There is good evidence that the more closely related plant species are, the more similar are aspects of their morphology and chemistry (and thus the cues that are recognized by a particular agent), and the higher the risk of off-target damage (Briese, 2002). However, in practice the centrifugal-phylogenetic method used in host specificity testing of weed biological control agents is largely based on taxonomic circumscription (Briese, 2002). For example, host specificity tests with *Hypericum* relied on infra-generic taxonomy, and from an “estimated phylogeny” based on this it was later concluded that species closely related to *H. perforatum* are not necessarily better hosts for *A. hyperici* than more distantly related species (Willis, 1994). A better estimate of evolutionary relationships is provided by phylogenetic methods, and in weed biological control using an agent with a very narrow host-range, it becomes particularly important whether a taxonomic grouping contains several distinct monophyletic lineages that have not been recognized (Briese, 2002). An improved phylogeny based on molecular methods may more accurately reflect the evolutionary relationships among *Hypericum* species, and together with the more sensitive bioassay methods developed during the current project, might improve our understanding of the host range of *A. hyperici*. We might then be able to explain the apparent paradox of sub-specific variation in mite-susceptibility in *H. perforatum* when other species in the genus appear to be suitable hosts.

If sub-specific variation exists in the suitability of *H. perforatum* for *A. hyperici*, it follows that we might have only a partial picture for other species in the genus *Hypericum*; some that were not suitable hosts in the host specificity tests may actually contain forms that are suitable hosts, and vice versa. While this may not be a significant issue for introduced

species of low economic importance, it suggests that it may be prudent to investigate the importance of sub-specific variation in native non-target plants, for which risk has come under stronger scrutiny over the last few decades (Briese, 2002). We do not know, for example, whether variation in mite-susceptibility exists (by chance) in the Australian endemic species *H. gramineum* and *H. japonicum*, and until we do we cannot assess the risk of omission of highly susceptible forms from host-specificity tests.

Studies of variation in the target weed may improve predictions of success of biological control programmes (Burdon & Marshall, 1981; Chaboudez & Sheppard, 1995), and are also informative for ecological studies and an integrated approach to management (McFadyen, 2002). While molecular studies are expensive, they are probably far less expensive than the failure of a biocontrol program. The current results indicate that molecular studies should be considered for agent taxa with a reputation of sub-specific variation in host range (e.g. mites and pathogens) where there is a high likelihood of variation in the target weed. Molecular studies can also be used to examine general problems with control or difficulty with agents with good track records; for example, the repeated failures of *Cecidochares connexa* (Strathie & Zachariades, 2000) and a number of other agents on the form of *Chromolaena odorata* found in South Africa (von Senger *et al.*, 2000). As in the case of *H. perforatum* and *A. hyperici*, alternative methods of assessing variation (e.g. morphological) may not provide the information necessary to address these issues. We can only rule out genetic variation when predicting outcome if an agent's host range is known to cover the weed diversity found in the introduced range.

8.1.3.2 Hypotheses of genetic variation and success

The argument surrounding the use of breeding system to predict success in weed biocontrol (Burdon & Marshall, 1981; Chaboudez & Sheppard, 1995) depends on whether breeding system provides a good first approximation of genetic diversity and population

structure. At first glance, the results of the present molecular and mite bioassay studies appear to support Burdon and Marshall's (1981) basic premise that weeds with low genetic diversity are more easily controlled biologically; in Australian *H. perforatum* populations, a high degree of apomixis appears to have produced a largely clonal intra-population structure that is likely to result in consistent local control where the genotype is a suitable host for *A. hyperici*. However, on a wider geographic scale, the implicit assumption that apomixis results in low genetic diversity and, therefore, success in control did not hold, because multiple introductions have resulted in significant genetic diversity and a range of mite-susceptibility among plant populations, and failure to establish on resistant *H. perforatum* genotypes means that overall success with *A. hyperici* is unlikely. Thus, for Australian *H. perforatum*, breeding system was a poor indicator of genetic diversity and control success. The results therefore support the argument that predictions of success based on breeding system alone are only likely to be valid in the case of clones (Chaboudez & Sheppard, 1995).

It is clear that breeding system is one of the factors that must be considered in weed biological control because it greatly determines the way plant populations are structured. However, it is also evident that considerable variation may exist in the home range of facultative apomicts and even clonal plants (Asker & Jerling, 1992; Nogler, 1984; Stebbins, 1950), and that the number and type of introductions will also have a significant impact on genetic variation and hence biocontrol success. Predicting success is not a simple matter, because of the complexity of plant-arthropod interactions that determine host range (Section 8.1.3.1) and the influence of so many other factors (Chaboudez & Sheppard, 1995). It is unfortunate that despite a paucity of quantitative information to substantiate them, the theories of Burdon and Marshall (1981) have been incorporated into biocontrol dogma, and as a result control programs for some sexually reproducing weeds

have not been funded because they have unjustifiably been pre-judged as difficult to control (Chaboudez & Sheppard, 1995; McFadyen, 2000).

8.2 Future research and prospects for control of *H. perforatum*

8.2.1 Impact assessment and distribution

While it seems clear that *A. hyperici* will fail to control resistant *H. perforatum*, it is premature to predict the long-term effectiveness and overall success of the mite on genotypes belonging to different susceptibility classes without ongoing field evaluation of impact and distribution. Photographic records over a seven-year period indicated a reduction in the density of an *H. perforatum* population that contained the highly susceptible A5 and A7 genotypes (Fig. 5.12), but more comprehensive impact studies with populations known to contain A4, A5 and A7 genotypes have been limited to relatively short periods (1.5- 4 years), and reduction in plant density has only been reported at one site containing the A5 genotype (Sections 5.1.4.3 and 5.5.2). To date, impact assessments have not included the four medium susceptibility genotypes; the only information available was that establishment on the population at Tuena, NSW (A12/A19/A20) required repeated releases and mite populations increased relatively slowly (Section 5.5.2). Recently at the Cassilis field site (NSW), known to contain the resistant A1 genotype, several plants were reported to have sustained populations of *A. hyperici* (Richard Arnott pers. com), and an investigation is needed to determine whether those individuals belong to the susceptible A4 genotype also present in that region.

Distribution maps of *H. perforatum* genotypes prepared during this study are currently being used in weed management decision-making. Quarantine inspection of Victorian collections of *A. hyperici* is presently underway prior to release of the mite in Western Australia (Katherine Batchelor, CSIRO, pers. com.). Optimal control is anticipated on the

highly susceptible A5 genotype found across the south of the State, but it remains to be seen what level of control will be achieved on the medium-susceptibility A14 genotype near Bridgetown (Fig. 4.21).

The scattered distribution of resistant and medium-susceptibility genotypes across the country provides foci for their spread and the replacement of susceptible genotypes. Monitoring for this and future incursions would require more accurate distribution maps of *H. perforatum* genotypes. Given the high degree of apomixis and the population structure found during the present study, the identification, mapping and monitoring of resistant plants are likely to be of more use than the study suggested by Jupp *et al.* (1997) to evaluate the extent of outcrossing and selection of resistance in the field, because the spread of mite-resistance is far more likely to be human-mediated via seed (Section 1.3.1) than by pollen flow. As a first step in increasing the resolution of distribution maps, molecular tools could be used to re-examine sites with repeated failure or slow establishment, and to re-evaluate the suggested causes of failure at 27 of the 148 original release sites (Mahr *et al.*, 1997). For example, drought was initially the suspected cause of repeated failure of mites to establish at the Weddin State Forest population (CSIRO, 1995), which was subsequently shown to contain the resistant A2 genotype. There is particular cause for concern that resistant genotypes may be spread deliberately by commercial production for the herbal industry of high-hypericin genotypes that are also mite-resistant (for example, A1 and A2 genotypes), despite individual States having regulations prohibiting movement of *H. perforatum* (see www.weeds.org.au/noxious.htm; for examples see www.agric.wa.gov.au or www.dpiwe.tas.gov.au). An increased risk of future illegal importations of new genotypes is also likely, since hundreds of Internet sites offer mail-order *H. perforatum* seed.

8.2.2 Management options

Expanding the agent gene pool is the next logical step when testing has shown that mismatch had occurred. The collection of new mite strains offers a cost-effective method of control of the more widespread resistant genotypes and of avoiding their spread to replace susceptible genotypes, and should have high priority (Cullen *et al.*, 1997). Resistant genotypes used as trap plants offer a relatively straightforward form of collection of suitable strains of *A. hyperici*, if they exist. The application of molecular techniques may simplify the process by locating the geographic origins of resistant genotypes, and a good starting point would be to examine genotypes in existing collections in Europe; for example, the 113 accessions amassed from botanical gardens across Europe, breeder's stock and ecotypes from personal collections (Matzk *et al.*, 2001). Commercial producers and existing crops may also be a good source of plant genotypes and mite strains. The history of human immigration in areas with resistant *H. perforatum* genotypes could also be considered; for example, where the broad-leaved mite-resistant A10 genotype exists near Orange, early settlers included a significant number of Cornish immigrants (www.members.optushome.com.au/kevrenor/fed_01.pdf).

An estimation of the proportion of genetic variation already in Australia would provide a useful decision-making tool for the management of *H. perforatum*. The estimation of allele frequencies using co-dominant molecular markers, together with an estimation of population size in the native range, would allow us to approximate the proportion of diversity already existing in Australia. Informed decisions could then be made as to the potential success of future management options, such as searching for new mite strains, based on the risk posed by future introduction of new genotypes.

Until biotypes of *A. hyperici* are found for control of resistant *H. perforatum* genotypes, integrated management involving grazing, herbicides and cultural methods offers the only

option for their control, and extra effort in those areas is warranted to prevent further spread. Furthermore, the impact of *A. hyperici* on susceptible genotypes is ultimately uncertain and, because biological control may fluctuate over time due to environmental conditions, integration with other methods is still regarded as essential (Mahr *et al.*, 1999).

8.2.3 Ecological studies

It is tempting to ask whether mismatch of *H. perforatum* genotypes has also occurred with other agents. It has been proposed that “until recently this variation (in morphology and biochemistry) has not had serious ramifications for the biological control of the weed” (Jupp *et al.*, 1997), but we cannot be certain unless we study the genotypes and agents involved in particular release attempts. For example, genetic variation was not likely to be implicated in the difference between good control with these agents in Western Australia and limited control in Victoria (Briese, 1985) (Briese, 1997a) because we can be reasonably certain that the A5 genotype was common to both regions. However, mismatch may have been involved the problematic establishment and spread of *Agrilus hyperici*, localized damage with *Zeuxidiplosus giardi* gall midge, and complete failure of establishment of various *Chrysolina* and *Anaitis* species (Table 1.1).

Molecular methods offer the opportunity to re-examine past studies for evidence that they included different *H. perforatum* genotypes. For example, it is possible that genetic diversity has confounded conclusions of variation in plant habit due to soil types at Bright, Victoria. Type A infestations on deep soil were taller and suckered less, were more susceptible to stresses such as defoliation, and were easier to control than Type B infestations on poorer shallow soils (Clark, 1953b). In a later study over a seven-year period the dynamics of four populations were examined and differences found in characteristics such as density of crowns, stem length, vegetative reproduction, and flower and fruit production (Briese, 1997b). However, during the current project, different

genotypes of the same age under standardized glasshouse conditions varied noticeably in susceptibility to water stress or fungal infections, stem number and degree of suckering. All genotypes in the above studies are unknown with the exception of the A7 genotype found at the Pierce's Creek site (Briese, 1997b), which was included in the present study. In future ecological studies of Australian *H. perforatum*, DNA-based methods offer the most reliable method of discriminating among the many genotypes, but where this is not possible at the time, precise records of study site locations and comprehensive reference collections (e. g., seed collection, herbarium specimens and frozen tissue) should be taken.

8.2.4 Mechanism and genetic control of resistance

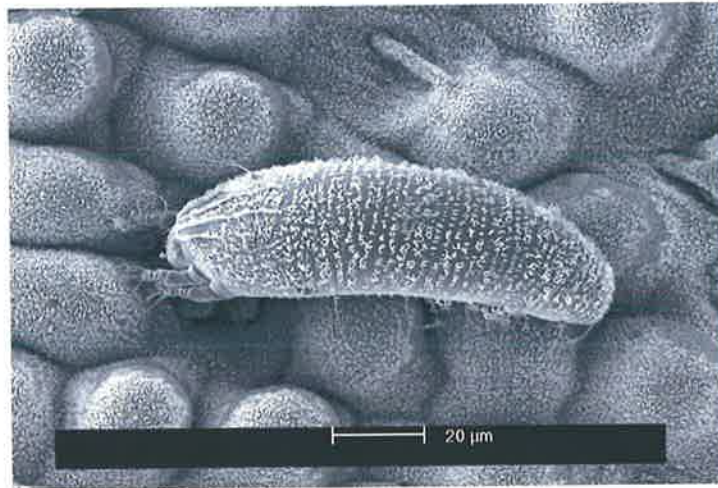
The mechanism for resistance to *A. hyperici* in *H. perforatum* may entail the presence or absence of physical characteristics (Chapter 6) or secondary chemicals, with the probable exception of hypericin (Chapter 7). Comparison of susceptible with resistant genotypes of *H. perforatum* and *Hypericum* species may help elucidate the system(s) involved.

Resistance genes have generally been studied in crop plants, thus it is of theoretical interest to investigate the genetic control of resistance in a natural ecosystem. The range of mite-susceptibility seen in the present study and the evidence from ploidy variation is not inconsistent with an hypothesis of single gene control, which could be tested by marker segregation in the progeny of classical crosses of resistant and susceptible genotypes. The diploids found to have low and high susceptibility in the present study (Section 5.4.7) present an excellent opportunity to pursue this in the future since they are likely to reproduce primarily by sexual means, which requires simplified screening compared with tetraploids.

8.3 Conclusions

Molecular methods are revolutionizing biological control by making it easier to identify agents and target weeds, and describe population structures and phylogenetic relationships (IOBC, 2002), in order to understand the complex relationships between target weeds and biocontrol agents and manage their diversity (Sheppard *et al.*, 2002). The DNA-based methods developed during this study were successfully applied to the identification of individual plants in studies of the reproductive biology, population genetic structure and host suitability of the target weed *H. perforatum* to the biological control agent *A. hyperici*. Breeding system and number of introductions were found to contribute, respectively, to relatively low intra-population and high inter-population genetic variation in this facultative apomict. Demonstration of agent mismatch to a number of genotypes, as a result of sub-specific variation in host-suitability, has added to our understanding of agent establishment and efficacy in this weed, and quantitative data on genetic population structure enabled the examination of hypotheses of biological control success. The AFLP and RFLP multilocus methods can be used in a scientific approach for future monitoring and impact evaluation, and for precise host matching to other strains of *A. hyperici*.

Biological control is encountering difficult times, with perceived low success rates compounded by an increased perception of environmental risks (Sheppard *et al.*, 2002). Neither view should apply to the Australian *H. perforatum* biological control program with *A. hyperici*. The agent is highly specific to the majority of genotypes of the weed and will provide a level of control over the bulk of infestations, and there are good prospects for future success by fine-tuning to achieve control of resistant genotypes. Environmental risks are minimal because the host range of the agent is limited and off-target effects negligible (Willis *et al.*, 2002), and the tools are available to verify that a new strain of *A. hyperici* or any other agent presents an acceptably low risk to native *Hypericum* species.



“The thing to know is when the battle is lost, but not the war”

Mr Richard Arnott, Grazier, Coolah, New South Wales (Arnott, 1997)



Appendix A. *Hypericum perforatum* accessions

Hypericum perforatum accessions used in DNA analysis and bioassays. Co-ordinates without brackets were supplied by the collector; those in brackets are taken from the closest named geographical feature in the Gazetteer of Australia 2001. Accession names provided by collectors are surrounded by double quotation marks. Date = day.month.year. Abbreviations for collectors are provided in a key below.

Identifier	State ^a	Co-ordinates	Accession notes	Collection ^b	Date	Collector
Adel	NSW	(35°17'S, 148°03'E)	Adelong	B	1989	MC
Alex	Vic	37°10'26"S, 145°39'38"E	Alexandra	I	15.7.1996	FM
Aven	Vic	36°53'41"S, 145°16'23"E	Avenel	I	17.7.1996	FM
Beec	Vic	(36°21'S, 146°41'E)	Beechworth historic park	B	1999	FM
Bemb	NSW	(36°36'S, 149°34'E)	Bemboka, approx. 5 km out on road to Bega, near bridge	B	1989	MC
Bena	Vic	36°57'07"S, 147°43'12"E	Benambra	I	28.8.1996	FM
Bong	SA	(38°00'S, 140°57'E)	Bonganditj Native Forest Reserve, 1.2 km N of Donovans	I	14.4.1997	GM
Broc	WA	(33°58'S, 115°45'E)	Brockman Hwy, 5 km E of Nannup	I	22.5.1996	SL
Bund	SA	(33°17'S, 138°36'E)	Bundaleer Pine Forest, 13 km S Jamestown	I	28.7.96	MS
Burr	NSW	(32°39'S, 149°06'E)	Burrendong Park, west side of Burrendong Dam	T	1.4.1997	GM
CaFl	NSW	(35°34'S, 149°27'E)	Captains Flat, 1-2 km NE on road to Queanbeyan	B	1989	MC
Cana	-	-	Richters The Herb Specialists, packaged herb seed from Canada	B	20.5.1996	JS
Cass	NSW	(32°00'S, 149°59'E)	Cattle Ck, 15 km N of Cassilis	B	8.3.1996	PL
Cast	Vic	37°01'14"S, 144°18'06"E	Mt. Alexandra, Castlemaine	I	8.8.1996	FM
CaTa	Vic	as above	Castlemaine, tall plant	I	1999	FM
Chin	WA	34°23'S, 116°27'E	Cnr Chindalup Rd and Tone River Camp Rd, Manjimup Shire	I	23.5.1996	SL
CooJ	NSW	31°45'S, 149°52'E	'Birmam Wood', property of grazier Richard Arnott, 20km NE of Coolah	B	1993	PJ
Cool	NSW	as above	as for CooJ	B	1989	MC
Cowr	NSW	(33°49'S, 148°41'E)	Cowra	B	1994	MC
Craw	SA	(34°43'S, 138°56'E)	Mount Crawford Forest	F	28.3.2000	GM
Cudg	NSW	?	Cudgong River Park, eastern side of Burrendong Dam, 35 km from Mudgee	Q	2.4.1997	GM

Brid	WA	50H0414736, 6249313	Daniels Rd, now Forrest Park Ave, Bridgetown/Greenbushes Shire	I	23.5.1996	SL
Eldo	Vic	(36°19'S, 146°31'E)	Eldorado	B	1993	FM
Flow	Vic	37°18'56"S, 145°19'03"E	Flowerdale	I	18.7.1996	FM
Inve	NSW	(29°46'S, 151°07'E)	Inverell	B	25.2.1997	PL
Karr	WA	(34°12'S, 115°06'E)	Karridale, 1 km W on Bushby Rd	I	17.4.1996	JS
Merr	NSW	?	Merrendee, 2 km from Cudgegong Park site, towards Mudgee	Q	3.4.1997	GM
Mont	France	-	Montpellier	B	3.1999	SB
MuCo	NSW	(32°34'S, 149°35'E)	Mudgee Common, 2 km E of Mudgee towards Mt Frome	T	3.4.1997	GM
MudJ	NSW	32°36'S, 149°35'E	Mudgee Common	B	1993	PJ
MudL	NSW	(32°34'S, 149°35'E)	Old Grattai Rd, 7km from Mudgee, 3 km from Mudgee Common	B	3.1996	PL
MuSh	NSW	(32°34'S, 149°35'E)	"Mudgee Short" (= "Mudgee Intermediate") growing with "Mudgee Tall" approx 10 km out of Mudgee on road to Gulgong	B	1989	MC
MuTa	NSW	(32°34'S, 149°35'E)	"Mudgee Tall", see MuSh	B	1988	MC
Nann	WA	(33°58'S, 115°45'E)	Folly Rd, Nannup, Nannup Shire	I	22.5.1996	SL
Nels	Vic	38°02'59"S, 141°01'19"E	Nelson	I	26.7.96	FM
Ora1	NSW	(33°16'S, 149°06'E)	"Orange 1" (= "Duntry League") Orange Golf Course	B	1989	MC
Ora2	NSW	(33°16'S, 149°06'E)	"Orange 2", Agriculture Research and Veterinary Centre, Forest Rd, Orange	B	1987	MC
OraK	NSW	(33°16'S, 149°06'E)	David Kemp's property, Orange	B	4.3.1996	RR
Pier	ACT	(35°22'S, 148°55'E)	Pierces Creek Pine Forest	F	1.2000	TW
Ronk	WA	34°27'S, 116°12'E	Property owned by Alan Ronk, Manjimup Shire	I	23.5.1996	SL
ScCk	SA	35°05'18"S, 138°40'26"E	Scott Creek, corner of Dorset Vale Rd and Matthews Rd	B	8.2.1996, 10.5.97	GM
ScCk-1	SA	35°05'18"S, 138°40'26"E	Scott Creek, corner of Dorset Vale Rd and Matthews Rd	I	22.3.1996	GM
ScCk-2	SA	35°05'18"S, 138°40'26"E	Scott Creek, corner of Dorset Vale Rd and Matthews Rd	I	22.3.1996	GM
Smit	ACT	(35°17'S, 149°05'E)	Smiths Paddock, Tony Willis' field site 3, adjacent Rani Dve, Black Mountain	F	1.2000	TW
SESA	SA		Bonganditj Native Forest Reserve, 1.2 km N of Donovans	I	16.8.1996	RS
Talm	NSW	35°55'S, 147°24'E	Talmalmo	B	1993	PJ
Tuen	NSW	(34°01'S, 149°19'E)	3-5 km S of Tuena, on Common	B	1989	MC
TueJ	NSW	34°01'S, 149°25'E	1 km E of Tuena, on private property (demography study site)	B	1993	PJ
TurB	NSW	(33°04'S, 149°41'E)	Turon River, Sofala, broad leaf	B	27.3.1996, 25.3.1997	PL

TurN	NSW	(33°04'S, 149°41'E)	Turon River Sofala, narrow leaf	B	22.4.1996, 25.3.1997	PL
Viol	Vic	(36°38'S, 145°44'E)	Violet Town	B	1993	FM
Wedd	NSW	(33°58'S, 148°02'E)	Weddin State Forest, 25 km SW of Grenfell	T	1.4.1997	GM
Wonn	Vic	(37°16'S, 146°52'E)	Wonnangatta	B	1993	FM
Wyan	NSW	(35°13'S, 148°20'E)	Wyangala Dam, 29 km SE of Cowra	B	1993	PJ

^aState: ACT = Australian Capital Territory, NSW = New South Wales, SA = South Australia, Vic = Victoria, WA = Western Australia.

^b Collection method: B = bulked seed from multiple plants, I = separate seed lots collected from randomly distributed individuals, T = separate seed lots collected along a transect through the population, Q = separate seed lots collected from individuals defined by quadrat coordinates, F = fresh plant material from discrete individuals.

[†] Montpellier was the original site of collection of *Aculus hyperici* in Southern France. Canadian samples were confiscated from a Perth Garden Show by Agriculture Western Australia.

Key for collectors

Initial	Collector	Contact
SB	M. Stefane Böttcher	CSIRO Entomology, European Laboratory, Campus International de Baillarguet, 34980, Montferrier-sur-Lez, France
MC	Dr. Malcolm Campbell	Agricultural Research and Veterinary Centre, Forest Rd, Orange, NSW, 2800
PJ	Mr. Paul Jupp	Animal and Plant Control Commission, Primary Industries & Resources SA, GPO Box 1671, Adelaide, SA, 5001
SL	Ms. Sandy Lloyd	Agriculture Western Australia, Locked Bag 4, Bentley DC, WA, 6983
PL	Mr. Paul Lutschini	NSW Agriculture, 90 Market St, Mudgee, NSW, 2850
FM	Mr. Franz Mahr	Keith Turnbull Research Institute, PO Box 48, Frankston, Vic, 3199
GM	Ms. Gwen Mayo	Dept. of Applied and Molecular Ecology, Waite Campus, University of Adelaide, PMB 1, Glen Osmond, SA 5064
RR	Assoc. Prof. Rick Roush	CRC Weed Management Systems, Waite Campus, University of Adelaide, PMB 1, Glen Osmond, SA 5064
RS	Mr. Richard Sage	District Council of Grant, 7 Charles St, Port MacDonal, SA, 5291
JS	Dr. John Scott	CSIRO Entomology, Floreat Park Laboratories, Wembley, WA, 6014
MS	Ms. Mary Simon	PO Box 134, Jamestown, SA, 5491
TW	Dr. Tony Willis	CSIRO Plant Industry, GPO Box 1600, Canberra, ACT 2601

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