The conservation biology of *Hemigenia exilis* (Lamiaceae), a serpentine endemic from the arid zone of Western Australia

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Abstract

This study was instigated by the re-discovery in 1995 of *Hemigenia exilis*, a rare serpentine endemic in the arid zone of Western Australia. It addressed issues pertinent to five areas of conservation biology of the species.

Levels and patterns of genetic diversity were quantified using RAPDs. *H. exilis* had a genetic diversity of 0.38 and a heterozygosity of 0.27, which is higher than the levels found in most rare, long-lived shrubs. Over 80% of the genetic variation was partitioned within populations, typical of long-lived out-crossing perennials. Importantly, the species was differentiated into a northern and southern provenance, with implications for rehabilitation of the species after mining.

The next investigation placed the species into a phylogenetic context within the Australian subfamily Chloanthoideae, by analysing *rbcL* sequences of 28 West Australian Lamiaceae species, including *Hemigenia exilis*, and a range of species belonging to Teucrioideae and other subfamilies. Within the tribe Prostanthereae, *H. exilis* was sister to a clade encompassing *Hemigenia*, *Hemiandra* and some *Westringia* species, suggesting that further analysis is required to clarify the phylogenetic relationships within that clade. A second clade consisted of *Prostanthera*, supporting the monophyly of this genus. *Microcorys subcanscens* appeared as the sister to Prostanthereae. In the tribe Chloantheae, the genera *Dicrastylis*, *Cyanostegia* and *Lachnostachys* were monophyletic. The position of *Pityrodia* was not resolved. Teucrioideae encompassed the Australian taxon *Spartothamnella teucriiflora*, and several other genera, with *Teucrium racemosum* at the base. Monophyly of *Teucrium* was not supported.

Aspects of seed and germination ecology of *H. exilis* were addressed in a series of experiments, designed to deliver effective conservation and restoration of the species. Viable seed averaged 25%, while 10-seed weight averaged 19 mg. The seeds possessed a combination of physiological and mechanical dormancy, which was alleviated by application of 30 µM gibberellic acid (GA_3), or removal of seed coat-endosperm complex.

Germination was highest at temperatures between 15 °C to 20 °C, indicating that the species is predisposed to germination in autumn or spring, rather than in summer. Dormancy was furthermore alleviated by seed burial under field conditions, and by dry storage for 12 months of the native soil seed bank. Germination was 2%-8% after 3 months of burial and increased to 20%-30% after 30 months, depending on genetic provenance. The increased germination was related to a reduction in seed coat strength and changes in seed coat morphology. No decline in viability was observed, suggesting that the species may form highly persistent soil seed banks. Analysis of the natural soil seed bank showed that germination of H. *exilis* increased with storage, while germination of annuals decreased. Addition of GA_3 had no effect.

To identify potentially narrower ecophysiological tolerances that might explain the rarity of *H. exilis*, the endemic was compared with seven co-occurring long-lived shrubs, as well as three short-lived shrubs, and two tree species, all being widespread. The seasonal responses of *H. exilis* in terms of xylem potential and various aspects of photosynthesis were comparable to species with similar life forms, in that xylem potential, photosynthesis and stomatal conductance were reduced over summer, in concert with a reduced carbon demand, as growth stalled. Thus the physiological parameters chosen for this investigation did not explain the restricted distribution of *H. exilis*. At the study site, only the tree *Eucalyptus grasbyi* and some short-lived shrubs appeared to be unaffected by summer drought, which suggests that these species, unlike *H. exilis*, have access to water at depth, or to hydraulically lifted water.

The final component of the study identified suitable soil materials and seedling pre-treatments for rehabilitation of *H. exilis* after mining. In a trial on a waste rock dump, with drip irrigation installed for the first summer, the survival of *H. exilis* seedlings after two years was highest (69%) in waste rock material from the ferrugineous zone (FZ) (cf. 50% in topsoil (TS) and 38% in the FZ:TS mix). The FZ soils were higher in nickel and cobalt and lower in most nutrients, but had higher penetrability, than topsoil. Pre-treating seedlings with salicylic acid, an anti-desiccant, did not enhance seedling survival. These results suggested that the species was adapted to arid conditions, and to a stressful edaphic environment. Plants on the waste dump all commenced flowering and set seed within 2

years. Recruitment of seedlings was also observed. This development may be interpreted as the first step towards a self-sustaining population. Implications of this study for further ecological research of arid zone plants, and endemics in particular, are discussed.

In summary, the knowledge gained in this study, spanning an understanding of high genetic diversity and differentiation of the species to phylogenetic relationship, exact germination requirements, ecophysiological responses and specific rehabilitation requirements of *Hemigenia exilis* will contribute to the long-term conservation of this species.

Table of Contents

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Candidate's Declaration

One of the eight chapters contained within this thesis is presented as published work:

Chapter 2 — Mattner, J., Zawko, G., Rossetto, M., Krauss, S., Dixon, K., Sivasithamparam, K, 2002. Conservation genetics and implications for restoration of *Hemigenia exilis* (Lamiaceae), a serpentine endemic from Western Australia. Biological Conservation 107, 37-45.

Prof. Sivasithamparam and Dr Dixon, in their role as supervisors were involved in overseeing the research and provided constructive criticism on the manuscripts. Grace Zawko, Maurizio Rossetto and Siegy Kraus were involved in parts of the conservation genetics study.

With these provisos, the work presented in this thesis is entirely my own, unless specifically stated otherwise.

Chapter 1

General Introduction

Aspects of conservation biology, with reference to rare plants in the arid zone of Western Australia

The aims of conservation are to protect biological diversity and to maintain ecological processes and systems. This requires the maintenance of all the components of biodiversity through a natural system of patch dynamics, while at the same time, ensuring that rare and endangered species are not neglected. Successful conservation consists of five key scientific areas:

- Taxonomy and genetics, in order to recognise ecologically significant units and to set appropriate conservation priorities;
- Biogeography and evolution, to ensure that ecosystems, communities and species are adequately represented in reserves;
- Regeneration and replacement ecology, to understand various ways of reproduction, recruitment and plant establishment;
- Resource cycling, to understand the role of water and nutrients in plant establishment and growth; and
- Risk assessment, to devise and implement conservation practices that minimize the risk of extinction (Main 1996).

These tasks are particularly challenging in Australia, an island continent characterised predominantly by aridity, tectonic stability, lack of topography and ancient soils, which are highly weathered and nutrient deficient. These soils support some 25,000 vascular plant species, representing about 10% of the world's flora. Species endemism is very high at 85%. It is also a country which has experienced large-scale irreversible transformations over the short time span since European settlement approx. 200 years ago. The biggest factor impacting Australian ecosystems is large-scale clearing for agriculture (Hopper 1998).

Western Australia, which is 1/3 of the continent, contains between 10,000 and 12,000 vascular plant species, just under half the Australian total. The Western Australian flora is distributed across three biogeographic regions:

1. South-West Province with a Mediterranean climate, globally one of the hotspots of species diversity;
2. Northern Province characterised by a tropical climate; and
3. Eremaean Province, an arid zone with less than 250mm precipitation, containing ca. 2100 plant species, 14% of which are restricted to that province (Beard 1980, Hopper 1998).

This study addresses some of the conservation issues outlined above, focussing on *Hemigenia exilis*, a rare and endemic species restricted to serpentine outcrops in the arid zone of Western Australia. The study also includes some investigations into co-occurring species. The approach is based on a reductionist / utilitarian philosophy, and is significant in advancing the biological understanding of this species. Together with similar studies, it will contribute to a broader understanding of ecological processes taking place at the species, community and ecosystem level.

Definition of rarity

Plants are rare for a variety of reasons and in a range of ways. They may be rare because of anthropogenic influences, such as clearing of natural vegetation and destruction of habitats, or they may be naturally rare. 'Rare" may mean narrow geographical range, narrow habitat specialisation, or low local population density; or any combination of these. Species restricted to patches of specific habitats will have likely survived in isolated populations for a long time, and have evolved strategies to cope with this situation. Generally "sparse" species possess traits that enable them to tolerate low population density (Huenneke 1991).

"Rarity is the attribute of a vast number of species in all classes, in all countries" (Darwin 1859). In most biotas, many species are to some degree rare, depending on the criteria chosen. Few species tend to be very common and dominant, and some species are at an intermediate range of abundance. A species may be rare in at least seven different ways, based on their distributional patterns (Rabinowitz et al. 1986), with

factors including geographic range, habitat specificity and population size determining the nature of rarity. These factors influence the degree of risk that a rare species is vulnerable to different extinction processes.

Species of concern include those that have become rare, and have had their population size reduced as a result of human interference. Of greatest concern are species that are naturally rare AND whose populations have been reduced in size AND whose habitat is under threat. *Hemigenia exilis* falls into the category of naturally rare and endemic, being geographically restricted to the Northeast Goldfields (NEG) of Western Australia, and edaphically confined to serpentine outcrops. Since the species occurs, in some instances, on economically viable nickel deposits, some populations are additionally threatened by mining.

The role of genetic diversity in conservation of rare plants

Conservation genetics is the application of genetic tools to preserve species as dynamic entities (Frankham et al., 2002). The protection of genetic diversity, especially of rare plants and of small populations, has become a priority for conservation efforts over the last two decades. The long-term objective is to maintain evolutionary viability of the taxon by conservation management based on genetic knowledge.

Genetic variation is determined by three factors (Frankel et al. 1995): abiotic, biotic and population dynamics. Abiotic components include climate, soil and location. Biotic factors are represented by competition, symbiosis and predation. Population dynamics involve factors influencing genetic diversity including population size, mating system, mutation, dispersal mechanism and migration. Levels of genetic diversity in rare plants are generally low, especially when existing in small populations. This generalisation applies predominantly to species that have become rare because of human impact. In contrast, species that are naturally rare are likely to have existed for a long time and exhibit levels of genetic diversity high enough to cope with small population sizes and low rates of gene flow between populations over large distances.

Plants differ greatly in their levels and patterns of genetic variation. While generalisations such as "endemics are less diverse than widespread species" and "outcrossing species are more diverse than selfing species" (Hamrick & Godt 1996)

hold true in many cases, this does not apply universally. Low genetic diversity exposes species and populations to a high risk of threat and even extinction, as they lack the capacity to adapt to environmental pressures. In contrast, genetically diverse species and populations are protected against environmental perturbations, including demographic and environmental uncertainties, natural catastrophes and genetic stochasticity (Shaffer 1981).

Sampling and measuring genetic diversity

Conservation programs necessarily involve selection of a subset of the entire species by sampling. The Center for Plant Conservation (CPC) has developed guidelines to design scientifically sound and practical methods of data collection. The CPC recommends sampling from at least five populations of the species under investigation, and that samples be taken from 10-50 individuals per species (Center for Plant Conservation 1991). This sampling density will generally detect existing genetic variation.

Early methods to quantify genetic diversity were confined to the measuring of morphological and phenotypic variation. While these methods do not require advanced technology, they are often not sensitive enough to detect small but significant variations.

The application of molecular markers began with the development of isozymes (Lewontin & Hubby 1966). Isozymes are multiple forms of an enzyme, and are genetically controlled. The strengths of this technique include low cost, low tech, an informative number of available loci, and, importantly the co-dominant inheritance pattern. For most species, these strengths outweigh the drawbacks, which include low variability, potential lack of detection, and occasional difficulties in interpretation of banding patterns. Isozymes remain a valuable tool for measuring genetic variation and inferring evolutionary process.

The next technological advance in molecular techniques came with the application of restriction endonuclease techniques, DNA restriction fragment length polymorphism (RFLP) (Brown 1980). This technique was developed before the advent of PCR and produces co-dominant markers like isozymes. RFLPs are useful for population studies and diversity classification, provided that sufficient polymorphism can be detected. This

technique is generally more sensitive than isozymes and is highly reproducible. The drawbacks are high development costs, prior knowledge of some DNA sequence, and the need for large amounts of DNA.

With the invention of polymerase chain reaction (PCR) by Mullis and Faloona (1987), new procedures including random amplified polymorphic DNA (RAPD) (Williams et al. 1990) or arbitrarily primed PCR (AP-PRC) (Welsh & McCleland 1990) have become widely employed. Both technologies require only small amounts of DNA and use short, arbitrary primer sequences to amplify anonymous regions of DNA in a single PCR reaction. These techniques produce one, or a series of dominant bands (markers) that can be visualised on agarose or polyacrylamide gels. The current study will use RAPDs.

More recent is the development of amplified fragment length polymorphism (AFLP) (Vos et al. 1995). In this methiod, total DNA is digested with two endonucleases and two successive PCR amplifications using different sets of primers. As with RAPDs, the AFLP technique produces dominant markers and may produce non-homologous co-migrating fragments, but has the advantage of amplifying 100-200 bands in a single reaction. A further advantage of AFLP over RAPDs is the high reproducibility. However the drawback of this method is its high cost.

Microsatellites are one of the most recent tools in population genetic studies. The usefulness of this method lies in its potential for identifying numerous loci with moderately frequent alleles. The highly polymorphic nature of these loci allows the tracking of gene movements within natural populations

The role of phylogenetics in conservation

Molecular phylogenetics is based on the quantification of differences in DNA sequences and has only recently been applied to the theory and practice of conservation biology. There are at least four areas in which molecular systematics can contribute to the conservation of rare plant species:

- It can refine the species concept: concepts that incorporate evolutionary history and reflect phylogeny will be more useful for preserving diversity than those that do not;

- This method can identify lineages in need of conservation: analysis of conspecific populations may reveal multiple lineages requiring protection as evolutionary distinct units;
- It can help in setting conservation priorities: phylogenetic relationships, together with biogeography, assist in identifying areas where speciation is occurring; and,
- It can also detect hybridisation and evaluate its effect on the biology of rare species: hybridisation may lead to extinction of taxa, but conversely hybridisation of a rare species and a widespread congener may be one avenue to preserve the genepool of the rare species (Soltis & Gitzendanner 1999).

Integration of such phylogenetic information in establishing conservation priorities can lead to an efficient conservation strategy. The application of molecular phylogenetics will avoid incorrect 'lumping' of several distinct species into one recognised species, which might hinder the protection of endangered species. Conversely, 'splitting' of one species into two or more recognised taxa might lead to erroneous conservation decisions (Frankham et al., 2002).

Tools used in molecular systematics involve the sequencing of genes followed by the analysis of differences in these sequences. In the early 1990s, most studies relied on *rbcL* sequences, a chloroplast gene with a broad range of taxonomic utility in angiosperms, ranging from species / genus level to subclass. The gene *rbcL* codes for the critical photosynthetic enzyme RUBISCO and is located in the large single-copy region of the chloroplast genome. This gene was used in the first comprehensive classic analysis of 500 taxa (Chase et al. 1993) to elucidate phylogenetic relationships within angiosperms and extant seed plants. The application of *rbcL* spans a wide taxonomic range from species to subclass, but is most widely used at the family level. It is the gene chosen for this study. The enormous number of *rbcL* sequences that have accumulated over the past decade has led to an understanding of the molecular evolution of the gene itself, including general rate of evolution and evolution by codon position (Chase and Albert 1999).

A range of statistical methods is available to analyse DNA sequence differences. These include maximum likelihood, compatibility, invariants, distance and parsimony methods. Most phylogenetic studies apply parsimony methods, an approach that will remain important for the future. Parsimony is especially suited to the analysis of large

data sets involving many taxa. The computing speed of parsimony allows a more thorough examination of tree topologies than most other methods. Furthermore, it has demonstrated its ability to produce valid phylogenetic hypotheses (Lewis 1999).

Understanding seed dormancy and germination

An understanding of seed germination ecology is essential when addressing conservation of rare plants, as rarity and reproduction are inextricably linked. Knowledge of the factors controlling germination will assist in the propagation and restoration of rare species. Germination ecology contributes to a better understanding of biological concepts such as plant reproductive strategies, life history traits, adaptation to habitats, and physiological processes. These processes include seed development and maturation, aging, biochemical processes controlling dormancy and germination. Responses of seeds to environmental factors including temperature, soil moisture and others are additional areas of research. A comprehensive understanding of seed germination requires communication between a range of disciplines, such as plant anatomy, biochemistry, genetics and molecular biology, and physiology. The sum of these contributions culminates in an integrated approach to germination ecology (Bewley & Black 1994).

The first step in germination ecology is the determination of seed viability, that is the proportion of seeds, which have the potential to germinate. Expressing germination % as a proportion of viable seeds accurately reflects the germination potential of a species and allows the comparison across different studies. There is a range of tests to determine viability. The simplest one involves sectioning the seed to check for presence of firm, white endosperm. Other tests, including the tetrazolium test and testing for catalase activity are more involved and not necessarily more conclusive (Baskin & Baskin 1998).

The next steps in germination research involve a series of investigations to assess dormancy. The comprehensive classification scheme developed by Nikolaeva (1977) is the basis for most germination studies. Nikolaeva (1977) differentiates between inorganic dormancy, where unfavourable environmental conditions impose dormancy, and organic dormancy, where dormancy is associated with some factor of the seed itself. Seed dormancy can also be imposed by the seedcoat and/or the embryo (Bewley

& Black 1994). The investigations of this study address organic dormancy, specifically in shrubs from arid environments.

Organic dormancy may be endogenous, including physiological, morphological or morphophysiological types. Exogenous dormancy encompasses physical, chemical and mechanical mechanisms (Nikolaeva 1977). On a global scale, physical and physiological dormancy are the most important dormancy types in desert shrubs (Baskin & Baskin 1998). In Australia, a range of desert species has developed mechanical dormancy. Additionally, many species exhibit multiple dormancy traits (Bell 1999).

Seeds germinate when dormancy is released. This requires the removal of the barrier controlling germination, for example, a change in hormone levels to alleviate physiological dormancy, or the removal of mechanical restrictions to facilitate germination. Under natural conditions, physiological barriers will be removed during a period of after-ripening, for example, during storage in the soil seed bank. In the soil seed bank the seed is exposed to a range of influences, including diurnal and seasonal temperature fluctuations, wetting/drying cycles, and soil-seedcoat interactions (Baskin & Baskin 1998). Seeds from arid environments may require prolonged seed bank burial and influences of multiple environmental cues to alleviate dormancy.

Understanding dormancy and germination requirements of the study species *Hemigenia exilis* will assist in the development of successful propagation protocols. This type of information will enable rehabilitation of the species after mining, and will elucidate some of the processes determining the species' demography.

Ecophysiological approaches to understand rarity

Ecophysiology describes the physiological mechanisms that underlie ecological observations. This scientific discipline aims to provide causal mechanistic explanations regarding the factors controlling geographical distribution, abundance, survival, growth, and reproduction of plants. It may provide the answer as to why and how a particular species grows in certain habitats and not in others (Lambers et al. 1998). The development of ecophysiology originated in observations such as a correlation between climate and life forms of plants. For example, many plants in hot, arid areas have small

thick leaves, an ecophysiological adaptation to minimize heat load under high temperatures (Ehleringer 1995).

Techniques employed in ecophysiological studies consist of measurements of the microenvironments of plants, often at the individual leaf level, to elucidate water relations and patterns of CO_2 assimilation. Rates obtained for carbon gain and loss can indicate growth rates and nutrient allocation. The integration of these data with plant water relations and mineral nutrition provides additional insights into controls over plant functioning (Lambers et al., 1998)

Detailed knowledge of the biochemical basis of photosynthesis and respiration, and the molecular structures underpinning the differences in key photosynthetic and respiratory proteins has been essential in explaining why plants grow in certain habitats. Apart from physiological factors, historical and biotic components, and the interaction between all three, determines the distribution of plants.

Plants respond to stress by a reduction in physiological processes (Lambers et al. 1998). Factors causing stress include drought, low nutrient availability, high levels of heavy metals and other influences. Plants resist stress by avoiding or tolerating. An example of stress avoidance in arid areas includes the development of deep roots, or production of pubescent leaves. Tolerance to drought stress includes sclerophyllous leaves with a low water content, of by drought-deciduous foliage. Many, but not all of these adaptations, are genetically controlled.

This study elucidates seasonal responses of a range of plant life forms in term of water stress and photosynthesis. The rarity of *Hemigenia exilis* is potentially explained by a narrower ecophysiological tolerance to water stress in summer, compared to a potentially more robust pattern in widespread species with similar life forms.

Developing a restoration protocol after mining in an arid environment

In the past, restoration was based largely on experience accumulated by practitioners who used successful projects as templates for future work. In many cases, restoration has been successful without detailed knowledge of the functioning of the system to be restored. The integration of this approach into a more formal, scientific way will

improve restoration success and enable a better understanding of the underlying processes (Diggelen et al., 2001).

Restoration goals can be defined within three categories:

- Reclamation, often targeting highly disturbed sites, attempts to increase biodiversity per se. This approach does not focus on rare and endangered species;
- Rehabilitation, which aims at the re-introduction of at least some ecosystem functions. This approach aims at a natural-looking artificial landscape, and does not focus on biodiversity; and
- True restoration is the most ambitious goal and plans to reconstruct an entire ecosystem, including ecosystem functions with its typical suite of species, plant communities and structure (Pfadenhauer 2001).

After having decided on the level of restoration, analysis of the area to be restored is carried out. In the context of mining in the arid zone of Western Australian, most restoration sites fall into one of two categories: rock waste dumps or tailings dams. This study focuses on rock waste dump as a result of lateritic nickel mining. These artificial landscapes have some similarity with natural landscapes, namely rocky ridges and breakaways on one side, and salt lakes and playas on the other. A possible restoration strategy logically may consist of re-creating communities in the artificial environments that resemble those in their natural equivalents. While in most landscapes, a complete return to an original situation is unlikely, successful restoration sites mimic the natural equivalents eventually and become self-perpetuating (Hobbs & Norton 1996).

In the arid zone, time scales involved in achieving restoration are long (decades rather than years) and variable. The success, even in the short term, is influenced by variables that cannot be controlled, such as irregular weather patterns and heterogeneity of rock waste following mining. Other factors such as impact of wildlife or soil fertility can be manipulated. Drought can be alleviated by irrigation. Due to the long time frame involved, there exist no universally accepted measurements to quantify the success of restoration projects.

The pre-requisite for successful restoration of rare species is a thorough understanding of the biology of the plant, and of its capacity to adapt to a novel, artificial environment

resulting from mining. It requires the knowledge of the plant's edaphic preferences, and the impact that this novel situation has on plant establishment, growth and reproduction. This knowledge will only be gained after long-term monitoring of the newly created populations (Hobbs & Harris 2001).

The study species and its natural environment

The genus *Hemigenia* belongs to the tribe Prostanthereae, which is one of the two tribes in the Australian subfamily Chloantheae of the cosmopolitan family Lamiaceae. The section *Hemigenia* includes 24 Western Australian species, nine of which are currently undescribed (B. Rye, pers. comm.).

Hemigenia exilis was first collected by the British botanist Spencer le Marchant Moore in 1895 and described in 1899 (Moore 1899). The species was listed as "presumed extinct" until the rediscovery in 1995 during a botanical survey around a major lateritic nickel deposit in the Northeast Goldfields (NEG) of Western Australia, ca. 800km northeast of Perth. Subsequent flora surveys have located over 50 populations, mostly associated with serpentine, ultramafic outcrops. *Hemigenia exilis* is a multi-stemmed, divaricately branched shrub up to 1.5m tall. Flowers are light to dark mauve with a five-lobed calyx (Fig. 1). Flowering has been noted throughout the year, with the peak intensity in the cooler months, following rain. Very few seedlings have been observed, but the species has the capacity to re-sprout if disturbed. Plants appear slow-growing and long-lived.

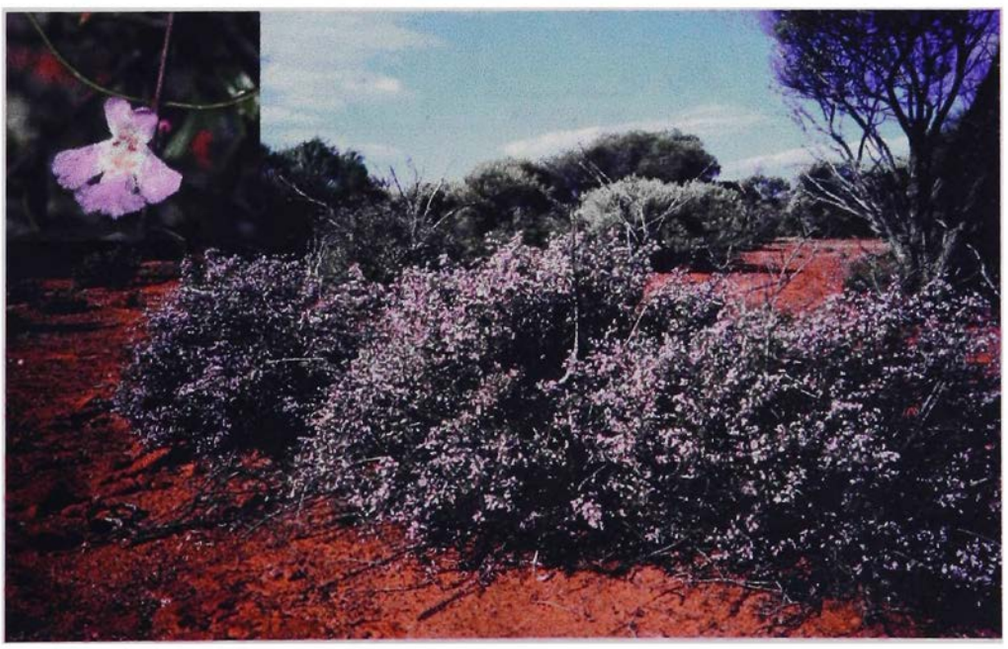

Fig. 1: *Hemigenia exilis* in its natural habitat. Inset: close-up of flower.

Populations of *Hemigenia exilis* have been recorded only in the Northeast Goldfields, in a northwest-southeast trending line from north of Leinster to southeast of Leonora (Fig. 2). One of the study sites, C2, may correspond with the location of the original collection by Spencer Moore in 1895. The species prefers consolidated lateritic caprock and breakaway environments with ultramafic bedrock and consolidated lateritic and ferricretic caprock, which is dark red-brown and rich in iron oxide.

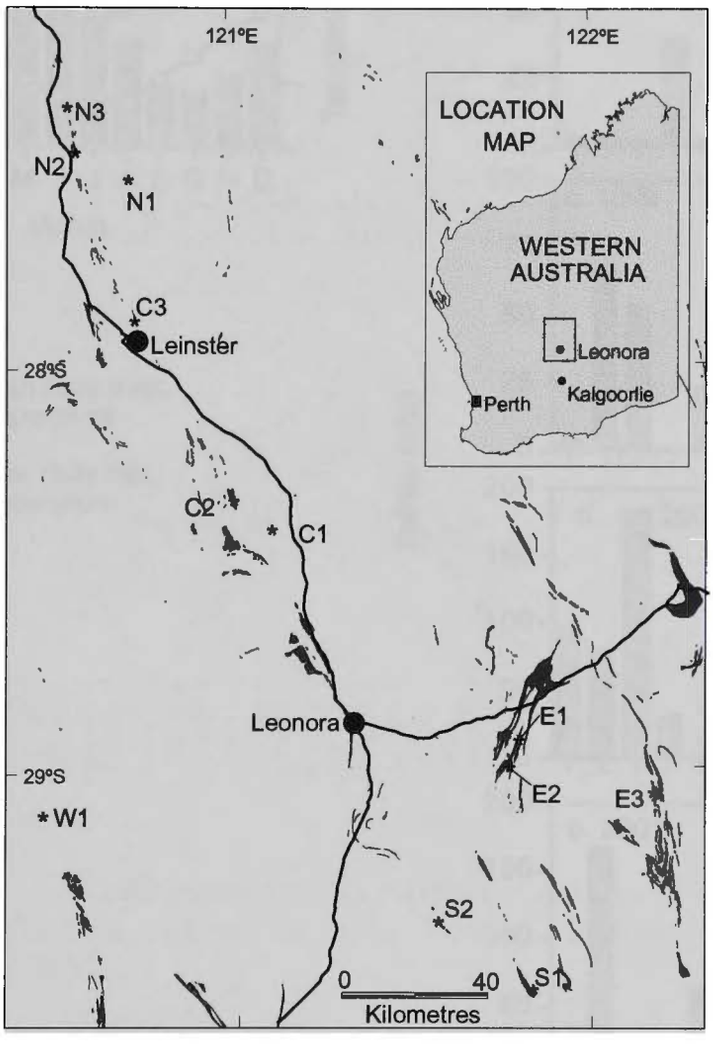

Fig. 2: Geographic distribution of *Hemigenia exilis*. Shown are 12 out of over 50 populations. Most populations are associated with serpentine geology (shaded areas).

The Northeast Goldfields (NEG) are part of the Eremaean Botanical Province. The climate is typical of warm deserts, with a mean annual rainfall of 230mm, (Fig 3a). However, the amount of rain and its seasonal distribution is highly variable, as illustrated in the years 1998-2001 (Fig. 3b-e). The small mining town of Leonora, lying approximately in the centre of the NEG, receives 54% of the annual rain in summer and 46% in winter. The mean monthly temperature profiles vary little throughout the NEG, with a mean maximum temperature in summer of 37°C and a mean maximum

temperature in winter of 21°C (Fig. 3a). The high diurnal fluctuations in temperature reflect the lack of cloud cover and an unhindered flux of incoming and outgoing radiation (Arnold 1963).

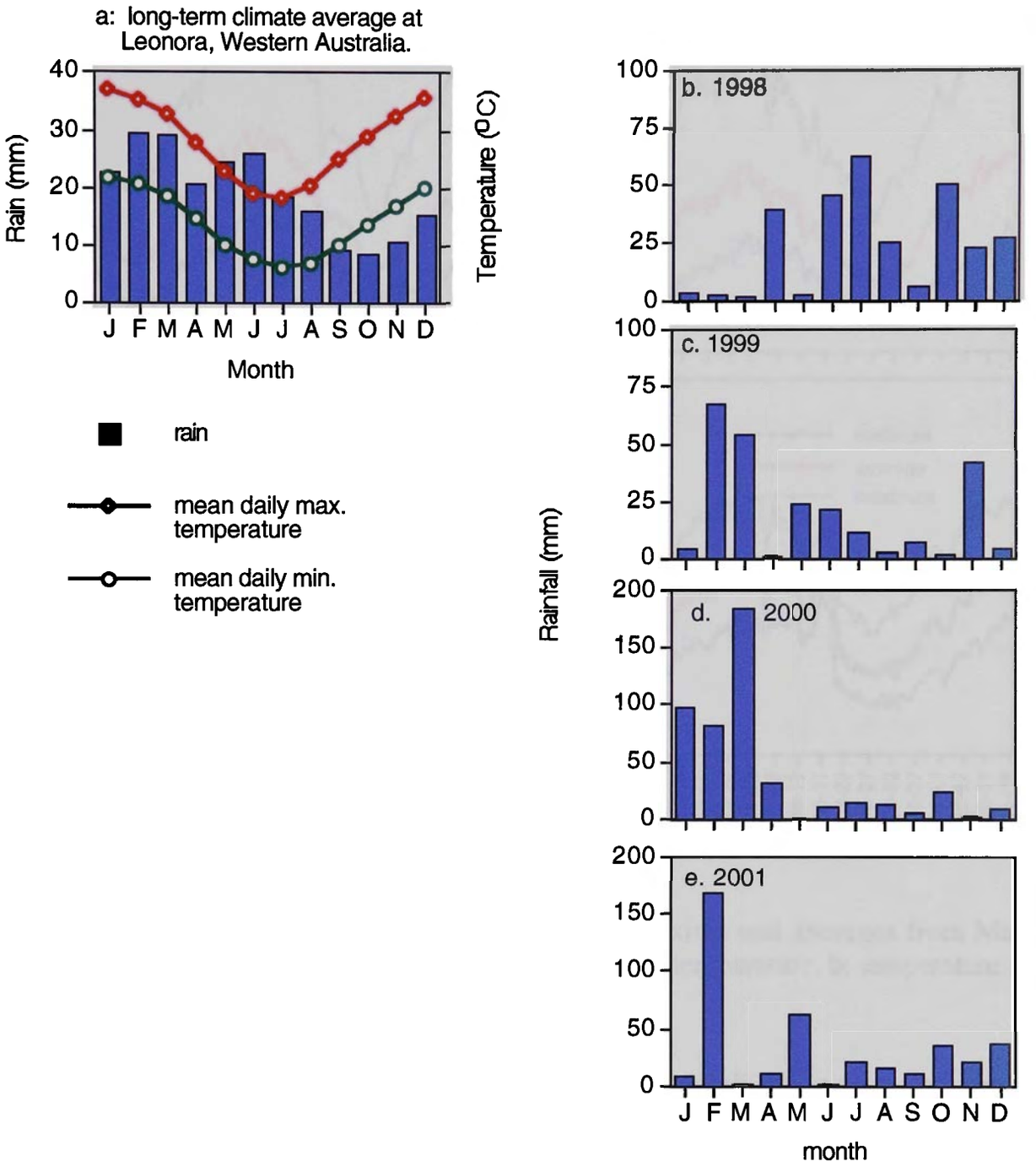

Fig. 3: Climate data of the Northeast Goldfields at Leonora. a: long-term climate averages, b-e: annual rainfall patterns for the years 1998-2001. Note different scales on y-axis.

Soil temperatures have been measured at one of the study sites from May 1997 to January 2002 using Tinytag® dataloggers. At the surface (Fig. 4a), temperatures over 70°C have been recorded in a dry summer, while in winter, frosts occur irregularly. Diurnal temperature fluctuations of 50°C are common, especially in summer. In winter,

fluctuations are less pronounced. Subsurface temperatures have been measured at 2cm depth (Fig. 4b) and show an attenuated pattern.

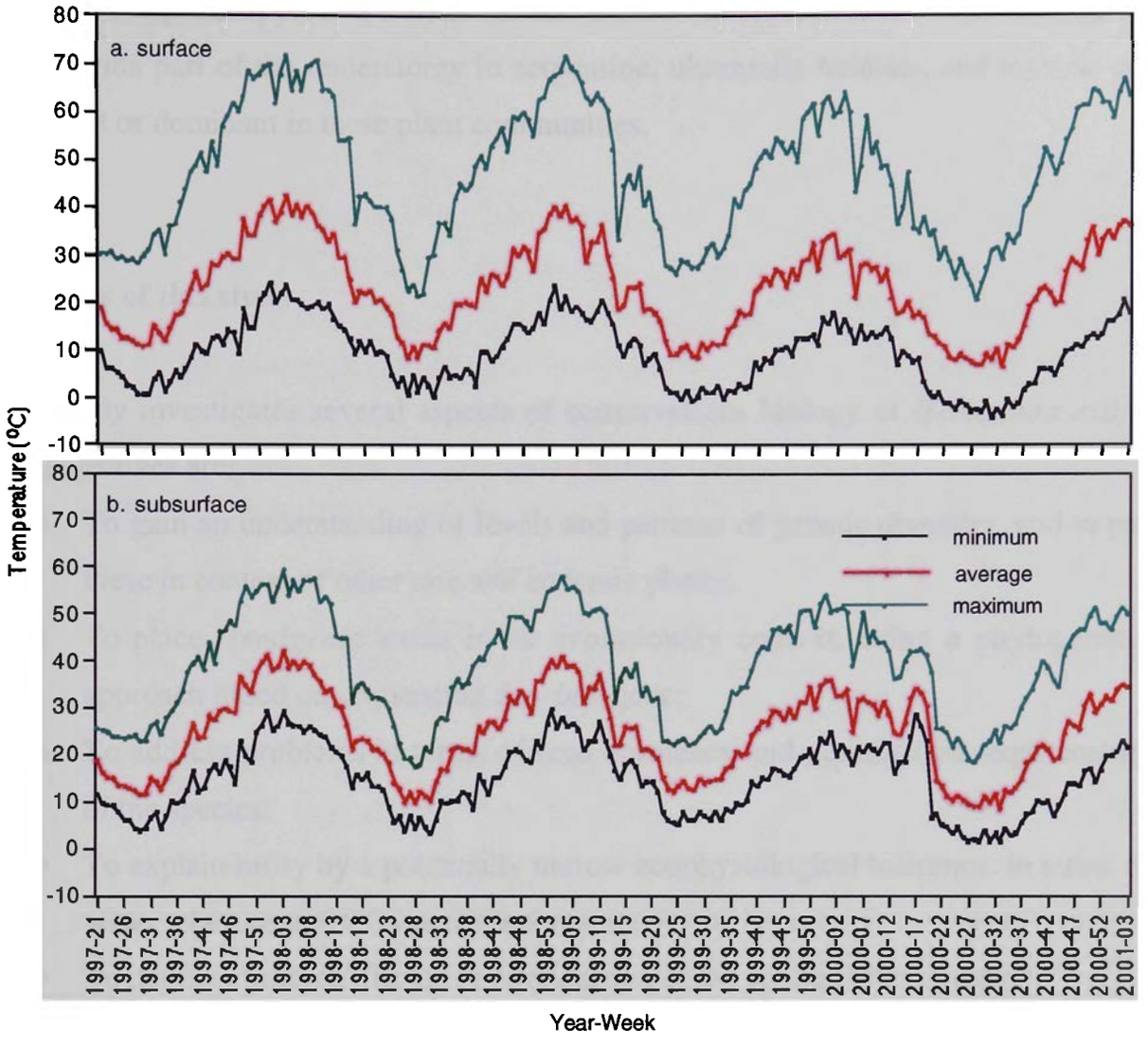

Fig. 4: Soil temperatures, depicting weekly minima, maxima and averages from May 1997 to January 2001, measured near Leonora. a: surface temperature, b: temperature at a depth of 2cm.

The environment of the NEG is characterised by high variability in terms of water, radiation and availability of nutrients. These resources vary across the landscape and may be locally abundant or scarce. Plants living in these environments show adaptations in growth form and growth strategies.

The region is dominated by mulga shrubland, which is characterised by *Acacia aneura* (a small tree or a tall bush) over an understorey of low shrubs. Plant density is low and approximately 85% of ground is bare. The most common growth form is sclerophylly, which is an adaptation to both low nutrient and water availability. These features allow plants to persist despite prolonged periods of drought, and are common in plants such as

Acacia and *Eucalyptus*. Other adaptive features include leaf and stem succulence (in some *Maireana* and all *Halosarcia* species), glaucous leaves and buds (*Atriplex* spp.), hairs (*Solanum*, *Ptilotus* and others), salt exudates (*Frankenia* spp.) resinous leaves (some *Eremophila* spp.) and leafless plants (*Casuarina* spp.) (Beard 1980). *Hemigenia exilis* forms part of the understorey in serpentine, ultramafic habitats, and may be co-dominant or dominant in these plant communities.

The aims of this study

This study investigates several aspects of conservations biology of *Hemigenia exilis*. The objectives are:

- To gain an understanding of levels and patterns of genetic diversity, and to put these in context of other rare and endemic plants;
- To place *Hemigenia exilis* in an evolutionary context, using a phylogenetic approach based on sequencing the *rbcL* gene;
- To address problems in terms of seed dormancy and germination requirements of the species;
- To explain rarity by a potentially narrow ecophysiological tolerance, in terms of water relations and CO_2 assimilation; and,
- To devise a protocol for the re-introduction of the species after mining.

Chapter 2

Conservation genetics and implications for restoration of *Hemigenia exilis* (Lamiaceae), a serpentine endemic from Western Australia

Abstract

Hemigenia exilis (Lamiaceae) is a rare plant endemic to serpentine soils of the Goldfields of Western Australia. The species was presumed extinct until 1995, when it was re-discovered on a nickel ore deposit. To delineate the origin and extent of seed collection for rehabilitation after mining, and to identify the impact of removing one population due to mining, levels and partitioning of genetic variation and differentiation were assessed. Twelve populations were sampled for DNA fingerprinting using the random amplified polymorphic DNA (RAPD) technique. Ten primers produced 89 bands, 97% being polymorphic. Genetic diversity within populations ranged from 0.197 to 0.409, averaging 0.38 at the species level, which is high compared to most other endemic species. Heterozygosity within populations ranged from 0.355 to 0.431, averaging 0.27 over the species. AMOVA partitioned over 80% of the total variation within populations. Multidimensional scaling revealed weak but significant differentiation into a northern and southern provenance. Despite selective sampling, the genetic data provided useful information for the management of *H. exilis*. For restoration, seed should be collected from a range of habitats of several populations, while keeping the two provenances separate. This strategy is likely to maintain high genetic diversity and locally adapted populations.

Introduction

The Goldfields of Western Australia are part of the Yilgarn Block, a granitoid craton nearly 3 billion years old and one of the oldest continental nuclei of the world. The land has not been affected by glaciation since the Permian, and the surface has been stable since the Miocene. Consequently, Western Australia possesses one of the most ancient and eroded landscapes in the world. The modern Australian flora has developed in isolation over the last 45 million years, resulting in a rate of species endemism ranging from 30% to 80% (Hopper *et al.*, 1996). Evolution during this long period is likely to have lead to high genetic diversity and genetic differentiation.

Hemigenia exilis S. Moore (Lamiaceae), a formerly presumed extinct species, was re-discovered in 1995 during a botanical survey around a lateritic nickel deposit, 800km northeast of Perth. Subsequent searches, specifically targeting serpentine outcrops, have located over 50 populations. The species is now listed as "rare but not threatened" (Western Australian Herbarium, 1998). Populations have been recorded only in the Northeast Goldfields, a hot and arid region comprising c. 90 000 km^2 (Fig. 1).

Most *H. exilis* populations are associated with Archaean outcropping serpentine rock, which is rich in iron, magnesium, nickel, cobalt and chromium, but low in calcium and major plant nutrients (Brooks, 1987). Serpentine soils often support vegetation specifically adapted to the high levels of metals and low levels of macronutrients. It was first observed in the 16th century that serpentine outcrops support distinctive vegetation. This relationship has been studied in increasing detail in terms of plant ecology, geobotany, biogeochemistry (Kruckeberg, 1992, Brooks, 1987), and lately genetics (Furnier & Adams, 1986; Mayer *et al.*, 1994; Westerbergh & Saura, 1994; Bachmann & Hombergen, 1997; Williamson *et al.*, 1997; Mengoni *et al.*, 2001; Oline *et al.* 2000; Wolf, 2001). Many plant species worldwide have now been recognised as serpentine endemics. *H. exilis* co-occurs in many populations with the known serpentine endemic *Hybanthus floribundus* (Cole, 1973), supporting the idea *H. exilis* could be assigned a serpentine-endemic status.

H. exilis grows in a range of habitats, eg. along rocky, exposed ridges, on slopes, and along creek lines where the soil is deeper and moister than in the other habitats. It grows as an understorey species in the shade and shelter of *Acacia aneura* trees, or as a (co)dominant species in low shrubland receiving full sun all day. The species appears to be slow growing and long lived. Flowering plants range in size from 30-250cm in height. Plants flower profusely in spring, attracting butterflies as potential pollinators. Although seed set is high, only 20-30% of seed contains embryonic tissue (Cochrane *et al.*, 1999).

Despite several years of above average rainfall during the 1990s, few seedlings have been found (pers. obs.). Field observations indicate that *H. exilis* may be clonal. Many young plants appear to be connected by a shared root system, and damaged plants resprout readily from the base of the stem. By definition, vigorous vegetative and poor sexual reproduction are characteristic traits of clonal plants.

Mining will impact conservation of one of the *H. exilis* populations located on a commercially viable nickel ore deposit. Additionally, the species is targeted for rehabilitation after mining. For conservation purposes, it was important to firstly quantify the degree and pattern of genetic diversity of the species in general and of the impacted population in particular. The species was expected to exhibit high levels of genetic diversity as a result of high pressures from a diverse and arid environment (Linhart & Grant, 1996; James, 2000). As most Lamiaceae are outcrossing (Judd *et al.*, 1999), most of the genetic diversity of *H. exilis* was expected to be partitioned within populations (Hamrick & Godt, 1996).

Secondly, as most serpentine outcrops produce differentiated plant populations (Linhart & Grant, 1996), genetic differentiation resulting in provenances was expected. A provenance is a local genotype, resulting from adaptive variation, which is suited to a particular environment (Frankel *et al.*, 1995). Generally, plants recruited from local seed sources possess increased fitness over non-local genotypes. Mixing of provenances may lead to reduced fitness, weak hybrids and outbreeding depression, thus compromising the long-term viability of local populations. Only when populations are genetically impoverished or sterile does mixing of provenances have a beneficial effect (De Mauro, 1993; Frankel *et al.*, 1995). The genetic integrity of rehabilitated populations of *H. exilis* should be maintained by delineating provenances.

To elucidate population genetics aspects of *H. exilis*, we have chosen the Random Amplified Polymorphic DNA method (RAPD) (Welsh & McCleland, 1990; Williams *et al.*, 1990). This PCR-based technique produces a large amount of dominant multilocus markers and has been used widely in population and phylogenetic studies of plants, fungi, bacteria and animals (Haig, 1998).

The present study is one of the few population genetic investigations of Australian arid zone plants (Coates & Hnatiuk, 1990; Sampson *et al.*, 1995; Li, 2000, all investigating taxa belonging to the Myrtaceae), and the first one focussing on a species of Lamiaceae, and on a rare Australian serpentine endemic. We discuss the consequences that removal of a population will have on the genetic structure of the species, and develop recommendations for restoration after mining.

Materials and Methods

Sampling procedure

Shoot material, consisting of young but fully expanded leaves, was collected in October 1998 from 12 out of a total of c. 50 populations, encompassing the entire geographic range of distribution of approx. 90,000km^2 (350km NS by 250km EW) (Fig. 1), and a range of habitats. The plant material was dried in sealed polycarbonate vials containing activated silica gel to prevent degradation of DNA until DNA extraction. Populations were also assigned to 5 geographic regions: north, central, south, east and west. Within each region, populations were 1-30km apart. In each population, up to 8 individuals were sampled, encompassing neighbouring and distant plants. Details of each of the 12 sampled populations are summarized in Table 1. The small but carefully selected sample size aims to provide results of the genetic structure of *H. exilis* on which management strategies can be based.

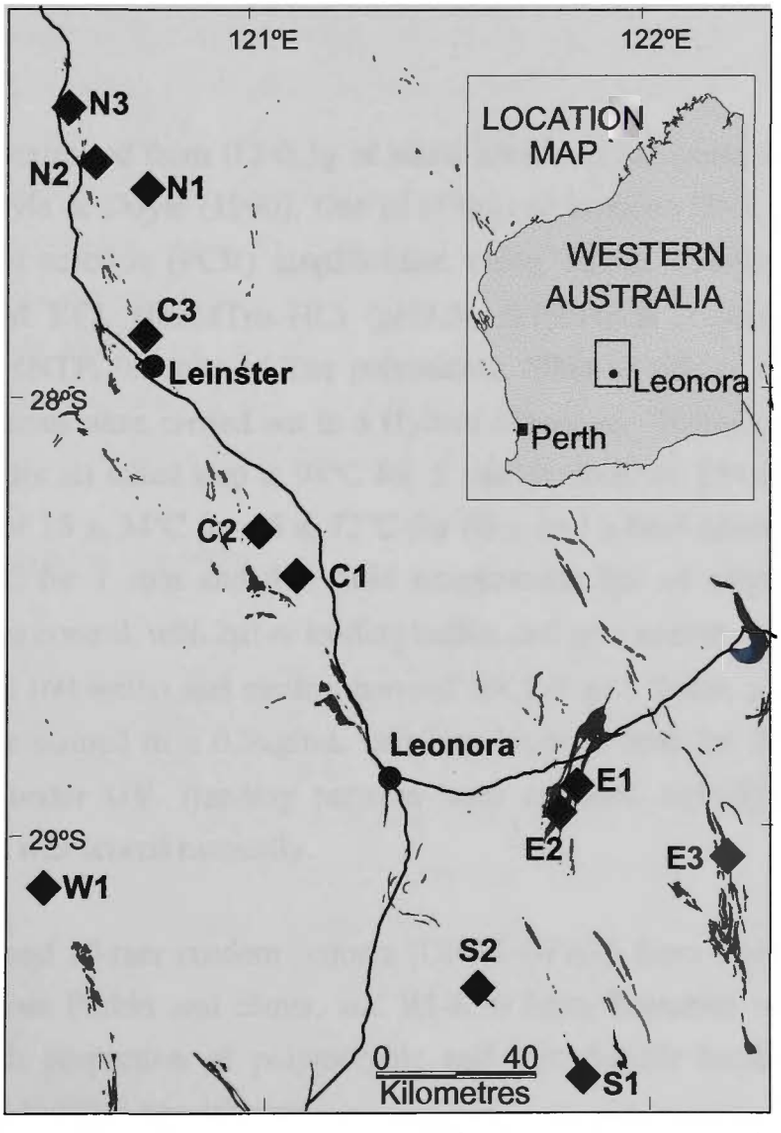

Fig. 1: Geographic distribution of sampling sites for *Hemigenia exilis*, including presence of serpentine geology (shaded).

Table 1: Details of populations sampled for measuring genetic diversity of *Hemigenia exilis*.

Name of population	Geographic region	Longitude (E)/ Latitude (S)	Approx. size of population	No. of samples included in RAPDs
N1	North	120°43.2' / 27°31.7'	100	3
N2	North	120°34.2' / 27°27.8'	250	6
N3	North	120°33.6' / 27°23.7'	350	6
C1	Central	121°07.2' / 28°22.7'	350	6
C2	Central	121°00.0' / 28°20.5'	600	8
C3	Central	120°43.8' / 27°52.6'	150	3
E1	East	121°47.4' / 28°57.0'	300	6
E2	East	121°46.8' / 28°58.9'	500	8
E3	East	122°11.4' / 29°03.7'	200	4
S1	South	121°49.2' / 29°32.5'	3	3
S2	South	121°34.2' / 29°22.7'	1	1
W1	West	120°27.6' / 29°07.2'	150	3

RAPDs

Total DNA was extracted from 0.2-0.3g of silica-dried material using the CTAB method described by Doyle & Doyle (1990). One µl (15ng) of template DNA was subjected to polymerase chain reaction (PCR) amplification using 12.5µl RAPD reaction volume containing 50mM KCl, 10mMTris-HCl (pH9.0), 0.1%Triton X-100, 2.0mM $MgCl_2$, 0.2mM of each dNTP, 0.5units of Taq polymerase, 50ng of primer, and 7µl of sterile water. Amplifications were carried out in a Hybaid Omnigene Thermocycler with heated lid programmed for an initial step at 94°C for 5 min to denature DNA, followed by 35 cycles of 94°C for 15 s, 34°C for 45 s, 72°C for 60 s, and a final extension step at 72°C for 10 min, 37°C for 1 min and 4°C hold temperature. 5µl of amplification product, including negative control, with 2µl of loading buffer, and size marker, were loaded onto a 1.5% agarose gel (60 wells) and electrophoresed for 2.5 to 3 hours at 70 V in 1xTBE buffer. Gels were stained in a 0.5µg/mL ethidium bromide bath for 20 min and bands were visualised under UV. Banding patterns were captured digitally, and presence / absence of bands was scored manually.

A number of 9- and 10-mer random primers (OPA1-OPA15 from Operon Technologies kit A, and a-f from Perkin and Elmer, and R1-R10 from Bresatec) were screened for producing a high proportion of polymorphic and reproducible banding patterns. Ten primers (Table 2) fulfilled this criterion.

Data analyses

The RAPD technique produces dominant multilocus markers. Statistical methods developed for co-dominant markers have been modified for use with RAPD or AFLP markers. For the purpose of this study we assumed absence of non-homologous co-migrating fragments. To avoid distortion of results due to problems regarding reproduciblity (Williams *et al.*, 1990; Jones *et al.*, 1997), only reproducible patterns were included in the data analysis, resulting in 57 samples.

Genetic diversity was calculated on the presence/absence (1/0) of bands, chosing the Nei&Li (1979) similarity index (S), where $S=2m_{xy}/(m_x+m_y)$, m_{xy} being the number of shared markers between two samples, while m_x and m_y are the numbers of markers for each sample. The estimate of diversity was then calculated as D=1-S. We estimated heterozygosity, averaged over all loci at the population and species level using the Bayesian approach developed by Zhivotovsky (1999). With dominant markers, bands derived from heterozygotes can not be distinguished from bands resulting from homozygotes. This poses statistical problems when estimating allele frequencies. Lynch & Milligan (1994) developed a procedure to reduce bias in parameter estimates. However, this may lead to severely biased estimates under some circumstances (Isabel *et al.*, 1999). Zhivotovsky (1999) developed a Bayesian approach for dominant markers, resulting nearly unbiased estimates. Also, his approach is less sensitive to sample size, while in outcrossing species departure from Hardy-Weinberg equilibrium is negligible. Bayesian methods have been applied successfully in a number of genetics studies (Krauss 2000). Correlation between population size, sample size and % of population sampled with measures of genetic diversity was calculated by simple linear regression in Statview®.

Partitioning of genetic diversity

To partition diversity within and among populations, as well as among geographic regions, Analysis of Molecular Variance (AMOVA) was performed using the Arlequin software (Schneider *et al.*, 1997), which was based on the original AMOVA adapted for dominant data (Stewart & Excoffier, 1996). AMOVA converted the 57 x 57 inter-individual Euclidean distance matrix into an equivalent analysis of variance. The relevant variance components were extracted, and significance levels were computed. AMOVA was additionally applied *a posteriori*, partitioning diversity among the two provenances.

Genetic differentiation and geography

The ordination technique Multidimensional Scaling (MDS) was employed to visualise the overall genetic similarities of populations and regions. MDS is an iterative process that

rearranges objects in an n-dimensional space (here, n=3), to arrive at a configuration that best approximates their original genetic distances. This is achieved mathematically by a steepest descent algorithm, which reduces the 'stress' of the final configuration (Kruskal, 1964). For MDS, a matrix based on clustering, UPGMA and squared Euclidian distance was employed using Statistica®. Fisher's exact test of population differentiation, developed for diploid/dominant marker data (Raymond & Rousset, 1995), was performed to statistically test the significance of genetic differentiation. Correlation between genetic and geographic distance was calculated in Statview®.

Results

The 10 primers produced 89 bands, ranging in size from 200 to 1900 basepairs. 9-mer primers produced an average of 9.6 bands, while decamers produced 8.2 bands on average (Table 2). Diagnostic bands for a single population were not observed, while one band was present only in the two populations (E1, E2) occurring on the Murrin Murrin South nickel ore body. Five bands were diagnostic for the South and East regions, and two bands were present only in North and Central regions.

Table 2: Summary of *Hemigenia exilis* RAPD bands obtained with 10 primers.

ID of primer bases of extension in ()	GC content (%)	total no. of bands	average no of bands / plant	range of bands/plant	approx. band size range (bp)
R1 (CCC ACC AAC)	66	10	3.9	1-7	300-1100
R2 (CCC TCC TTC)	66	11	3.5	1-7	200-1600
R3 (GGG TTG TGG)	66	8	4.0	1-7	300-1400
R5 (GGG TGG TTG)	66	9	2.8	1-5	450-1600
R7 (GGG TGG TGG)	77	10	4.1	2-6	300-1300
opa1 (CAG GCC CTC C)	70	8	2.7	1-6	520-1750
opa2 (TGC CGA GCT G)	70	6	2.6	1-4	750-1900
opa3 (TGC CGA GCT G)	60	8	2.9	1-6	600-1500
opa13 (CAG CAC CCA C)	60	11	3.4	2-7	600-1500
d (CAG GCG CAC A)	70	8	4.7	1-8	650-1800
total		89	34.6	20-44	

Levels of genetic diversity

97.7% (87/89) of bands were polymorphic over the entire species. Six bands were >90% monomorphic (present in 52-56 individuals / 57). Polymorphism on a population basis ranged from 45.5% for the smallest and completely sampled population to 91.7% or one of the larger populations, averaging 72.0%. Populations encompassed 52% to 81% of all bands, averaging 66% (Table 3). Levels of polymorphism increased significantly as the number of samples per population increased (r^2=0.884, p<0.0001); they increased only weakly with larger populations (r^2=0.701, p=0.0013), while decrease in polymorphism with higher sampling % was also weak (r^2=0.372, p=0.046).

Nei and Li (1979) genetic diversity within populations ranged from 0.197 to 0.409 and averaged 0.378 over the entire species (Table 3). Diversity within populations was not significantly affected by population size (r^2=0.421, p=0.031), percentage of population sampled (r^2=0.409, p=0.0341) or number of samples (r^2= 0.532, p=0.011). No clonality was detected, as each plant sampled was genetically distinct, even when growing within 30cm of each other (as in population S1).

Total species heterozygosity (H_T) averaged 0.267 over all 89 bands (Table 3). At the population level, it varied between 0.355 (C2) and 0.417 (N1) and averaged 0.389. No significant relationship was detected between heterozygosity and population size (r^2= 0.613, p=0.004), percentage of population sampled (r^2=0.156, p=0.229), but was significantly reduced as number of samples increased (r^2=0.902, p<0.0001). Levels of diversity of the population impacted by mining (E1) were similar or lower than in the neighbouring population.

Table 3: Genetic diversity of *Hemigenia exilis* calculated at the population, region and species level. *denotes populations at the margin of the range, # denotes population impacted by mining.

Name of population	% polymorphic bands	Genetic diversity	Bayesian heterozygosity (no of bands)
N1	60.4	0.300	0.417±0.005 (48)
N2	81.7	0.363	0.372±0.011 (60)
N3 *	82.5	0.357	0.369±0.012 (57)
C1	78.7	0.321	0.379±0.011 (60)
C2	86.8	0.325	0.355±0.013 (68)
C3	57.7	0.270	0.409±0.004 (53)
E1 #	80.3	0.329	0.364±0.010 (65)
E2	91.7	0.409	0.369±0.012 (72)
E3 *	64.1	0.237	0.399±0.008 (57)
S1*	45.5	0.197	0.415±0.005 (54)
S2 (solitary plant)			
W1*	63.0	0.317	0.413±0.007 (46)
Average for population	71.5	0.311	0.389±0.007 (58)
Average for species	97.7	0.378	0.267 (89)

Genetic differentiation and geography

MDS of the 57 individuals revealed that *H. exilis* separates into 2 genetic provenances: a Northern one comprising the North and Central regions, and a Southern one comprising the South and East regions. (Figure 2). The provenances are not completely distinct, rather they form a continuum. The exact test (Table 4) showed that the differentiation was weak but significant. The designation of the West region is intermediate, possibly because only a few individuals of one population were sampled. There was no relationship between genetic and geographic distance (r^2=0.086).

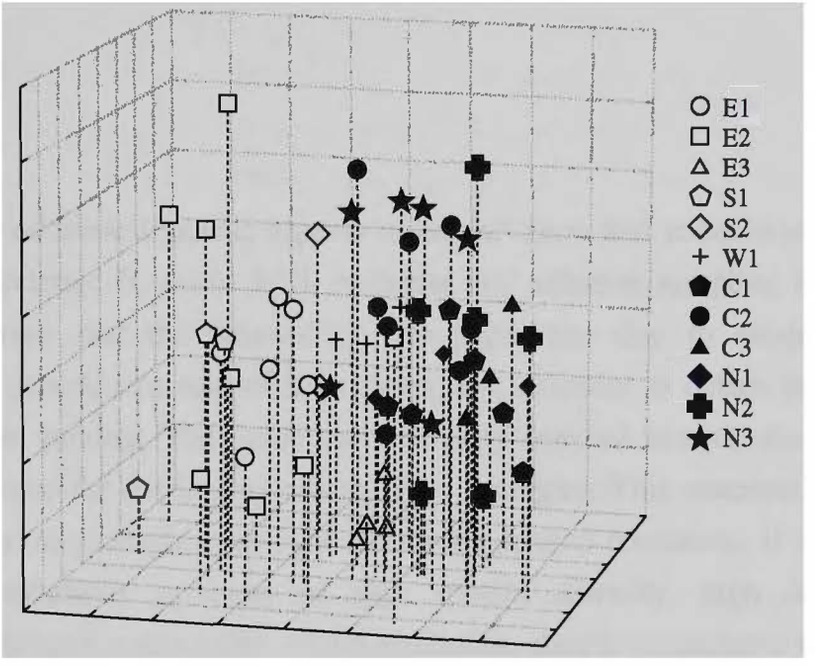

Fig. 2: MDS plot of the genetic relationship among 57 individuals of *Hemigenia exilis*, resolving the two provenances.

Table 4: Fisher's exact test of population differentiation into the 5 geographic regions.

	North	Central	West	South	East
North	*				
Central	0.768	*			
West	1.000	1.0000	*		
South	0.272	0.458	1.000	*	
East	0.0001	0.000	1.000	0.999	*

Results over loci: X-sq=113.65, d.f.: 178, overall p = 1.0

Partitioning genetic diversity

AMOVA partitioned 83.9% of the total variation within populations, 8.5% were partitioned among populations within the 5 regions and 7.6% among regions (Table 5). AMOVA applied *a posteriori* to partition genetic variation among the two provenances showed a similar pattern with 77.4% of the total variation partitioned within populations, 14.1% among populations within the two provenances and 8.5% among the two provenances.

Table 5: Analysis of molecular variance (AMOVA) using 89 RAPD loci, partitioning genetic variability within and among populations as well as among 5 regions (North, Central, South, West, East).

Source of variation	d. f.	SS	Variance component	% of variation
Among regions	4	107.65	1.01	7.6
Among populations within regions	7	115.96	1.13	8.5
Within populations	45	503.29	11.18	83.9
total	56	726.90	13.32	

Discussion

In this study we addressed genetic aspects of conservation and restoration issues of the rare serpentine endemic *H. exilis*. With moderate and selective sampling it was possible firstly to determine that the removal of one population due to mining would not compromise the genetic structure of the species, and secondly to define provenances for rehabilitation after mining. The small and carefully selected sample size provided the information required for developing management strategies. This outcome is particularly relevant to conservation studies that have to rely on limited resources. If the results had contradicted expectations in terms of high species diversity, high diversity within populations and at least some degree of differentiation, then it would have been necessary to sample more intensively.

Genetic diversity was high, as evidenced by a species polymorphism of 97%, a mean genetic diversity of 0.378 and a heterozygosity of 0.267. Diversity of the population impacted by mining was similar to the non-impacted population nearby. About 80% of the total variation was partitioned within populations, and, importantly, genetic differentiation into a Northern and a Southern provenance was detected. Contrary to expectation, the species was not clonal.

Population genetics of *Hemigenia exilis*

Total genetic diversity is generally higher in widespread than in endemic species (Hamrick & Godt, 1996), however no differences have been found when comparing rare and widespread congeners (Gitzendanner & Soltis, 2000). In the absence of congeneric data, comparison of *H. exilis* diversity to RAPD and allozyme surveys (Bussell, 1999; Hamrick & Godt, 1996) of plants with similar life traits indicated that diversity and heterozygosity of *H. exilis* are high. This comparison should be treated cautiously, as allozymes and

RAPDs may (Aagaard *et al,* 1998) or may not be (Fritsch & Rieseberg, 1996) similarly sensitive. Furthermore, heterozygosity is highly correlated within a genus (Gitzendanner & Soltis, 2000). Heterozygosity of *H. exilis* populations is generally high, suggesting that the Bayesian estimates are robust. Serpentine specialists often have higher genetic diversity than generalist species, as a result of small-scale heterogeneity typical of serpentine soils (Linhart & Grant, 1996). Other factors, including climate, seed aborting lethal polymorphisms (James, 2000), high innate diversity of the genus (Gitzendanner & Soltis, 2000), and a diverse, long-lived seed bank (Baskin & Baskin, 1998) may also contribute.

Partitioning of diversity is influenced by breeding system, such that selfing species partition about 50%, whereas outcrossing species partition on average 20% among populations (Hamrick & Godt, 1996; Bussell, 1999). About 16% of the variation of *H. exilis* is contained among populations, suggesting an outcrossing mating system. Outcrossing is further suggested by field observations of flowering plants attracting butterflies, which may be involved in pollination. Outcrossing and insect pollination are common in Lamiaceae (Judd *et al.*, 1999). The high degree of diversity partitioned within populations of *H. exilis* may also be an indication of gene flow, despite the naturally fragmented distribution and the large distances between populations. These distances appear to be overcome by the pollinator. To avoid distorted estimates of partitioning caused by uneven sampling (Hamrick & Godt, 1996), an average of 5 plants per population were sampled, including neighbouring and distant plants, and sampled throughout the entire range of distribution. Values obtained for *H. exilis* fit expectations for outcrossing species, therefore we have confidence in our sampling strategy and analysis.

Hemigenia exilis is genetically differentiated into a Northern and a Southern provenance. Differentiation is weak but significant, possibly as a result of genetic drift combined with sporadic gene flow. The provenances might not represent diverging evolutionary processes but simply populations divided by a distance making gene flow less common. Differentiation coincides with a gap of 90km in the species' distribution, despite the presence of serpentine outcrops, a distance larger than occurring within the two provenances. A major drainage feature (Lake Raeside) and a wide ridge ("The Terraces") in the generally flat topography characterize this area where *H. exilis* is absent, and possibly constitute additional obstacles to gene flow. Serpentine outcrops commonly produce strongly differentiated plant populations (Linhart & Grant, 1996; Kruckeberg, 1992). Strong differentiation has been observed in many serpentine endemics (Mayer *et al.*, 1994, Rajakaruna & Bohm, 1999; Westerbergh & Saura, 1994; Williamson *et al.*, 1997; Wolf, 2001; Oline *et al.*, 2000). While genetic differentiation is often proportional to the distance between populations, breeding system, genetic drift, and environmental

heterogeneity, which affect phenological traits may also contribute (Linhart & Grant, 1996). For example, variability in soil moisture leads to differential flowering, which in turn poses barriers to gene flow and enhances genetic differentiation, especially when populations are isolated. These phenological differences have been observed in populations of *H. exilis*.

This study used only molecular data to determine provenance, assuming an association between marker diversity and adaptive, polygenic traits. However, several studies have shown that selective differentiation of polygenic traits might cause little differentiation of the underlying loci, as genes, markers and traits will each behave differently in the adaptive divergence of populations (McKay & Latta 2002). Therefore, extrapolating from one type of variation to another must be done with caution. Conversely, low marker differentiation does not necessarily imply a low quantitative trait divergence (Lynch et al. 1999). The degree of differentiation in quantitative traits typically exceeds that observed in neutral marker genes, although the difference between the two measures is less pronounced in DNA based marker techniques (Merilä & Crnokrak 2001). For conservation of present and future evolutionary potential of species genetic diversity, the combination of as much ecological information and molecular genetic markers is desirable (McKay & Latta 2002).

Managing *Hemigenia exilis* for conservation and mine site restoration

The aims of conservation and restoration programs are to establish and maintain long-term viable populations, to restore ecosystem functions and processes, and, more recently, to preserve and restore the evolutionary potential of the species by maintaining or recovering natural levels and patterns of genetic diversity (Frankel *et al.*, 1995; Lesica & Allendorf, 1999). Populations with high diversity are given higher priority than genetically depauperate ones. Differentiated populations, or provenances, need to be protected as separate entities (Montalvo & Ellstrand, 2000).

Conservation aims to protect as many diverse populations as possible. However, there may be overriding reasons that lead to the loss of plants. In this case, at least one population, E1, is threatened by mining, being situated on a commercially viable nickel deposit. A similar scenario might apply to C1. At both sites, there are other populations nearby, which contain similar levels and patterns of diversity, thus mitigating the loss of genetic diversity.

In restoration, provenance, resulting from intra-specific genetic differentiation, has become increasingly an issue. Provenance has a long history in forestry (see review by Langlet 1971). As early as in the 18th century, foresters noticed differences growth rate, disease resistance and frost tolerance in tree plantation originating from a range of seed sources. In the mid 1800s, the Swedish government cautioned against the use of foreign seed for

timber plantations, while in the 1930s the US government recommended local seed collected from specified "seed collection zones" be used for reforestation (Montalvo & Ellstrand 2000). The importance of provenance in restoration and rehabilitation of natural ecosystems has only recently been recognized (Millar & Libby 1991, Knapp & Rice 1994). Increasingly, ecologists advocate genetic surveys to delineate provenances before embarking on seed collection. The aim is to maintain the genetic integrity of reinforced and restored plant communities, with beneficial consequences for population fitness (Lesica & Allendorf, 1999, Frankel *et al.* 1995).

Provenances represent genetic adaptations to a local environment. Co-adapted gene complexes make local plants uniquely suited to a specific environment. This adaptation is increasingly significant as the environment becomes more stressful. Serpentine outcrops are one example of extreme environments, and serpentine endemics appear to be particularly well adapted to or even dependent on conditions at a very local scale (Linhart & Grant, 1996; Wolf 2001). The "home site advantage" as illustrated by increase fitness has been demonstrated in trees from temperate (Tibbits & Hodge, 1998), tropical (Khurana & Singh, 2001) and arid (Roupsard *et al.* 1998) environments, in grasses (Knapp & Rice, 1994), shrubs (Montalvo & Ellstrand, 2000) and herbaceous annuals and perennials (Wolf, 2001; Keller *et al.* 2000). Mixing of provenances could lead to gene flow with a number of detrimental consequences. These include outbreeding depression, concomitant with loss of fitness due to lack of local adaptation. The introduction of non-local provenances could swamp the local provenances through gene exchange, and may result in hybrids with reduced vigour (Lesica & Allendorf, 1999). Only in genetically depauperate or sterile populations may a foreign genotype increase genetic diversity and fitness (De Mauro 1993).

Based on conventional population genetics theory, restoration of *H. exilis* should aim at encompassing maximum genetic variation and maintaining local adaptation. Consequently, seed should be collected from as many habitats as possible of several populations within a provenance. Just a few populations may encompass a very high proportion of adaptive variation coding for topographic, biotic and edaphic heterogeneity (Lesica & Allendorf, 1999). The two provenances need to be kept separate as local genotypes. They may be genetically adapted to different local environments, and therefore are generally more successful than non-local genotypes in terms of fitness. This seed collecting strategy would ensure that genotypes adapted to a range of soil types, soil moisture regimes and temperatures are represented in the new populations. Adaptability to a wide range of soil conditions is particularly important, as soil profiles constructed on rehabilitated mine dumps will be dissimilar to the native habitat, and will pose a novel and potentially stressful edaphic environment.

Chapter 3

Phylogeny of Chloanthoideae (Lamiaceae) based on *rbcL* sequences

Abstract

Sequences of *rbcL* were analysed to resolve phylogenetic relationships in Lamiaceae subfamilies Chloanthoideae and Teucrioideae. Monophyly of Chloanthoideae was not supported, due to *Tectona* being the only taxon situated outside, towards the base of Lamiaceae. The tribe Prostanthereae received moderate support, with *Microcorys subcancescens* being sister to the remaining tribe. Chloantheae emerged as a weakly supported clade, within which *Dicrastylis*, *Lachnostachys* and *Cyanostegia* appeared monophyletic. The position of *Pityrodia* remains unresolved. Teucrioideae form a clade (bootstrap <50%), with the Australian taxon *Teucrium racemosum* as sister to the remaining taxa.

Introduction

Lamiaceae are a large cosmopolitan family, consisting of nearly 260 genera and 7000 species. The family encompasses economically important species which are used for essential oils and medicinal purposes, for herbs and spices, and for ornamentals, while a few have edible roots and one species, *Tectona grandis*, is a valuable tropical hardwood (Judd et al. 1999).

Bentham (1876) originally circumscribed Lamiaceae as consisting of eight tribes, including the exclusively Australian Prostanthereae. His classification encompassed most of the Lamiaceae genera known at that time. The next revision by Briquet (1895-97) differed only little from the original in that his eight subfamilies consist of five tribes and three subtribes *sensu* Bentham, while the three remaining tribes *sensu* Bentham are subsumed in an enlarged subfamily Lamioideae. Junell (1934), in a comparative study of Lamiaceae and Verbenaceae in terms of gynoecial morphology, pointed out strong similarities between Lamiaceae and all Verbenaceae except the subfamily Verbenoideae. He suggested, then unsuccessfully, transferring most subfamilies (including Chloanthoideae) to Lamiaceae and limiting Verbenaceae to Verbenoideae.

A subsequent revision by Erdtman (1945) simplified the classification, based on two types of pollen: taxa of the subfamily Lamioideae have tricolpate pollen shed at the 2-celled stage, where as members of the Nepetoideae are characterised by hexacolpate 3-celled pollen. The next reclassification by Wunderlich (1967) was based on pollen, ovule, embryo sac and seed morphology, splitting Erdtman's Lamioideae into four subfamilies (including Prostantheroideae and a newly enlarged Lamioideae), and his Nepetoideae into two subfamilies.

A comprehensive cladistic analysis of Lamiaceae and Verbenaceae using morphological and anatomical characters (Cantino 1992) suggested that in the traditional classifications, Lamiaceae were polyphyletic. The study also demonstrated that Prostanthereae (Lamiaceae) are most closely related to Chloanthoideae (Verbenaceae). Furthermore, a clade cutting across traditional family and subfamily boundaries, including *Teucrium* (Ajugoideae/Teucrioideae, Lamiaceae) and *Spartothamnella*

(Chloanthoideae, Verbenaceae) emerged. These poly- and paraphyletic relationships were confirmed by analyses of gene sequences (Chase et al.1993, Wagstaff et al. 1995, Wagstaff & Olmstead 1997, Cantino et al. 1997, Wagstaff et al. 1998), and by biochemical systematics (Grayer et al. 1999, Pedersen 2000).

Cantino et al. (1992) proposed a new classification of Lamiaceae and Verbenaceae, essentially following Junell's suggestion, thus rendering both families monophyletic, while making other adjustments at the subfamily and tribal level. Support for the monophyly of the Lamiaceae comes from *rbcL*, *atpB* and 18S rDNA sequences (Wagstaff & Olmstead 1997, Savolainen et al. 2000, Soltis et al. 2000).

This study will adhere to the classification by Cantino et al. (1992) and call it the "CHW classification". Although there are more recent phylogenetic studies (see above) and more compact classifications (Judd et al. 1999) which propose similar changes to the re-classification of Lamiaceae and Verbenaceae, they did not include sufficient taxa of Chloanthoideae, the focus of this study, to define groupings within that subfamily. Additionally, the CHW classification attempts to fulfill the criteria for a useful phylogenetic systematic treatment postulated by De Queiroz & Gauthier (1994).

Within Lamiaceae the CHW classification recognises eight subfamilies: Ajugoideae, Lamioideae, Nepetoideae, Scutellarioideae, Teucrioideae (subsumed by Judd et al. 1999 into Ajugoideae), Pogostemonoideae (sunk by Judd et al. 1999 into Lamioideae), as well as Viticoideae (transferred from Verbenaceae), plus they included the Australian Chloanthoideae. The latter was variously recognised as a separate family Chloanthaceae (Hutchinson 1959, Munir 1979), or as a subfamily or tribe in Verbenaceae (Bentham 1876, Briquet 1895).

Chloanthoideae, sometimes referred to as Prostantheroideae, are endemic to Australia and with over 170 species account for 80% of Australian Lamiaceae (Hedge 1992). They may be monophyletic (Olmstead et al. 1998) or paraphyletic (Wagstaff et al. 1998). The subfamily contains the tribes Prostanthereae and Chloantheae. Prostanthereae encompass c. 200 species of six genera, namely *Hemiandra*, *Hemigenia*, *Microcorys*, *Westringia*, and the monotypic genera *Eichlerago* (provisionally sunk into *Prostanthera* in the CHW classification) and *Wrixonia*. This tribe was identified in earlier morphological taxonomic treatments (Bentham 1876, Briquet 1895-97, Junell

1934, Erdtman 1945, Wunderlich 1967), and has been shown to be monophyletic in recent analyses (CHW classification, Conn 1992, Olmstead et al. 1998). Chloantheae contain 8-9 genera: *Chloanthes, Cyanostegia, Dicrastylis, Lachnostachys, Mallophora, Newcastelia, Pityrodia, Physopsis* (and *Denisonia* provisionally subsumed into *Pityrodia* in the CHW classification), of which at least two genera are poly- or paraphyletic (Cantino 1992, Olmstead et al. 1998). Relationships within the tribes require further study.

Teucrioideae are represented in Australia by *Spartothamnella* and *Teucrium*, which share a similar pollen and flower morphology (Abu-Asab & Cantino 1993, Cantino 1992). In previous classifications, *Spartothamnella* was included in Chloantheae (Munir 1979) or Viticeae/Ajuginae (Junell 1934). The position of *Teucrium* has been ambiguous and has variously been assigned to Ajugeae and Teucrioideae (Conn 1992, Wagstaff et al. 1998, Wink & Kaufmann 1998).

In this study, phylogenetic relationships within Chloanthoideae and two Australian taxa from Teucrioideae are examined within the broader context of Lamiaceae using the *rbcL* gene. This gene is located in the large single copy region of the plastid genome and codes for the large subunit of RUBISCO, a critical photosynthetic enzyme functioning as the major carbon acceptor. It was the first gene to be sequenced directly from plastid DNA, and has been widely sequenced largely as a result of near-universal PCR primers being made freely available (Zurawski et al. 1981). The gene is highly conserved, has a slow and known rate of change, contains only rare insertions/deletions, and varies in length from 1428-1434 basepairs (Soltis & Soltis 1999). It has been used at a wide taxonomic level throughout the plant kingdom, including angiosperms, ferns, bryophytes and algae. In angiosperms, it is widely used at the family level and below, and it has also been successfully employed at the species and generic level, especially when sequencing past the 3′ end of the gene (Soltis & Soltis 1999). Its lack of length variation means that it can be easily aligned at the suprafamilial level (Chase & Albert 1999).

The original purpose of employing *rbcL* in phylogenetics of angiosperms was to evaluate interfamilial patterns within closely related groups (Chase et al. 1993). Reasons for generating further sequences include resolution of previously ambiguous clades and taxa, refinement of infrafamilial and intraordinal relationships, and addition of *rbcL*

sequences to other data for combined (or separate) analysis, thus improving resolution and support of clades, detecting true phylogenies, and decreasing computing time (synergistic effect) (Chase & Albert 1999, de Queiroz et al. 1995, Judd et al. 1999, Savolainen et al. 2000). The new phylogenetic trees may then serve as a basis for revised classifications (APG 1998, Chase & Albert 1999, Judd et al. 1999).

Following on from Chapter 2, this chapter aims to identify species closely related to *Hemigenia exilis* for future population genetic studies, in order to put the high levels of genetic diversity found in *H.* exilis into context. The closely related species might belong to *Hemigenia*, or other genera including *Hemiandra*, *Microcorys* and *Westringia*. Furthermore, this molecular analysis encompassing a large number of taxa will contribute to a revised classification of Chloanthoideae that reflects the evolutionary history of this subfamily more accurately. Of particular interest are the phylogenies of the poly- and paraphyletic genera in Prostanthereae and Chloantheae, as well as the position of the monotypic genus *Wrixonia*.

Materials & Methods

Sampling

Twenty-six species were collected from the Kings Park & Botanic Gardens nursery and garden beds, representing five genera of the tribe Chloantheae and six genera of the Prostanthereae, in addition to *Spartothamnella teucriiflora* and *Teucrium racemosum* of Teucrioideae. Fresh shoot tips were dried in activated silica gel, to ensure that DNA would not degrade (Chase & Hills 1991).

DNA sequencing

Total DNA was extracted from 0.3 – 0.5g of silica dried leaf material using the 2x CTAB method of Doyle & Doyle (1987), purified on a 1.55g/ml CsCl gradient followed by dialysis. The *rbcL* gene was amplified with the forward primer 1F (5´-ATGTCACCACAAACAGAAAC-3´) which matches the first 20 base pairs (bp) of the exon, and the reverse primer 1460R (5´-TCCTTTTAGTAAAAGATTGGGCCGAG-3´) that matches a downstream ribosome control site (Fay *et al.*1998). To improve amplification of potentially recalcitrant samples, 1µl of 0.4% aqueous bovine serum albumin (BSA) was added per sample (Savolainen et al. 1995). The total reaction volume was 100µl. PCR conditions consisted of 28 cycles of: 1 min of denaturation at

94°C, 30 s of annealing at 50°C, 1 min of extension at 72°C; it concluded with 6 min at 72°C for final extension. PCR products were cleaned using QIAquick columns following the manufacturer's instructions. Dideoxy cycle sequencing was performed during 26 cycles of: 10 s of denaturation at 96°C, 5 s of annealing at 50°C, 4 min of extension at 60°C. It included dye terminators and one of the primers 1F, or 1460R or the internal primers 636F (5´-GCGTTGGAGAGATCGTTTCT-3´) or 724R (5´-TCGCATGTACCTGCAGTAGC-3´) (Fay et al. 1998), to provide generous overlap and complementary pairs of sequences. Cycle sequencing products were cleaned with sodium acetate and absolute alcohol. The products were sequenced directly on an ABI 373A automated sequencer according to the manufacturer's protocols. Sequence output files were edited in Sequence Navigator and assembled in AutoAssembler (Applied Biosystems Inc.).

Phylogenetic analysis

Sequences were aligned manually with the *rbcL* gene of *Nicotiana tabacum*. As *rbcL* contained no insertions or deletions in the taxa of this study, alignment was unambiguous. Additional sequences for Lamioideae, Pogostemonoideae, Viticoideae, Nepetoideae, and the Chloanthoideae *Tectona* and *Congea* were obtained from GenBank and included in the analysis. In the phylogenetic trees, GenBank sequences are recognised by the appendage of a combination of letter(s) plus numbers.

Sequence data were analysed with the software package PAUP version 4.0 for Macintosh (Swofford 1998), using the parsimony algorithm. The most-parsimonious trees were obtained by performing 1000 replicates of RANDOM taxon addition, using equal weights and subtree pruning-regrafting (SPR) branch swapping, with only five trees held at each step (NCHUCK=5). The trees collectively found in these 1000 replicates were then used as starting trees for new searches using SPR swapping until more than 10000 trees had been saved.

Internal support was evaluated by using bootstrap resampling (Felsenstein 1985). One thousand bootstrap replicates were performed using the SPR swapping algorithm with simple taxon addition and only 10 trees held at each step.

Additional statistics, including consistency index (CI) and retention index (RI) were performed to measure support for the whole tree. The CI measures overall homoplasy,

and equals the minimum number of genetic switches/number of actual switches. However, measures of CI may be inflated when autapomorphies are present, or only few taxa are analysed. Its range may also be limited. These shortcomings render the CI useless when comparing different data sets (Judd et al. 1999). The RI overcomes the shortcomings of the CI by taking into account the maximum number of changes that could have occurred on an unresolved tree. It equals max. length – actual length / max. length – min. length (Judd et al. 1999). RI values are thus more informative as they measure the amount of structure or phylogenetic signal retained on optimal trees (Farris 1989, Savolainen et al. 2000).

Results

The heuristic search resulted in 10000 equally parsimonious trees with 1456 steps in length, with a CI of 0.43 and a RI of 0.62. Lamiaceae are monophyletic in this analysis (80% BS). Within Lamiaceae, all most parsimonious trees contained several clades (Fig. 1): tribes Prostanthereae (C1, 64% BS) and Chloantheae (C2, 59% BS), Teucrioideae excluding *Cyclonema* (C3, 54% BS), and Lamioideae including Pogostemonoideae (C4, 75% BS). Teucrioideae are polyphyletic if *Cyclonema* is included, and Pogostemonoideae is paraphyletic to a monophyletic Lamioideae *sensu stricto* in this analysis. Viticoideae were polyphyletic. Nepetoideae is monophyletic (99% BS) and is sister to all other Lamiaceae.

Within C1 Prostanthereae, three subclades (SC) were apparent: SC1 *Microcorys subcanescens* alone, SC2 *Prostanthera*, SC3 *Hemiandra*, *Hemigenia*, *Microcorys exserta* and *Westringia* (the "HHMW clade"). SC1 was weakly supported as sister to SC2 and 3 (59% BS). SC2 including *M. exserta* received <50% BS, and *M. exserta*, SC2 and SC3 formed an unresolved trichotomy in the bootstrap consensus tree. *Westringia*, *Hemigenia* and *Hemiandra* were not resolved as monophyletic. In SC3, *Prostanthera* received moderate support (77% BS). When a partial sequence for *Wrixonia prostantheroides* was added to the analysis, this species was embedded in *Prostanthera* (data not shown), but resolution of the pattern of relationships will require additional data.

Within C2 (59%BS) relationships were not clearly resolved. However, *Cyanostegia* (63% BS), *Dicrastylis* (96% BS) and *Lachnostachys* (89% BS) were monophyletic. *Newcastelia hexarrhena* was sister to *Lachnostachys* and *N. chrysophylla* (=*Physopsis chrysophylla*) was sister to *Dicrastylis*. *Pityrodia* spp. were interspersed between these groups in individual trees, and the clade collapsed to a polytomy in the strict consensus tree (arrows). There was no evidence of monophyly of *Pityrodia*.

Teucrioideae including the Australian species *Teucrium racemosum* formed the third clade (C3, <50% BS). Excluding *T. racemosum* the remainder of C3 received 54% BS. Three clades emerged within Teucrioideae: SC1 (83% BS) comprising the Australian and New Zealand genera *Spartothamnella*, *Teucridium* and *Oncinocalyx*, SC2 (86% BS) consisting exclusively of *Teucrium* (of Mediterranean distribution), and SC3 (<50% BS) encompassing *Ajuga*, *Caryopteris*, *Clerodendrum*, *Tetraclea* and *Trichostemma*. The relationships between these three clades were not resolved.

The fourth clade represented Lamioideae plus Pogostemonoideae (C4, 75% BS). The fifth clade crossed taxonomic boundaries, including some Viticoideae, some Chloanthoideae and *Cyclonema* of Teucrioideae (C5; <50% BS). Nepetoideae formed the sixth clade (C6, 99% BS). Other members of Viticoideae fell in unresolved positions among these clades.

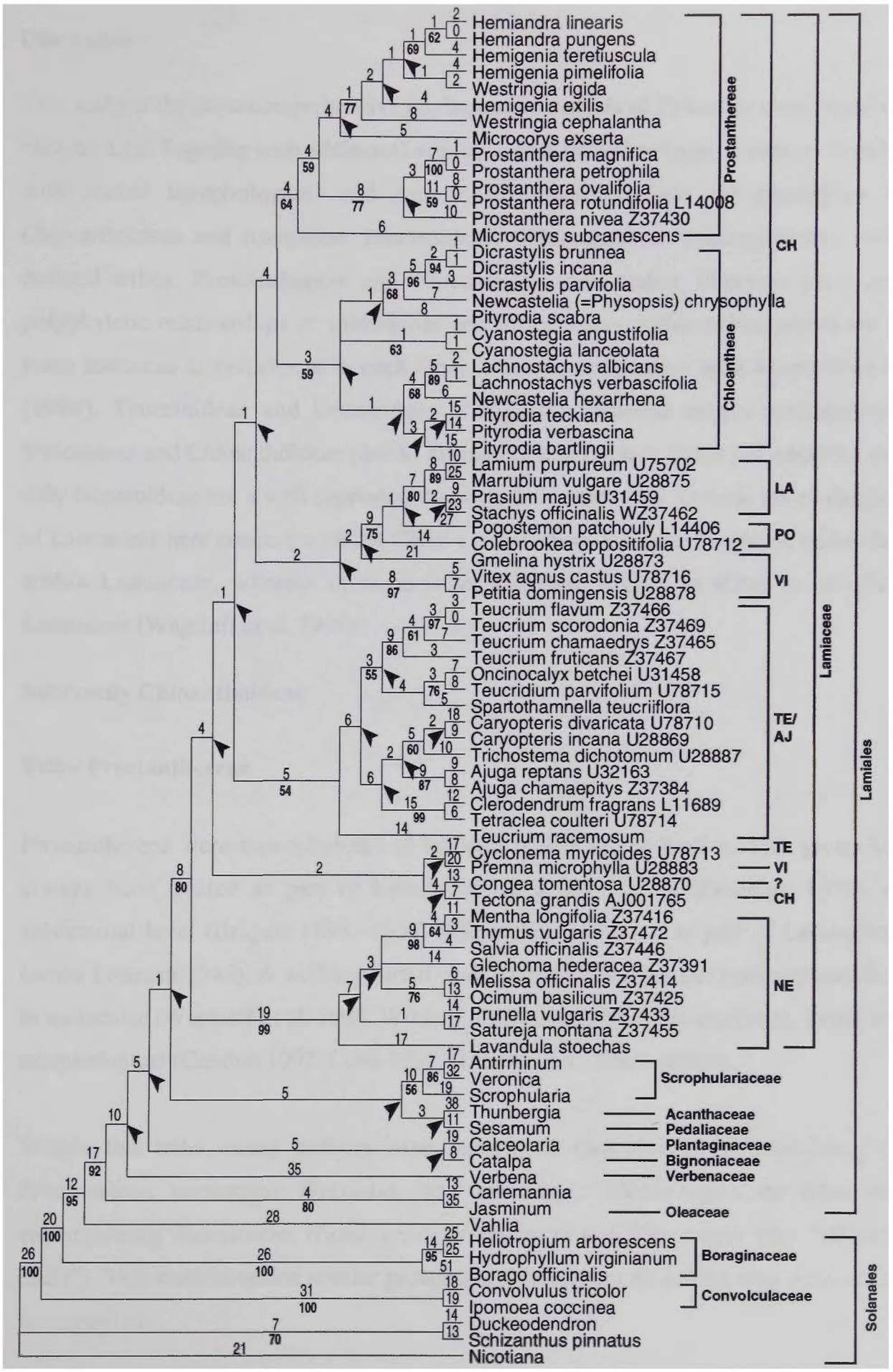

Fig. 1: One of 10000 most parsimonious trees. Number of nucleotide changes are shown above and bootstrap support percentages below the branches. Branches not present in the strict consensus tree are indicated by arrows. CH=Chloanthoideae, LA=Lamioideae, PO=Pogestemonoideae, VI=Viticoideae, TE/AJ=Ajugoideae, NE=Nepetoideae.

Discussion

This study is the most comprehensive phylogenetic analysis of Chloanthoideae based on *rbcL* to date. Together with additional sequence data from GenBank, it concurs broadly with recent morphological and genetic investigations into the phylogeny of Chloanthoideae and Australian Teucrioideae. Chloanthoideae encompass two well-defined tribes, Prostanthereae and Chloantheae. All studies illustrate para- and polyphyletic relationships of subfamilies and some genera within tribes, which are in some instances at variance with each other. Here, in congruence with Wagstaff et al. (1998), Teucrioideae and Lamioideae + Pogostemonoideae appear paraphyletic, Viticoideae and Chloanthoideae (due to *Tectona* falling in clade 5) are polyphyletic, and only Nepetoideae are a well supported monophyletic subfamily. Overall the phylogeny of Lamiaceae here concurs with the CHW classification, but here *Congea* is embedded within Lamiaceae, whereas in some other analyses, it falls as sister to all other Lamiaceae (Wagstaff et al. 1998).

Subfamily Chloanthoideae

Tribe Prostanthereae

Prostanthereae were monophyletic, in agreement with recent studies. This group has always been treated as part of Lamiaceae, at a tribal level (Bentham 1876), at subfamilial level (Briquet 1895 –1897, Wunderlich 1967), or as part of Lamioideae (*sensu* Erdtman 1945). A well-supported clade at this tribal level has been substantiated in molecular (Wagstaff et al. 1995, Wink & Kaufmann 1996, Olmstead et al. 1998) and morphological (Cantino 1992, Conn 1992, Rimpler et al. 1992) studies.

Within this tribe, many authors have recognised two clades, one consisting of *Prostanthera*, monotypic *Wrixonia* (and monotypic *Eichlerago*), the other one encompassing *Hemiandra*, *Hemigenia*, *Microcorys* and *Westringia* (the "HHMW clade"). This study revealed similar groupings, although not all genera were resolved as monophyletic.

A robust prostantheroid clade consisting of *Prostanthera* (encompassing the tricolpate pollen in *P.* section *Prostanthera* and the hexacolpate pollen in *P.* section *Klanderia*), *Wrixonia* and *Eichlerago* emerged consistently based on synapomorphies in pollen

morphology (Cantino 1992, Abu-Asab & Cantino 1992, 1993). Synapomorphies in calyx morphology, reversals in anther appearance and autapomorphies (Conn 1992, Cantino 1992, Rimpler et al. 1992) further delimited this clade. In some studies, *Wrixonia* appeared as a sister to *Prostanthera*, making *Prostanthera* monophyletic (Conn 1992, Rimpler et al. 1992), while in Cantino's (1992) analysis and our preliminary data, *Wrixonia* is nested within *Prostanthera*. It is therefore probably appropriate to treat *Wrixonia* in *Prostanthera*.

A second group within Prostanthereae is the HHMW clade (hemigenioid clade *sensu* Cantino 1992, *Microcorys* clade *sensu* Conn 1992, and Olmstead et al. 1998). This group was delineated by similarities in androecial morphology (dimidiate stamens with an elongate connective, Cantino 1992, Rimpler et al. 1992), pericarp structure (Ryding 1995), and anther morphology (Conn 1992). Morphologically this clade was less stable than the prostantheroid clade (Cantino 1992, Conn 1992). In contrast, in the *ndhF* analysis (Olmstead et al. 1998) this clade had 100% bootstrap support.

Relationships within the HHMW clade were variable: morphological analyses supported a monophyletic MW subgroup (Conn 1992, Cantino 1992), which was not supported by *ndhF* analysis (Olmstead et al. 1998) or this *rbcL* study. A palynological investigation (Abu-Asab & Cantino 1993) separated *Hemiandra* from the other genera in the clade. Cantino (1992) hypothesized that *Hemigenia* may be a paraphyletic group that had given rise to *Hemiandra* and the MW clade. This MW clade was stable, sharing similar staminodes (Conn 1992). Alternatively, *Hemiandra* or an unresolved HH had given rise to the remaining genera in that clade (Conn 1992), or W formed a strongly supported sister to HHM (Olmstead et al. 1998).

Results in this study differ from the above findings in that *M. subcanescens*, a species not included in previous studies, is sister to all other Prostanthereae sampled. At the next lower level however, we do obtain a HHMW clade where *M. exserta* is sister to HHW, and HH is polyphyletic or unresolved (similar to Conn 1992). The situation of *Microcorys* in this study appears to be anomalous, especially when taking into account that both species belong to the same morphological section (Conn 1992, Blackall & Grieve 1981). As the present study seems to be at variance with other studies, *M. subcanescens* should be re-sequenced; alternatively, analysis of combined data sets

might clarify the situation, or a closer morphological analysis might reveal small but significant differences.

Tribe Chloantheae

Chloantheae have been variously regarded as a subfamily (Briquet 1895) or tribe (Bentham 1876, Junell 1934) belonging to Verbenaceae, or as a family (Hutchinson 1959, Munir 1979). Already Junell (1934) noted a close relationship between many members of Verbenaceae and Lamiaceae, due to shared similar gynoecial architecture (forming a continuum ranging from gynobasic to terminal style). This observation contributed substantially to the re-classification of the chloanthoid clade to the Lamiaceae at the tribal level, while at the same time giving the name to subfamily Chloanthoideae in the CHW classification. Chloantheae share a range of morphological characters with Lamiaceae, including similarities in inflorescence, ovules attachment, style and stigma architecture, pollen exine and hairs (Judd et al. 1999).

Cantino (1992) referred to this group as Physopsideae and Chloantheae (Chloanthoideae), belonging to Verbenaceae, but shortly afterwards this group was assigned to Lamiaceae in the CHW classification. This reclassification was provisional, but has since received qualified support from subsequent studies (Wagstaff & Olmstead 1997, Olmstead et al. 1998, Wagstaff et al. 1998). The reclassification achieved the important goal of monophyly of Lamiaceae *s. l.* and Verbenaceae. The chloanthoid / physopsioid clade in Cantino's (1992) morphological cladistic analysis encompassed all genera of the tribe Chloantheae and revealed several poly- and paraphyletic genera. The clade was however not strongly supported. Confirmation for the relationships within the tribe comes from *ndhF* (Olmstead et al. 1998) analysis and our *rbcL* study.

These studies all agree that *Pityrodia* is polyphyletic and that *Lachnostachys* and *Newcastelia* form a clade. The latter is possibly supported by biochemical systematics (Pedersen 2000). *Dicrastylis* and *Mallophora* (a genus with only two species) are closely related based on *ndhF* analysis and morphological traits (Olmstead et al. 1998), leading to the proposition to subsume *Mallophora* into *Dicrastylis* (B. Rye, pers. com). Morphological and *ndhF* data concur in the position of *Pityrodia halganiacea* being sister to all Chloantheae (Cantino 1992, Olmstead et al. 1998). Based on morphological differences, *P. halganiacea* has recently been reclassified as *Brachysola halganiacea*, which together with *B. coerulea* (formerly *Chloanthes coerulea*) form the new, small

genus (Rye 2000). Operculate pollen was hypothesized to be a synapomorphy delineating a clade encompassing *Lachnostachys-Newcastelia-Dicrastylis-Mallophora* (LNDM) (Cantino 1992). In contrast, this study enlarges the LNDM clade by adding paraphyletically *Pityrodia*, *Cyanostegia* and *Physopsis*, and supporting the reclassification of *N. chrysophylla* as *Physopsis chrysophylla* (Rye 1996).

Subfamily Teucrioideae (*sensu* CHW)

The circumscription of Teucrioideae has varied over time. In the CHW classification it encompasses over 20 genera and achieved monophyly of the subfamily, based on clades that emerged in a previous analysis (Cantino 1992). Its membership cuts across traditional familial and tribal boundaries and encompasses genera of previously widely divergent taxonomic positions, but has substantial support for monophyly (Cantino 1992). Teucrioideae also contain *Ajuga*; consequently, if the subfamilies are combined, the name Ajugoideae would apply as this is the older name and would take precedence under the International Code of Botanical Nomenclature (Cantino et al. 1992, Wagstaff & Olmstead 1997).

Teucrium has always been treated as part of Lamiaceae. The position of *T. racemosum* at the base of this subfamily indicates that *Teucrium* is polyphyletic and that *T. racemosum* is not related to the species in the Mediterranean region, the center of *Teucrium* diversity (Cantino 1992).

Spartothamnella, which since the CHW revision belongs to the subfamily Teucrioideae, traditionally belonged to a chloanthoid group within Verbenaceae (Bentham 1876, Briquet 1895, Munir 1979, Blackall & Grieve 1981). This classification was based on similarities in corolla, stamen and anther morphology. However Junell (1934) suggested that the genus belong to the tribe Viticeae of Labiatae, subtribe Ajuginae, near *Teucrium*. Support for the close relationship between *Spartothamnella* and *Teucrium*/Teucrioideae plus *Ajuga* comes from verrucate pollen sculpturing (Abu-Asab & Cantino 1993, Cantino 1992), and *ndhF* sequence analysis (Olmstead et al. 1998). The Teucrioideae-*Spartothamnella* clade, which emerged in this *rbcL* analysis, is additionally supported by similarities in pollen, flower bud and calyx morphologies (Cantino 1992, Abu-Asab & Cantino 1992).

Taxonomic implications of this study

Cantino's 1992 study and our preliminary results suggest that *Wrixonia* should no longer be treated as a distinct genus but should be included in an enlarged *Prostanthera*. This suggestion is in agreement with de Queiroz & Gauthier (1994) who advocate that monotypic taxa are redundant and should be subsumed by existing larger taxa to which they are most closely related. The abandoning of the principle of exhaustive subsidiary taxa results in explicit, universal and stable nomenclature and leads to an efficient classification.

Future work

Molecular systematics is a powerful tool to study the phylogeny of organisms. The aim of a phylogenetic approach is to obtain monophyletic groupings. Subfamily Chloanthoideae contains many clades with para- and polyphyletic taxa. Before reclassifying these problematic taxa to achieve monophyly, well-supported phylogenetic trees will need to be constructed. Relationships in the HHMW clade and the evolutionary position of *Pityrodia* remain ambiguous. These phylogenetic relationships may become clearer by additional research. Phylogenetic analyses of the small genera *Denisonia*, *Mallophora*, *Hemiphora* and *Eichlerago* might lead to these taxa being subsumed into *Pityrodia*, *Dicrastylis*, *Chloanthes* and *Prostanthera* respectively, while detailed analysis of *Physopsis* and *Newcastelia* is expected to substantiate differences between the two genera. Separation of *Pityrodia halganiacea* and *Chloanthes caerulea* into the new genus *Brachysola* might also receive support from phylogenetic investigations. An exhaustive study of the genus *Chloanthes* remains outstanding.

Clarification of currently ambiguous relationships can be achieved by morphological analyses, or by sequencing further plastid loci (e.g. *matK*, *ndhF*, *trnL*) or nuclear loci (e.g. ITS, 5S rDNA spacer), to elucidate relationships between closely related genera and species. Combined analysis of several data sets, molecular or otherwise, will result in more strongly supported clades. If several analyses show the same topologies for ambiguous taxa, then reclassification may be warranted.

Chapter 4

Basic germination requirements *of Hemigenia exilis*

Abstract

This chapter explores the basic germination requirements of *Hemigenia exilis*. The production of viable seed averaged 25.3% (±1.2), with a mean 10-seed weight of 19 mg (±0.6). Incubating intact seeds at lower temperatures (15 and 20°C) and high GA_3 concentration (30 μM) achieved best germination (41%). Higher temperatures (30°C) reduced germination, indicating that *H. exilis* may have a natural preference to germinate during cooler months in autumn and spring. Seeds exhibited a combination of physiological and mechanical dormancy. Physical dormancy alone was excluded, as fresh and aged seed imbibe water. Similarly, no endogenous chemical inhibitors could be isolated by tissue isolation and recombination testing. Physiological dormancy was overcome by the addition of a high concentration of GA_3. Mechanical dormancy was alleviated by seed coat and endosperm removal, resulting in 100% germination. Exposure to heat (80°C to 120°C) and aerosol smoke was less successful in promoting germination.

Introduction

Knowledge of seed biology, especially of germination ecology, is essential for successful propagation and conservation of rare plant species. An example is the serpentine endemic *Hemigenia exilis* (S. Moore) (Lamiaceae), which is restricted to the arid northeast Goldfields of Western Australia (Paczkowska & Chapman 2000). This species is subjected to impact from mining, and knowledge of its germination ecology is needed for rehabilitation. Information regarding the propagation of this species from seed is limited (Cochrane et al. 1999). A detailed understanding of the germination ecology will benefit the propagation of this species for revegetation and horticultural purposes. Furthermore, such information will help in determining the factors restricting natural seedling recruitment, and shed some light on the underlying factors influencing the rarity of the species.

The seed biology of agricultural, horticultural, weedy and silvicultural species growing in temperate regions is generally well understood (Baskin & Baskin 1998). Much less is known about the seed biology of wild plants of little economic importance, particularly when these species are from harsh environments. Most studies regarding seed biology of plants from the arid zone focus on grasses and annual species, with a geographical emphasis on North America (Baskin & Baskin 1998) and the Middle East (Gutterman 1993). Germination ecology of certain Australian arid zone plants has been investigated by Mott & Groves (1981), Hacker (1989) and Plummer et al. (2001), with emphases on grasses and annual Asteraceae. Only a few perennial species that are important in rehabilitation of degraded lands or mine sites, or serve as livestock fodder, have received attention (Langkamp 1987, Richmond & Chinnock 1994, Letnic et al. 2000).

Most shrubs in desert environments produce orthodox, desiccation tolerant, dormant seed. Nikolaeva (1977) classifies two types of dormancy, endogenous and exogenous. Endogenous dormancy may be physiological, morphological or a combination of both. Exogenous dormancy includes physical, chemical and mechanical types. In arid zone species, physical and physiological dormancy are equally important, with only very few species producing non-dormant seed (Baskin & Baskin 1998).

Dormancy in plants from arid areas ensures that germination and seedling establishment take place at times when soil moisture is not limiting. The high temperatures and

drought conditions that dormant seed experiences over summer may lead to a gradual alleviation of dormancy and primes seed that is ready to germinate with the onset of the cooler wet season (Kigel 1995). In some species, a certain amount of rain is needed to leach chemical inhibitors from the seed. The degree of dormancy within a species may depend on genetic, maternal and environmental factors (Gutterman 1993, Bewley & Black 1994).

Orthodox seed needs a period of after-ripening under dry conditions before dormancy is broken. Processes taking place during after-ripening include changes in plant hormones (for example a decrease in levels of abscissic acid (ABA) and an increase in gibberrelins (GA)). Other changes in seed coat integrity also occur, to enhance permeability to water, and lead to embryo growth that is less mechanically restricted (Bewley & Black 1994).

The stimulatory effect of applied growth regulators such as GA on germination of dormant and non-dormant seed of many species, including all cereals, has been widely reported since the mid 1960's (Jones & Stoddart 1977, Bewley & Black 1994). Approximately 100 gibberellins exist, their conjugates, GA_3, GA_4 and GA_7 being the forms most widely used in dormancy and germination studies (Bewley & Black 1994). Levels of GA are high in developing seed, and decrease as the seed matures, while ABA increases and maintains dormancy. Gibberellic acid increases again just at the onset of germination. Based on extensive research with GA deficient *Arabidopsis* and tomato plants, GA has been shown to be crucial for germination (Karssen et al. 1989). Gibberellins initiate hydrolysis of storage material, thus mobilising food reserves and reducing mechanical constraints imposed by the endosperm on the embryo (Hillhorst & Karssen 1992). Application of exogenous GA overcomes dormancy and any after-ripening requirements (Baskin & Baskin 1998), but it stimulates germination of intact seed with non-deep or intermediate physiological dormancy (Nikolaeva 1977).

High temperature plays a dual role in the germination ecology of desert species, by inducing dormancy and preventing germination events when conditions are unlikely to sustain seedling survival. At the same time, elevated temperatures accelerate the release of dormancy. In some cases, fluctuating temperatures, as they occur naturally, lead to a relief in dormancy (Kigel 1995), while other studies have shown that constant high temperatures are equally capable of releasing dormancy (Baskin & Baskin 1998). Dry

heat may break seed coat-imposed, physical dormancy as well as non-deep physiological dormancy, particularly in hard-seeded legumes, Cistaceae and Malvaceae (Baskin & Baskin 1998). Generally, an increase in temperature reduces the time required to break physical dormancy. The temperature optimum varies between species and ranges from 80 to 150°C, while the length of exposure to heat can vary from a few minutes to several days (Baskin & Baskin 1998).

Smoke, as a result of fire, has been implicated in breaking dormancy of species from fire prone habitats in mediterranean ecosystems of Southern Africa (de Lange & Boucher 1990, Brown 1993), California (Keeley & Bond 1997), and Australia (Dixon et al. 1995, Roche et al. 1997, Tieu et al. 2001). Some species from less fire prone habitats may also increase germination in response to smoke (Pierce et al. 1995, Adkins & Peters 2001). The mechanisms by which smoke breaks dormancy is not fully known, but it is thought that smoke constituents may induce ultrastructural changes in the seed coat (Egerton–Warburton 1998) and affect endogenous hormone levels (Gardner et al. 2001).

Very little is known about the germination ecology of Australian native Lamiaceae, particularly the arid zone taxa. This study was initiated with the aim to establish baseline characteristics of the germination ecology of *Hemigenia exilis* as the first steps towards developing an understanding of the restoration requirements of the species. We hypothesized firstly that seed germination characteristics vary between genetic provenances and the maternal plant. Secondly, *H. exilis* is a desert species that probably produces dormant seed with some degree of after-ripening that can be overcome by application of exogenous GA_3 or scarification (nicking) of the seed coat. Thirdly, germination would take place under temperature conditions typical for cooler months, since many sympatric taxa from the endemic range of *H. exilis* exhibit winter germination strategies. The nature of dormancy was investigated, with chemical, physical and/or mechanical factors being the potential agents. Methods for dormancy relief will be presented and implications for the restoration of the species discussed.

Material and Methods

Field observations on the phenology of *Hemigenia exilis*

Hemigenia exilis is a longlived shrub, growing in the Northeast Goldfields of Western Australia, in a hot and arid environment. The species has an affinity for ultramafic outcrops and may be categorised as a serpentine endemic. Observations of several populations since 1997 have shown that the species flowers profusely in late winter / early spring (August – October), attracting butterflies, which are the potential pollinators. The flowers are light purple or mauve, with faint markings in the throat. White-flowering plants also occur, but these are very rare. Flowers measure up to 1 cm in diameter and sometimes are lightly scented. Most plants appeared to flower profusely year after year, and only a few plants did not flower during the 4-year period of observation.

The 4 nutlets (hereafter called seeds) contained in each calyx mature in late spring / early summer (November / December), when seed changes from being soft, milky with a whitish / greenish seed coat to hard, maroon and dark brown. Mature seed measures approximately 2 mm in length and 0.5 mm in diameter. Dissection of the seed revealed a relatively hard seed coat and a thin layer of endosperm enveloping a large, fully differentiated embryo. As the seed matures on the mother plant, the calyces are dropped on the ground, sometimes containing the seeds. In other instances the seeds are dislodged while the calyx is still on the mother plant. Seed appears to be dispersed by gravity. Opportunistic flowering in summer has a low intensity, and then seed set is virtually nil. Very few seedlings have been observed in the natural habitats, despite a succession of years with above average rainfall. Plants re-sprout readily when damaged, and re-sprouting from roots has also been observed. Neither leaves, stems, calyces or seed seem to produce obvious volatile oils typical of many Lamiaceae. The plant is very rarely grazed, and seed predation has not been observed, neither at the pre- nor post-dispersal stages.

Experiment 1: Viability and weight of *Hemigenia exilis* seed

Seeds were collected in November 1997 from two pairs of sites in the Northeast Goldfields of Western Australia, sites E1 and E2 (121°47' East / 28°57' South) representing the southern genetic provenance, and C1 and C2 (121°07' E / 28°22' S) representing the northern provenance. Seed from four maternal lines at each site was

collected separately. Viability was determined by a flotation test, where seed was placed in a beaker containing 1 L of water with three drops of a surfactant (Tween 80). Floating seed was deemed empty and seed which sunk was considered filled, indicative of a viable embryo. Viability of the sunk seed was reconfirmed by the cut test (Roche et al. 1997), revealing white, plump intact endosperm. For subsequent routine determination of viability, the more convenient float test was therefore employed.

To elucidate differences in viability between maternal lines, populations and genetic provenances, seeds of four plants of two populations of the two provenances (16 plants in total) were examined, using 4 replicates of 100 seeds. Data were arcsin transformed prior to statistical analysis by ANOVA in StatView® v.5.0. To quantify differences in seed weight between maternal lines, populations, and genetic provenances, seeds of four plants of two populations of the two provenances were weighed, using six replicates of 10 seeds each. Data were analysed by ANOVA.

Experiment 2: Establishing optimal conditions for germination (temperature, GA, nicking)

Bulked seeds from site E1 which were collected in November 1997 and after-ripened at room temperature for 5 months were used in this experiment. Empty seed was separated by the flotation test (as described above). Only filled seeds were used for the experiment. To reduce contamination by fungi and bacteria, seeds were vacuum sterilised in a 1% (v/v) sodium hypochlorite solution (5 min vacuum on, 5 min off, 5 min on) and rinsed in sterile water. This technique has been shown to be not inhibitory to germination (Meney & Dixon 1988, Roche et al. 1997).

Four replicates of 25 intact seed each were tested under four constant temperature conditions (15, 20, 25, 30°C) and 5 GA_3 levels (0, 1, 3, 10 and 30 µM GA_3). The effect of nicking on germination was tested under the same temperatures in the absence of GA_3. Seeds were placed on sterile filter paper in petri dishes, moistened with sterile distilled water with and without GA_3 and were sealed with plastic wrap to reduce evaporation. Petri dishes were kept in the dark and wrapped in aluminium foil to eliminate potential photo-inhibition. Seed was exposed to white fluorescent light for a few hours once per week, when the trial was assessed. Germination was scored on a weekly basis over an 8-month period. Filter papers were moistened with sterile distilled water when necessary. Seeds were deemed germinated when the radicle protruded.

Germinants were counted and removed. Viability of ungerminated seed was determined by dissection. Over 90% of that seed contained firm white endosperm and embryo, which were considered viable. Final germination was viability adjusted. Arcsin transformed data were statistically analysed by ANOVA, quantifying effects of temperature, GA_3 levels and nicking on germination.

Experiment 3: Physical dormancy - imbibition

Physical dormancy is defined as the seed coat being impermeable to water. Consequently the seed cannot imbibe. Imbibition curves were established for bulked seed from population E1, comparing viable fresh seed and seed buried for 1.5 and 2.5 years. Three replicates of 10 seed were measured for dry weight, then suspended in nylon mesh baglets in aerated water. Seeds were then removed, patted dry and weighed every half hour for the first 4 hours, after which intervals were extended to 1, 2, 12 and 24 hours over a 9 day monitoring period. Differences in imbibition at day 9 were analysed by one way ANOVA.

Experiment 4: Chemical dormancy - endogenous inhibitors

Endogenous inhibitory compounds in the seed coat are often associated with the regulation of seed dormancy and germination (Baskin & Baskin, 1998). Consequently the presence of water- and alcohol soluble inhibitors in the seed coat of fresh *H. exilis* seed was evaluated in a bioassay on lettuce (*Lactuca sativa* L.). Seeds were sourced from a bulked sample of population E1 collected in 1998. One gram of seed coats of fresh empty seed was ground to a fine powder under liquid nitrogen and extracted by stirring for 3 h in 10 ml of methanol. The suspension was filtered through a Whatman 1PS filter paper. The solid material was extracted again with 10 ml methanol for 3 h, followed by extraction in 3 ml for 24 h. The filtered liquid was evaporated under vacuum in a rotavator. Water-soluble compounds were extracted using the same procedure, except double distilled water was used as the extraction medium.

The residue of the methanol extract was dissolved in 0.75 ml of methanol, which was poured onto filter paper in a petri dish, allowed to evaporate and re-moistened with 0.75 ml of 10 mM sodium bicarbonate buffer (pH 6.8). The residue of the water-soluble extract was dissolved in 0.75 ml of 10 mM sodium bicarbonate buffer (pH 6.8) and poured onto 2 layers of filter paper (Whatman No. 1, 70 mm diameter). Filter papers were divided into 3 sectors, each of which contained 10 lettuce seeds. As a control,

filter paper was moistened with the same volume of water or buffer only. The petri dishes were sealed with plastic wrap to prevent evaporation and incubated at room temperature. Seeds were scored after 48 h and recorded as germinated when the radicle was at least 1mm long.

Lipids were isolated from seed coats of fresh empty seeds using chloroform as the extraction solvent. Depending on availability, 0.05 g to 0.12g of seed coats from three individuals of population E1 were ground in 6-9 ml of chloroform : methanol (2:1) and centrifuged at 10,000 rpm for 10 min. The supernatant was decanted and partitioned with the same amount of 0.7% NaCl (binding the non-lipids) and again centrifuged. The chloroform fraction containing the lipids was transferred to a new tube, evaporated to dryness under argon to prevent oxidation, left under vacuum for two hours and subsequently weighed.

Experiment 5: Mechanical dormancy - embryo extraction

Fresh seeds collected from site E1 in November 1998 were separated into filled and empty seeds by the flotation test. Only filled seeds were used for the experiment. Seeds that had been aged for three and 12 months under field conditions at site E1 were also tested. After surface sterilisation, seeds were subject to five treatments: seed coat left intact, seed coat partially removed (=nicked), seed coat completely removed (=endosperm and embryo only, e+e), embryo excised (=emb), excised embryo in contact with seed coat (=emb+coat). There were 3 replicates with 10 seeds for each treatment. Seeds were placed in petri dishes on two layers of sterile filter paper, saturated with sterile de-ionised water and incubated in a 20°C±2 cabinet with 12 h photo-period provided by white fluorescent light. Germination was recorded weekly over 5 months. Final germination percentages were arcsin transformed and analysed by 2 way ANOVA to examine the effect of aging and treatments on germination.

Experiment 6: Evaluation of dry heat as a mechanism for dormancy relief

Seeds were collected from two populations, E1 and C2, representing the Southern and Northern genetic provenances. Only fresh, filled seed that was separated from the bulk by the flotation method was used in the experiment. Seeds were placed in paper envelopes and incubated at 25°C (control), 80°C, 100°C or 120°C for 30 min, 3 h, 2 days or 2 weeks in laboratory ovens. The 80°C treatment reflected temperatures found at the soil surface in summer in the natural environment of *H. exilis* (Fig. 4, Chapter 1). After

treatment, seed was allowed to equilibrate to room temperature. Four replicates of 25 seed each were sown in a soil mix of equal parts of pasteurised sand, composted sawdust and peat, and covered with a thin layer of sand. Punnets were incubated at 20-25°C at a 12 hour photoperiod. Germination was monitored weekly and scored for 14 weeks. Final germination percentages were arcsin transformed and analysed by 3-way ANOVA to investigate the effect of temperature, duration of heat exposure and genetic provenance on germination.

Experiment 7: Effect of smoke on germination of fresh *Hemigenia exilis* seed

Fresh filled seed bulked from the seed collection at E 1 was placed onto punnets filled with a soil mix as above and subjected to aerosol smoke for 1.5 h (Roche et al. 1997). Punnets were incubated at 20°C-25°C with a 12 hour photoperiod. Germination was monitored weekly for 14 weeks. Final germination percentages were arcsin transformed and analysed by 1 way ANOVA to determine the effect of smoke on germination.

Results

Experiment 1: Seed viability and weight of *Hemigenia exilis*

The flotation test successfully differentiated between filled seed containing healthy, that is, white, plump and firm endosperm, and empty seed. The majority of seeds (99%) that sunk to the bottom contained a healthy endosperm whereas 96% of seed that remained floating on the surface was empty. Thus the float test represented an efficient way to separate viable from empty seed.

Viability differed significantly ($p<0.001$) between maternal lines (Fig. 1a), ranging from as low as 6% to a maximum of 34% and averaging 28.8%. Differences in viability between populations and provenances were not significant ($p=0.09$ and $p=0.17$ respectively). Weight of 10 seeds differed significantly ($p<0.001$) between maternal lines (Fig. 1b), ranging from 12 mg to 27 mg and averaging 20 mg. Weight differences were also significant between populations ($p<0.001$), ranging from 17 mg for E1 to 23 mg for C1, and provenances ($p=0.006$), with 21 mg for the Northern and 19 mg for the Southern provenance.

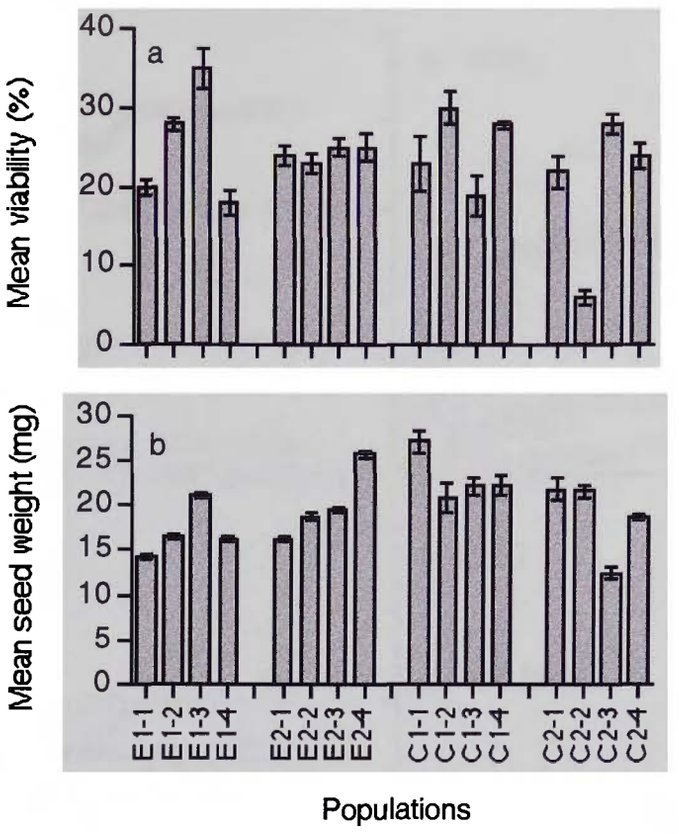

Fig. 1: Average viability (%) (±s.e.) (a) and weight (mg) (±s.e.) of 10 seeds (b) of 4 individuals in 4 populations representing two genetic provenances of *Hemigenia exilis* (E1 and E2 are southern provenance, C1 and C2 are northern provenance (Chapter 2).

Experiment 2: Establishing optimal conditions for germination (temperature, GA_3, nicking)

Temperature ($p<0.01$), GA_3 ($p<0.01$) and the interaction of temperature x GA_3 ($p<0.01$) showed significant effects on germination. The highest germination of 41% was achieved under a low temperature / high GA_3 regime (15°C / 30µM GA_3) (Fig 2.1a). The dormancy breaking effect of GA_3 was expressed to a greater degree at lower temperatures (Fig. 2.1a-d). Germination of intact seed without GA_3 reaches as maximum of 31% after 34 weeks at 25°C and was similar to nicked seed at that temperature (Fig. 2.1c). Nicking of the seed coat increased germination significantly ($p<0.001$), especially at the lower temperatures (Fig. 2.1a, b). At 25°C and 30°C, germination of both intact and nicked seed was similar (30% and 20% respectively) (Fig. 2.1c,d). Dissection of the non-germinated seed showed that more than 90% contained white plump endosperm, indicating that low viability was not the reason for lack of germination.

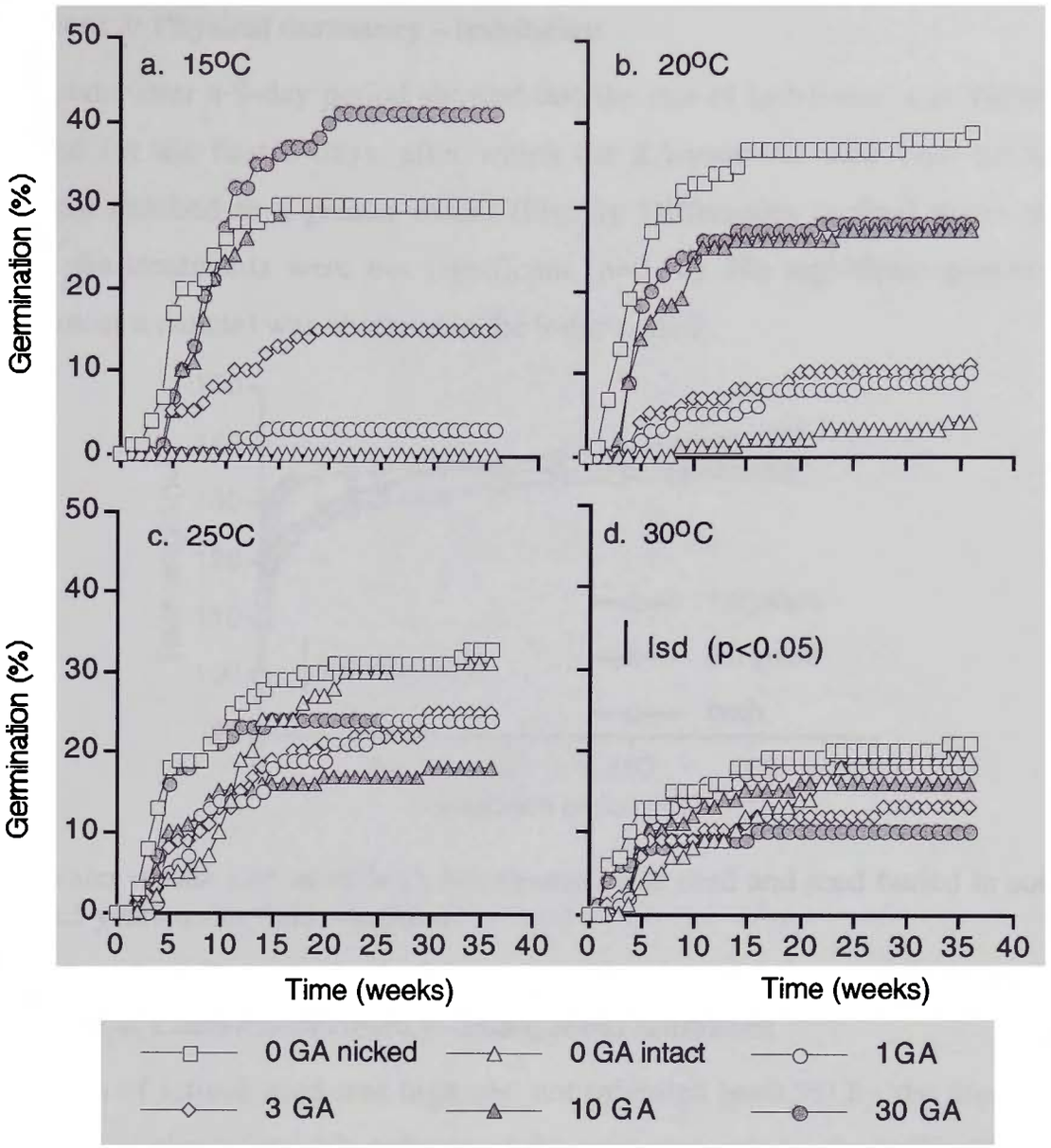

Fig. 2.1: Germination response (%) of *Hemigenia exilis* tested under 4 temperature regimes and 5 GA_3 concentrations (μM). The LSD bar applies to all 4 graphs.

Once the radicle started protruding, radicle elongation and unfolding of the cotyledons was accomplished within 4 days (Fig. 2.2)

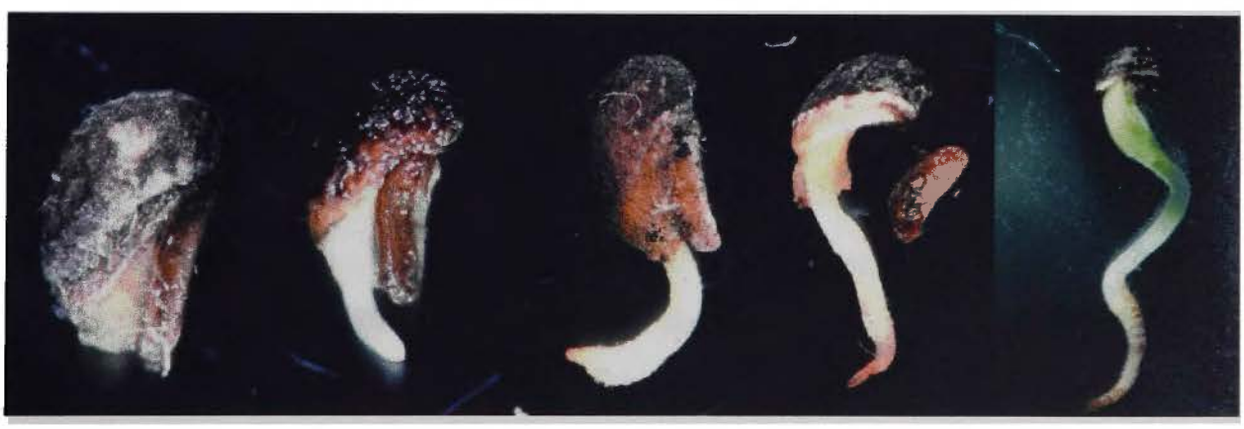

Fig. 2.2: Germination of intact seed of *Hemigenia exilis*, illustrating the gradual protrusion and subsequent elongation of the radicle, and unfolding of cotyledons.

Experiment 3: Physical dormancy – imbibition

Water uptake over a 9-day period showed that the rate of imbibition was highest for fresh seed for the first 5 days, after which the 2.5-year old seed from the burial experiment imbibed to a greater extent (Fig. 3). Differences in final water uptake between the treatments were not significant (p=0.18). No significant germination (protrusion of a radicle) was observed in the 9-day period.

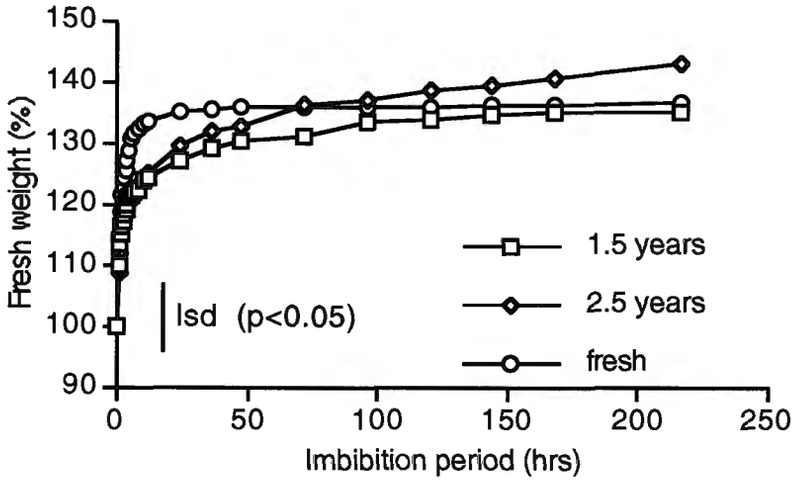

Fig. 3: Water uptake curves of fresh *Hemigenia exilis* seed and seed buried in soil for 1.5 and 2.5 years under field conditions.

Experiment 4: Chemical dormancy - endogenous inhibitors

Germination of lettuce seed was high and not inhibited (p=0.58) by the presence of either water or alcohol-soluble extracts of the seed coat, nor by the buffer (Table 1). Lipid content in the seed coat averaged 23.9% ±1.3%.

Table 1: Effect of water and alcohol-soluble extracts from seed coats of *Hemigenia exilis* on the germination of lettuce seeds.

Treatment	Germination(%)	s.e.
Control water only	100	0
Control buffer only	96.7	3.3
H. exilis water extract	96.7	3.3
H. exilis methanol extract	100	0

Experiment 5: Mechanical dormancy - embryo extraction

Seed coat treatment and storage period had a significant effect on germination (p<0.001). No germination took place in intact seed (Fig. 4), regardless of storage period. Partial removal of the seed coat (nicking) lead to increased germination with increased storage periods. This trend was enhanced when the seed coat was completely removed (Fig. 4). The presence of endosperm inhibited germination, with the effect

gradually diminishing over time. Extracted embryos germinated within 4 weeks in all 3 age classes. The seed coat alone had no inhibitory effect on germination. Seedlings produced from excised embryos developed normally and produced healthy seedlings in standard seedling raising mix consisting of equal parts of sawdust, sand and peat.

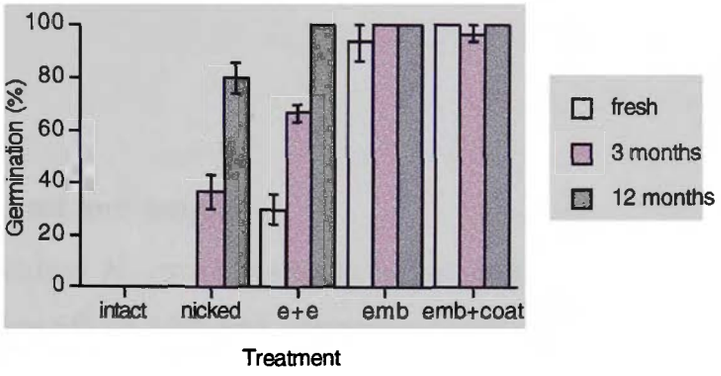

Fig. 4: Germination (%) (±s.e.) of *Hemigenia exilis* seed in relation to duration of burial (fresh, 3 months 12 months) and progressive removal of embryo covering structures (intact, nicked, endosperm+embryo (e+e), fully excised embryo (emb), and excised embryo with replaced seed coat (emb+coat).

Experiment 6: Evaluation of heat as a mechanism for dormancy relief

There was a significant difference between the two provenances ($p=0.001$) in response to the heat treatment. The southern provenance produced low numbers of germinants (Fig. 5a), regardless of treatment, whereas seed from the northern provenance showed higher levels of germination especially at 80°C for 3 h (Fig. 5b), and germinated over a wider range. However, the promotive effect is far less than addition of GA_3 or nicking (Fig. 2). Higher temperatures (100–120°C) reduced germination significantly ($p<0.0001$). Germination was also reduced ($p=0.006$) as duration of heat increased.

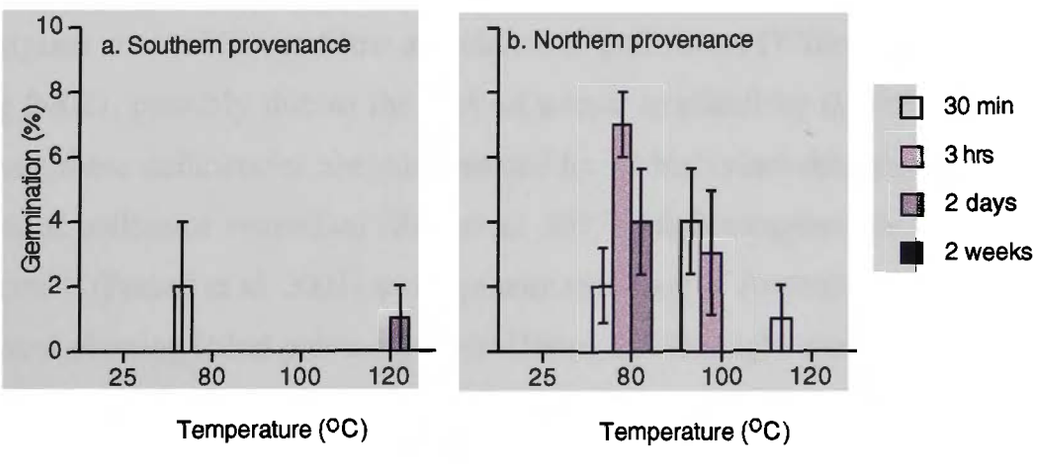

Fig. 5: Germination (%) (±s.e.) of *Hemigenia exilis* seeds sourced from two genetic provenances and subjected to heat (25°C, 80°C, 100°C and 120°C) over 30 minutes, 3 hours, 2 days and 2 weeks.

Experiment 7: Effect of smoke on germination of fresh *Hemigenia exilis* seed

During the four-month observation period, no germination was observed in the control treatment. Smoke increased germination significantly over the control to 8% (±1.4). Similar to the heat experiment, the effect of smoke was less than the effect of GA_3 or nicking.

Discussion

Variations in seed set and weight

Seed set for individual *H. exilis* plants is about one quarter of the total number of ovules, ranging from 6% to 34% and averaging 22.2%. At the population level, seed set ranges from 19.6% to 26.1%. These figures are slightly lower than reported by Cochrane et al. (1999), who noted the proportion of healthy *H. exilis* seeds ranging from 24% to 42% (mean=30%). Theses values are comparable to those for other insect pollinated species with a similar ovary structure (4 ovules/ovary) from dry environments, including *Dracocephalum* (Milberg & Bertilson 1997), *Phlomis* (Aparicio 1997) and *Merremia* (Willmott & Burquez 1996).

Pollination and seed set are influenced by a range of factors. These include environmental pressures and genetic factors, as well as an interaction between the two (Gutterman 1993, Mitchell 1997, Baskin & Baskin 1998). Low seed set in *H. exilis* may be due to the plant being a vigorous re-sprouter and thus having a minimal reliance on sexual reproduction alone (Bell et al. 1993). The lack of mortality of parent plants during the study period indicates that sexual reproduction may not be necessary in all years. Additional factors that contribute to low seed set include the lack of soil moisture (Choengsaat et al. 1998), and low abundance of pollinators (Willmott & Burquez 1996, Young 2002), possibly due to the lack of nectar availablility (Golubov et al. 1999). However, these deficiencies are compensated for by high plant densities, which increase chances of pollinator visitations (Roll et al. 1997). High temperatures inhibiting pollen tube growth (Prasad et al. 2001) and a genetic tendency of Australian arid zone plants to carry seed-aborting lethal polymorphisms (James 2001), might exacerbate low seed set.

In addition to the chemical constituents present in all plant tissues, seeds contain extra amounts of substances stored as food reserves to support early seedling growth. These are carbohydrates, fats and oils, and proteins, and other minor reserves. The major

storage reserves may be deposited within the embryo, usually the cotyledons, or within extra-embryonic tissue such as the endosperm, or within both types of tissues (Bewley & Black 1994). The significant differences in seed weight between the provenances may be due to the amount of storage reserves. These differences could be attributed to genetic differences and environmental conditions including mineral nutrition and soil moisture prevalent during seed development and maturation (Gutterman 1993). Higher seed weight, reflecting higher seed storage reserves, may improve seedling survival, a particularly vulnerable stage in plant establishment in a harsh environment. Consequently, seedlings from the Northern provenance may have a better chance of survival than seedlings from the South. Additionally, an increase in seed number is generally correlated with a decline in seed weight (Baskin & Baskin 1998), but this relationship does not apply to *H. exilis*. Future studies might investigate influences of seed weight on germination and survival, or document seed phenologies of the same mother plants over several years to investigate seasonal effects on reproductive performance.

Desert plants are efficient in resource allocation during several stages of their development. In absolute terms, they produce fewer flowers, fruits and seeds, seeds that are smaller and weights that are less than seed of their Mediterranean congeners. However, in relative terms, based on production of biomass, desert plants have higher reproductive fitness than their cousins from less demanding climates (Boaz et al. 1994). Quantification of seed weight and viability of *Hemigenia* species from the southwest of Western Australia might add support to this trend.

The role of temperature and GA in relieving dormancy

a. Temperature

The majority of desert shrubs in the northern hemisphere (Baskin & Baskin 1998) and in Australia (Jurado & Westoby 1992) produce dormant seed, and *H. exilis* is no exception. After-ripened, untreated seed of *H. exilis* germinated over a six-month period, which indicates a form of protracted dormancy. This figure is slightly higher than in a previous report (Cochrane et al. 1999). The slow germination rate (and viability exceeding 2.5 years, see Chapter 5) results in a persistent soil seed bank and ability to respond to varying environmental conditions. Slow germination of up to three months has been observed in several ornamental *Hemigenia* species, including some from arid zone environments in Australia (Elliot & Jones 1990).

At lower temperatures, dormancy in this study was released by addition of GA_3. Since seeds were incubated in the dark and were only occasionally exposed to light, the addition of GA_3 may have compensated for a potential light requirement (Baskin & Baskin 1998). Conversely, short exposure to light may have fulfilled that requirement. Nicking the seed coat also improved germination, pointing towards physical or mechanical dormancy mechanisms. Anecdotal evidence (pers. obs.) suggests that nicking at the apex of the seed increased germination to a lesser extent than nicking at the ventral side of the seed (Fig. 2.2). Depending on treatment, optimum temperature for germination of *H. exilis* ranges from 15°C to 25°C. Most of the germination curves follow the usual sigmoidal, positively skewed pattern (Bewley & Black 1994), often with low germination capacities. The temperature optimum lies within the range reported for warm desert shrubs, being 20-25°C (Baskin & Baskin 1998).

The temperature conducive to germination corresponds to the temperatures at the period of reliable rainfall (Bell 1999). Rainfall patterns in the northeast Goldfields (Leonora) show approximately equal probability of summer and winter rainfall (Bureau of Meteorology 2002). Summer rainfall results in germination of grasses (Mott & Groves 1981). Winter rains lead to germination of annual forbs such as everlastings (Plummer & Bell 1995) and perennials (Pringle et al. 1994). Temperatures achieving high germination of *H. exilis* under lab conditions indicate that in the field the species would germinate in spring or autumn. *Hemigenia exilis* is thus adapted to both summer and winter rainfall, provided the amount of rainfall is high enough to maintain soil moisture at adequate levels (Gutterman 1993, Baskin & Baskin 1998).

b. Influence of gibberellic acid

Hemigenia exilis responds positively to application of GA_3, reflecting a non-deep or intermediate physiological dormancy (PD). Although Nikolaeva (1977) postulated that light is a requirement for non-deep PD, this may be overcome by appropriate temperature treatments (Baskin & Baskin 1998). Addition of high concentrations of GA relieved non-deep PD in *H. exilis*. The amount of exogenous GA might decrease with an increase in the after-ripening period, that is, prolonged storage exceeding 2.5 years (see Chapter 5). GA enables the embryo to exert a greater growth potential and physical thrust to fracture constraining seed coats and other restricting covers. Gibberellic acid also facilitates endosperm breakdown and may counteract the effect of germination

inhibitors (Black 1996, Baskin & Baskin 1998). Added GA might have accelerated an after-ripening requirement for *H. exilis*, such as prolonged dry storage at room temperature or elevated temperatures.

Endogenous GA synthesis, leading to germination, is closely linked with phytochrome activity (Pons 1992) and temperature (Favier 1995). Hilhorst (1993) has developed a model of the physiological action of GA in breaking dormancy, which may be applied to *H. exilis* as follows: during the dormancy-breaking treatment, such as hot summer conditions, phytochrome receptors are formed in the cell membranes. When temperatures become favourable for germination (cooler), the membrane consistency changes. The receptors move to the surface where they are activated by nitrate. The activated receptor binds with the phytochrome, which itself becomes activated by light. Endogenous GA is synthesised after phytochrome activation, and binds with a GA receptor. The GA-receptor complex produces a signal that stimulates germination, and thus relieves dormancy.

Endogenous GA production may increase after a period of heat treatment (Favier 1995). Heat increased endogenous GA and subsequent germination in annual Asteraceae from the Australian arid zone (Choengsaat et al. 1998) and in a *Leptospermum* from the South African fynbos (Brits et al. 1995). However, the biochemical mechanism for heat-increased GA synthesis is not fully understood (Favier 1995).

Determination of the type of dormancy in seed of *Hemigenia exilis*

Hemigenia exilis responded to exogenous GA_3 and nicking, which alleviated non-deep physiological dormancy (PD). The seed responded to an even greater extent to removal of the seed coat, supporting the presence of a strong mechanical dormancy. This type of dormancy is caused by mechanical resistance of seed covers, including endocarp, mesocarp, hard woody fruit wall, endosperm, caryopses, and seed coats (Nikolaeva 1977, Brown & Bridglall 1987, Dekker et al. 1996, Black 1996, Baskin & Baskin 1998). This study has shown that removal of those tissues facilitated germination.

Embryo extraction techniques employed in this study demonstrated that there is a mechanical constraint to germination of *H. exilis* seeds caused by a seed coat in fresh and aged seed, and by the endosperm in younger seed. The fact that endosperm hindered germination of younger but not older seed indicates that physiological or

mechanical dormancy weakens during after-ripening. In seeds of tomato, capsicum and celery, endosperm weakening is promoted by an increase of endogenous GA synthesis during after-ripening and by some enzymes, while at the same time the embryo develops greater growth potential in the presence of GA (Hilhorst & Karssen 1992, Voigt & Bewley 1996, Baskin & Baskin 1998).

Chemical dormancy, caused by soluble inhibitors in the pericarp (Nikolaeva 1977) can be excluded in *H. exilis*, as water and alcohol-soluble extracts from the seed coats did not inhibit the germination of the bioassay species lettuce (*Lactuca sativa* var. Grand Rapids).

Physical dormancy, caused by the impermeability of the seed coat to water (Nikolaeva 1977), was suspected to cause low germination, as the fresh seed of *H. exilis* was waxy and oily, and stained paper envelopes in which the seed was stored. This type of dormancy may be ruled out, as even fresh seed was shown to be permeable to water. However, imbibition may be an experimental artifact, as the surfactant Tween 80 employed in the flotation test might have removed the compounds present in the originally impermeable coat of fresh seed. Alternatively, the permeability of fresh seed could be due to changes in permeability during the short period (2 weeks) of storage under ambient room conditions. Water-repellent substances may have disintegrated during that time.

Heat and smoke as agents for breaking dormancy

Dry heat treatments, at artificially high temperatures up to 140°C for short periods, or at temperatures occurring naturally (40-80°C) for extended periods, has increased germination of many desert shrubs (Baskin & Baskin 1998), and grasses and Asteraceae from the Australian arid zone (Mott 1974). Heat causes cracking in seed coats or acts on other embryo-covering structures. High temperature primarily alleviates physical dormancy (Nikolaeva 1977), although it also affects GA synthesis and regulates the breakdown of germination inhibitors (Favier 1995).

Heat promoted germination of *H. exilis* to a maximum of only 7% and was thus less successful in alleviating dormancy than the addition of 30 µM GA_3 at 15-20°C (up to 41% germination) or embryo excision (100% germination). Similarly, experiments by other researchers indicated that subjecting *H. exilis* seeds to 70°C for periods ranging

from 11 days to 6 months elicited only low germination (Cochrane et al. 1999). The two provenances showed differential responses to heat treatment, which may be attributed to genetic and/or environmental factors. The southern provenance remained virtually unresponsive, while the northern provenance increased germination to a maximum of 7% at 80°C for three hours. Higher temperatures and longer exposures reduced germination, possibly by inducing thermodormancy or by affecting ultrastructural, chemical and physiological processes (Bewley & Black 1994, Egerton-Warburton 1998). The lack of response to heat and the ability of fresh seed to imbibe, excludes physical dormancy from being important in *H. exilis*.

Aerosol smoke increased germination of *H. exilis* from 0% only to 9%, illustrating that smoke is not an efficient tool to alleviate dormancy in this species. Similarly low germination responses were obtained when treating the seed with smoke water (Cochrane et al. 1999). The exposure to smoke as a method to stimulate germination in non-fire prone species appears to be confined to annuals including *Mesembryanthemum*, *Nicotiana* and everlastings (Pierce et al. 1995, Preston & Baldwin 1999, Plummer et al. 2001). Smoke has been applied to two *Hemiandra* species from the southwest of Western Australia, which are closely related to the genus *Hemigenia*. *Hemiandra ramosissima* was smoke responsive, whereas *H. pungens* was not (Roche et al. 1995). Some dormant species require a combination of heat followed by smoke, as the physical changes induced by heat render the seed sensitive to the compounds of smoke. Alternatively, soil storage and smoke may alleviate dormancy (Keeley & Fotheringham 1998, Tieu et al. 2001). The interaction of heat, soil storage and smoke on germination of *H. exilis* may be useful areas for future investigations.

In summary, *Hemigenia exilis* exhibits both physiological and mechanical types of dormancy, which can be partially relieved by the addition of GA_3, and fully released by seed coat removal. For propagation of this rare species, seed coat removal and embryo excision are suitable when seed is in short supply, assuring 100% germination in a short time. When seed supply is not limited, incubation for extended periods of surface-sterilised, intact seed at 15°C / 30µM GA_3 or nicked seed at 20°C / 0 GA_3 achieves around 40% germination. Improvement of germination by addition of GA_3 is less labour intensive than mechanical manipulation of the seed coat and may be more practical for large scale rehabilitation programs using *Hemigenia exilis*.

Chapter 5

Prolonged seed burial and soil seed bank storage enhance germination of *Hemigenia exilis*

Abstract

To investigate the effect of soil burial on dormancy and germination, seeds of two genetic provenances of *Hemigenia exilis* were buried in their respective natural habitats. Results indicated that burial alleviated dormancy and improved germination. Germination was 2%-8% after three months of burial and increased to 20%-30% after 30 months of burial. The level of germination was also dependent on genetic provenance. This increase may be due to physiological and mechanical dormancy as described in Chapter 4. During burial seed surface morphology changed, concomitant with a significant reduction of seed coat strength from 7.7 N in fresh seed to 4.8 N in aged seed. Viability did not change during that period.

Storage of soil seed bank samples for 12 months increased germination of *H. exilis* 6-fold (from 15 seedlings m^{-2} in fresh soil to 100 seedlings m^{-2}) during the course of 1 year. In contrast, germination of annuals was significantly reduced from ca. 900 m^{-2} in fresh soil to 500 m^{-2} after 1 year of storage. These results are comparable to most other soil seed bank studies of arid zone plants. This study also showed that the majority of seeds, regardless of species, germinate in samples taken from the soil under the canopy of *H. exilis*, demonstrating the topochorous dispersal mechanisms of *H. exilis*. Results indicated that *H. exilis* benefits from prolonged aging in soil, which alleviates mechanical and physiological dormancy. It appears that the species is capable of building up a persistent seed bank.

Introduction

Seed banks consist of the reserve of viable, ungerminated seeds. They may be aerial seed banks, where seed is retained on the mother plant long after maturity, for example seeds from serotinous species belonging to Proteaceae (Lamont 1991). Seeds can also be stored in the soil seed bank, which are produced by plants that disperse seed at maturity (Thompson & Grime 1979). The soil seed bank consists of buried seeds, seeds on the soil surface, and seeds held in a litter or humus layer (Baskin & Baskin 1998). Seed banks may be of transient or persistent nature. Transient seed banks remain viable for less than one year, as opposed to persistent seed banks which maintain viability for periods greater than one year (Thompson & Grime 1979). An alternative definition has been proposed by Walck et al. (1996), who describe seed banks in terms of germination seasons: a persistent seed bank is represented by seeds that remain viable until the second (or subsequent) germination season. The difference between these two concepts is more apparent in an arid environment, where rainfall, which determines the germination season, may be infrequent and unpredictable.

Persistent seed banks are a strategy to survive a risky environment, ensuring that germination events are spread over several years or seasons. If the species does not produce seed in a certain year, for example because of drought, the presence of the seed bank ensures the species' survival and population stability, and avoids (or delays) extinction (Kalisz & McPeet 1992, Baskin & Baskin 1998, Rice and Dyer 2001). Furthermore, seed banks contribute to genetic diversity of a population (Cabin 1996).

The persistence of the soil seed bank is influenced by the longevity of the seed, secondary dormancy, decay and predation (especially of larger seed) (Kigel 1995, Anderson & MacMahon 2001, Thompson et al. 2001). Most desert shrubs produce dormant seed which benefits from aging in the soil seed bank, before dormancy is alleviated. The soil storage requirement varies between species and ranges from a few months to several years (Baskin & Baskin 1998, Gutterman 1993).

In most arid environments, the majority of the soil seed bank extends from the soil surface to a depth of 2 cm and only very few species, generally the larger seeded ones, are capable of germinating from greater depths (Gutterman 1993, Kigel 1995). The benefits of soil burial are many fold: burial induces and maintains secondary dormancy,

it protects the seed from the inhibitory effects of light and predation, it provides a less stressful germination environment compared to the soil surface, and maximises the contact between seed and soil (Gutterman 1993, Bewley & Black 1994, Kigel 1995, Baskin & Baskin 1998).

Most studies of seed longevity of desert species have been conducted on seeds stored under ambient room conditions. Extrapolation to field conditions has been problematic. Ideally, longevity needs to be assessed in long-term burial experiments. Processes occurring during long-term burial include changes in dormancy status, *in situ* germination, death due to aging, or high moisture or relative humidity, death due to soil organisms (animals, fungi, bacteria), or fire (Meney et al. 1994, Harrington & Driver 1995, Baskin & Baskin 1998, Marone et al. 2000a). These factors do not impact on seed stored *ex situ*, where the environment can be controlled.

The germination studies conducted on *H. exilis* in Chapter 4 indicate that the species probably forms a persistent seed bank. This is typical for most desert shrubs, with few exceptions. Over 22 genera in the Lamiaceae with over 29 species are known to form persistent seed banks (Schutz & Milberg 1997, Baskin & Baskin 1998).

The first part of this study investigated seed dormancy following prolonged soil burial of two genetic provenances of *H. exilis*. Additionally, seeds from the southern genetic provenance were assessed for changes in seed coat morphology and strength. I hypothesised that firstly, dormancy is alleviated over time and that germination increases with duration of burial. Secondly, the two provenances differ in their responses to burial. Thirdly, the mechanical restrictions imposed by the seed coat may be weakened, thus alleviating mechanical dormancy.

In the second part of the study, the germinable seed bank taken from the field was quantified to determine the impact of soil storage on germination. Fresh soil samples included several generations of seeds, consisting of both transient and persistent seed banks (Baskin & Baskin 1998). It was expected that more *H. exilis* seedlings emerge from soil seed bank samples stored for 12 months than from fresh samples, similar to the trend observed in the seed burial study. Conversely, prolonged storage of soil might impact negatively on germination of seeds with shorter longevity. We also hypothesised

that *H. exilis* seed is gravity dispersed, that is, most seedlings should emerge from soil samples proximal to mother plants.

Material and Methods

Experiment 1: Responses of *Hemigenia exilis* to prolonged soil burial

Mature *H. exilis* seeds were harvested prior to their dispersal in November 1997 from two sites in the northeast Goldfields of Western Australia, site E1 (121°47' east / 28°57' south) representing the southern genetic provenance, and C1 (121°07' east / 28°22' south) representing the northern provenance. Seed was after-ripened for five months at room temperature until burial. Viability was determined using a flotation test, as described in Chapter 4.

Fifty filled seeds were placed with two tablespoon of washed silica sand in nylon mesh (0.1 mm) sachets (5x5 cm), resulting in 30 sachets, similar to the method employed by Tieu et al. (2001). All sachets were buried ca. 2 cm deep and ca. 50 cm apart in dry soil at the respective collection sites in autumn. Four to five sachets/replicates were retrieved after 3, 6, 12, 18, 24 and 30 months of burial. At site C1, no sachets could be retrieved at 30 months of burial, as animals had disturbed the experimental site.

Experiment 1.1: Changes in germination in response to soil burial and genetic provenance

Seeds from each retrieval were bulked, sterilised (Meney & Dixon 1988) and tested for germination in the absence and presence of GA_3 (30 µM). Seeds (20-25) were placed on moist sterile filter paper in petri dishes, incubated under dark conditions at 20°C and monitored weekly for germination over 6 months. There were 4-5 replicates, depending on the amount of seed available. Seeds that had not germinated were dissected to assess viability.

Viability adjusted germination percentages (VAG =raw germination % / viability, Roche et al. 1997) were arcsin transformed prior to statistical analysis by 3-way ANOVA, to investigate the significance of provenance, duration of burial and GA_3 on germination. Viability adjusted germination percentages are given in the figures.

Experiment 1.2: Changes in seed coat morphology during soil burial

Changes in seed coat morphology during soil burial were documented by field emission scanning electron microscopy (FESEM). From each retrieval at site E1, 10 seeds were placed on carbon conductive adhesive tape and evaporatively coated with carbon and gold. Samples were examined with the JEOL 6300F FESEM under an accelerating voltage of 3 kV.

Experiment 1.3: Changes in seed coat strength in response to soil burial

Fresh seeds, 1.5 and 2.5 year old seeds from the burial of site E1 were tested with a compression-testing device, Instron 4301, for determination of seed coat strength. Fifteen seeds of each age class were compressed at 2 mm per minute and the force (N) fracturing the seed coat was recorded. Data were analysed using 1-way ANOVA to test for significance of aging on seed coat strength.

Experiment 2: Soil seed bank study

In late summer (March) 1999 soil samples containing the natural seed bank, measuring 20 cm x 30 cm in area and 3 cm in depth (1800 cm^3), were collected at site C1 from around 4 bushes of *H. exilis*. Four samples per bush were taken from within the canopy, 4 samples each at a distance of 1 m, and 4 samples at a distance of 3 m, totaling 48 samples.

One half of each sample was stored for 12 months in the laboratory prior to planting out in April 2000; the other half was planted out immediately in April 1999. One half of each subsample was spread onto seedling trays containing germination mix (1:1:1 sawdust : sand : peat) and watered once with 1 L of 30 μM GA_3 solution. The other half of each subsample was spread and watered without GA_3. At each planting out period there were 96 seedling trays. Trays were incubated in an unheated glasshouse and were watered to prevent drying out. Seedling emergence was assessed weekly to fortnightly over a 10-month period. Seedlings were grouped into *H. exilis*, other perennial species and annual species. Where possible, each seedling was identified down to the species level. Germination was extrapolated to a $1m^2$ area.

Results

Experiment 1.1: Changes in germination in response to soil burial and genetic provenance

At both field sites, no seedlings or observable signs of germination were recorded in the sachets under natural conditions during the 30-month duration of the experiment.

At the southern site E1, prolonged burial increased germination in the greenhouse, to 22% within 30 months (P=0.027) in the absence of GA_3. Addition of GA_3 increased germination of seed stored for 12 months, but had no promotive effect on seeds stored for longer periods (Fig. 1a).

At the northern site C1, burial of seed significantly increased germination in the greenhouse to nearly 30% within 24 months (P<0.001). Addition of GA_3 increased germination of seeds buried for up to 3 months, but had no overall effect (P=0.08) on germination, and led to an average germination of 19% regardless of duration of burial (Fig. 1b).

Overall, the germination responses of the two provenances to soil storage and GA_3 were similar. Viability remained high during the 24-30 months burial, ranging from 90% to 100%. Most of the germination was completed during the first two months of incubation under laboratory conditions.

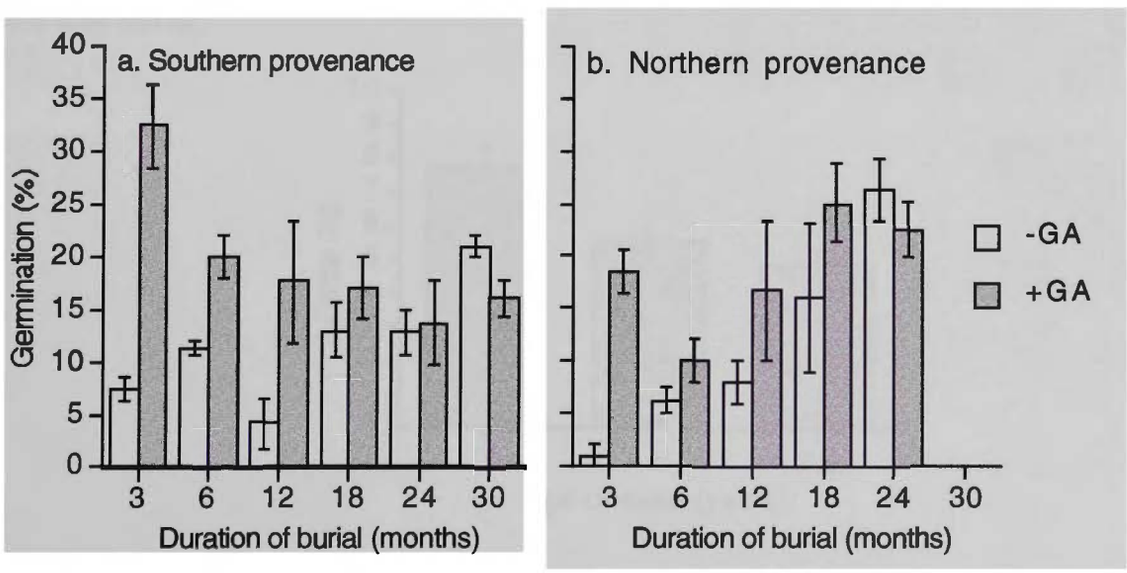

Fig. 1: Mean germination (±s.e.) of *Hemigenia exilis* from the seed burial experiment dependent on genetic provenance, duration of soil burial and GA_3. a: southern provenance E1. b: northern provenance C1 (data for 30 months not available due to predation).

Experiment 1.2: Changes in seed coat morphology during soil burial

Freshly picked seed could not be imaged due to the high oil or wax content interfering with the coating process. Shelf storage at room temperature for six weeks was required before scanning electron microscopy was possible. The seed coat of shelf-stored seed was smooth on the dorsal side, with polygonal depressions up to 200 μm in diameter (Fig. 2.1 A, 2.2A), and dome-like protrusions up to 10 μm in diameter (Fig. 2.2 B). The ventral side consisted of a lid-like elaiosome structure, which appeared rich in oil (Fig. 2.2 B). Only minor changes in seed coat morphology occurred over the first 6 months of burial (Fig. 2.1 C, D; Fig. 2.2 C, D).

Major abrasion occurred following 12 and 24 months of soil burial. The ridges separating the depressions became flatter, the topography of the dome-like protrusions became less pronounced, and layers exfoliated from the surface progressively. Furthermore, fissures appeared on the dorsal side and at the interface between ventral lid and the dorsal seed coat, and fungal hyphae invaded (Fig. 2.1 E-H, Fig. 2.2 E-H). The finer features of the seed coat became less discernible, but the general shape of the seed was preserved.

Experiment 1.3: Changes in seed coat strength in response to soil burial

Strength of the seed coat was reduced significantly ($P<0.001$) with prolonged soil burial, indicating reduced mechanical resistance to germination (Fig. 3). The pressure required for fracturing the seed coat was reduced by approximately 40% during the 2.5 year soil burial.

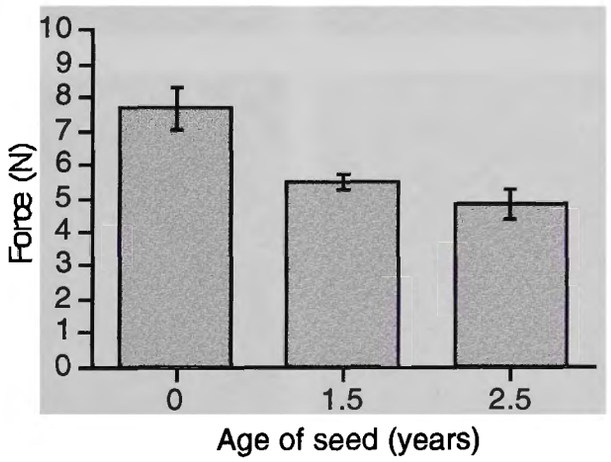

Fig. 3: Effect of prolonged soil burial on force (N) required to fracture the seed coat of *Hemigenia exilis* (mean N ±s.e.).

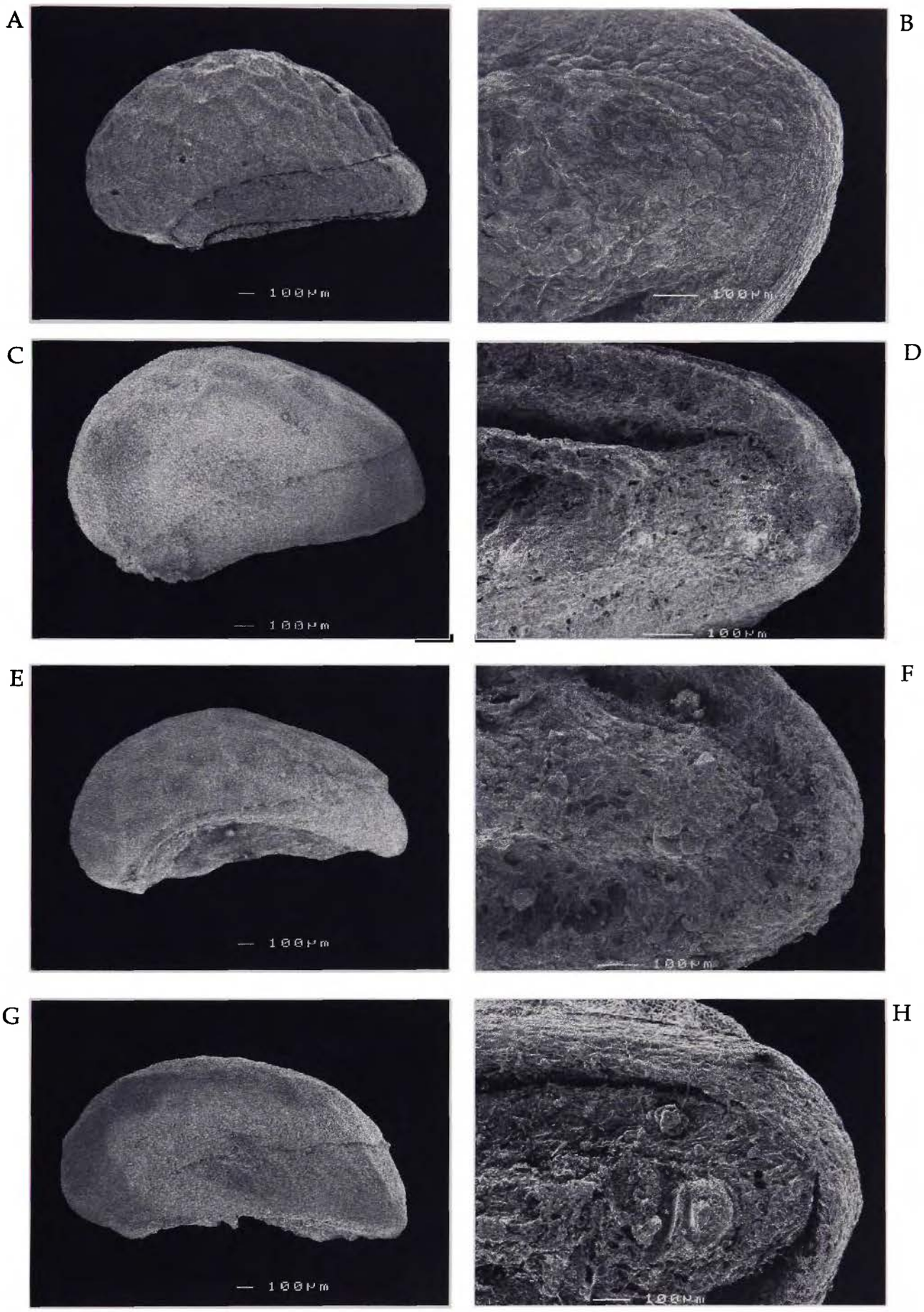

Fig. 2.1: Scanning electron micrographs of *Hemigenia exilis* seed buried for 0 (A,B), 6 (C,D), 12 (E,F) and 24 months (G,H). Overview images are on the left, and detailed images of the tip of the seed are on the right.

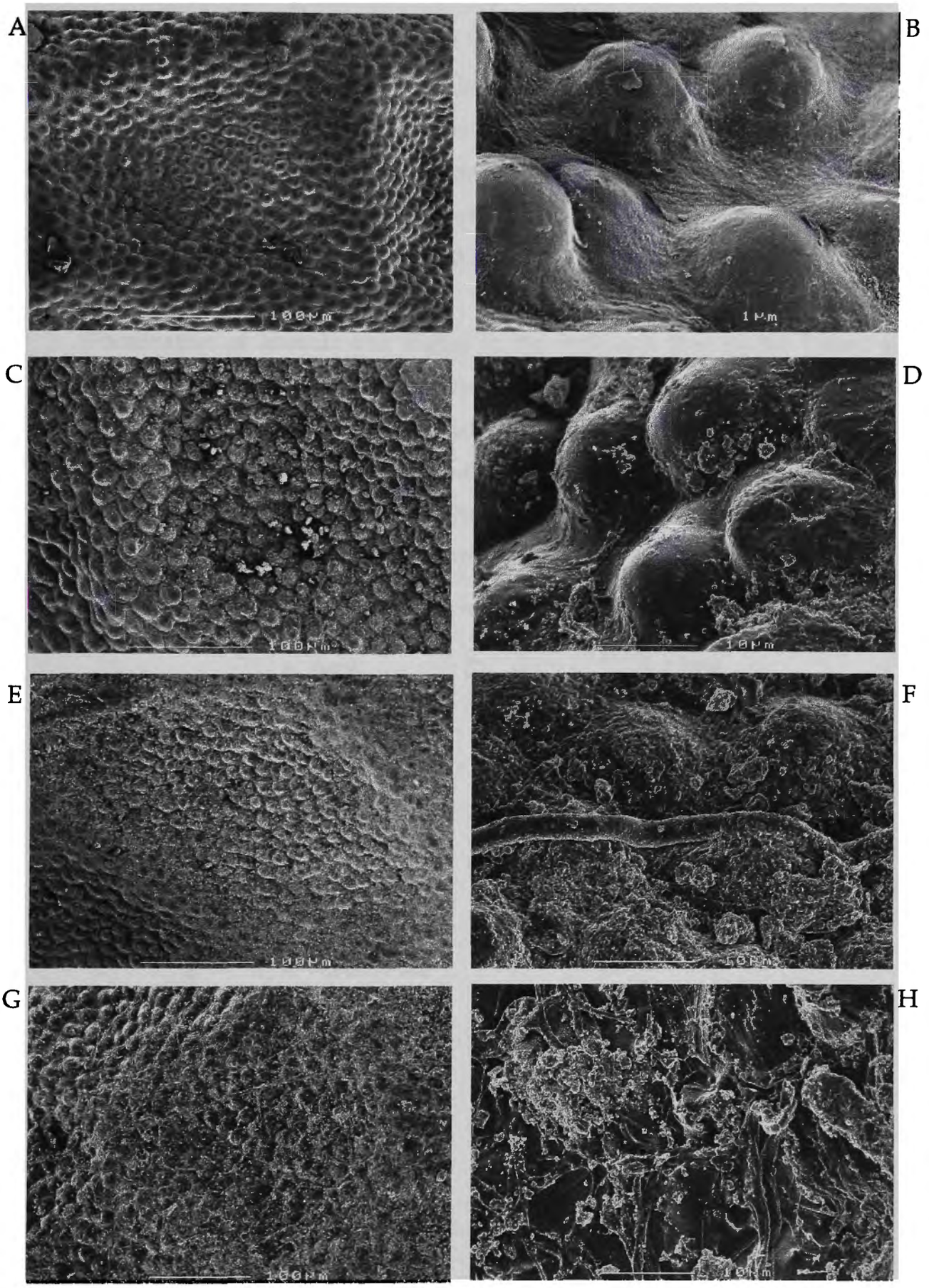

Fig. 2.2: Scanning electron micrographs of *Hemigenia exilis* seedburied for 0 (A,B), 6 (C,D), 12 (E,F) and 24 (G,H) months. Images of an entire depression on the seed coat are on the left, and detailed images of 4 protrusions are on the right.

Experiment 2: Soil seed bank study

The only perennial germinants observed during the 10-month period were *H. exilis*. Most of the germination occurred in the first 15 weeks, that is during winter (Fig. 4). More *H. exilis* seedlings emerged in the 1-year old soil (up to 101 seeds m^{-2} on average) than in the fresh soil (up to 15 seeds m^{-2} on average) (P=0.044). The number of seedlings was higher in samples collected within the canopy of the mother plant than at distance (P=0.005). Unlike in the soil burial experiment, addition of GA_3 did not increase germination of *H. exilis* (P=0.85).

Germination of annuals was higher in the fresh soil than in the 1-year old soil (P=0.035), and more germinants were recorded in soil samples closer to a *H. exilis* shrub (P=0.004). Annuals included species belonging to the Asteraceae (*Angianthus*, *Cephalipterum*, *Myriocephalum*, *Podotheca*, *Waitzia*), Portulacaceae (*Calandrinia polyandra*), Chenopodiaceae (*Dysphania*), Amaranthaceae (*Ptilotus*) and Poaceae. Addition of GA_3 had no effect on germination of annuals (P=0.33).

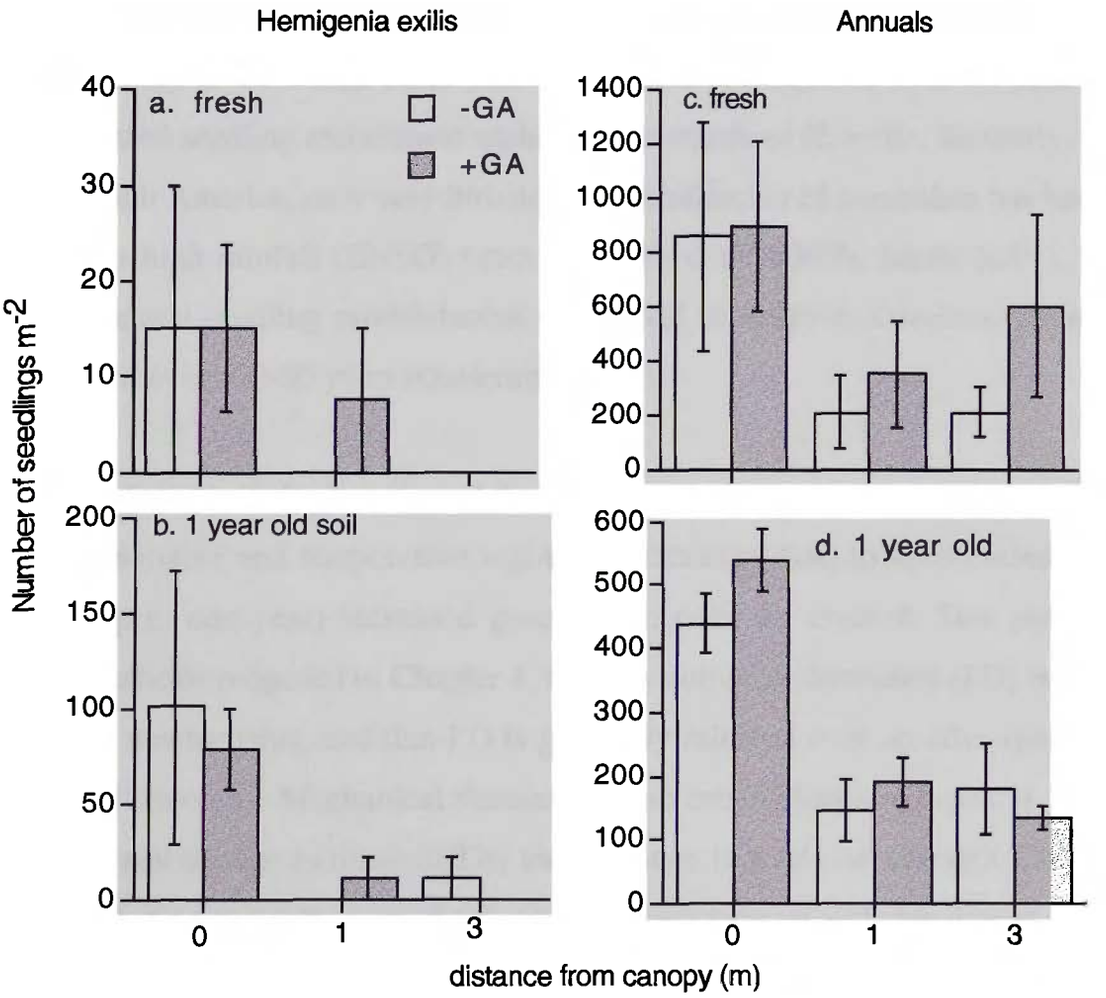

Fig. 4: Germination (seedling m^{-2}±s.e.) of *Hemigenia exilis* in fresh (a) and 1 year old soil (b), and annuals in fresh (c) and stored (d) soil in response to distance from a mother plant and GA_3 (note different scales on y-axes)

Discussion

Seed burial experiment

Prolonged seed burial in soil under natural conditions increased germination of *H. exilis* under laboratory conditions, indicating that dormancy was alleviated over time and by exposure to environmental factors such as temperature fluctuations, wetting/drying cycles and seed-soil interaction. Germination of the southern provenance increased 3-fold over the 30 month period, while germination of seeds from the northern provenance increased 8-fold within 24 months. The seeds have an after-ripening requirement of at least 12 months, which can be met by addition of GA_3. The requirement is more pronounced in the northern provenance than in the southern provenance.

The absence of seedlings in the burial experiment at both field sites may be attributed to unfavourable natural conditions (despite seasons with above average rainfall), which maintained dormancy in the buried seed. This observation is congruent with the lack of any observed natural seedling recruitment under or near stands of *H. exilis*. Similarly, in the deserts of South America, only very little natural germination of perennials has been observed even in high rainfall (ENSO) years (Marone et al. 2000b, Jaksic 2001). In another study, natural seedling establishment of the arid zone shrub *Artemisia sieberi* was only observed every 20-30 years (Gutterman 1993).

Germination of *H. exilis* took place over 2-3 months, even when seeds were incubated at a conducive moisture and temperature regime. Addition of GA_3 to seed buried for short periods (up to one year) increased germination over the control. This pattern supports the hypothesis proposed in Chapter 4, that physiological dormancy (PD) is one of the dormancy mechanisms, and that PD is gradually relieved over an after-rpening period of up to 12 months. Mechanical dormancy, also established in Chapter 4, was alleviated during soil storage as illustrated by the reduction in seed coat strength. One of the causes contributing to weakening of the seed coat was erosion and abrasion of seed surface features, leading to thinning and cracking of the seed coat. Activity of microorganisms might also have contributed by accelerating the erosional influences. The progressive morphological degradation concurred with enhanced germination. Long-term soil storage resulting in seed coat erosion and gradual dormancy relief has

been reported for a range of species from mediterranean environments (Tieu & Egerton-Warburton 2000), although Baskin & Baskin (2000) refute that soil-microbial interaction and / or abrasion by soil particles makes evolutionary sense.

Prolonged dormancy and gradual dormancy release, often over protracted periods of time, are typical of desert species forming persistent soil seed banks. Persistent seed banks occur in many plant families including Lamiaceae, and in many habitats, including deserts (Baskin & Baskin 1998). Similarly, *H. exilis* produced a persistent soil seed bank as demonstrated by the increased number of seedlings germinating from soil samples stored for one year.

In this study, dormancy relief as a result of long-term burial was faster in the northern (ca. 30% germination after 24 months) than southern provenance (ca. 15% after 24 months). This phenomenon may be an expression of a habitat-correlated pattern, which is exhibited by many widespread desert perennials (Meyer et al. 1995, Baskin & Baskin 1998).

High temperatures and fluctuations experienced by buried seed over successive summers may have alleviated dormancy in *H. exilis*, from an initially low level of 2%-8% to 20%-30%. Diurnal temperature fluctuations near the soil surface measured at the field site in summer ranged from 73°C to 20°C (Fig. 4 in Chapter 1). Many desert species require after-ripening at naturally high temperatures before germination (Gutterman 1993, Baskin & Baskin 1998). The duration of after-ripening ranges from a few months for many species, to more than one year for desert perennials including *Lesquerella fendleri* (Hyatt et al. 1999, Cabin & Marshall 2000) and *Atriplex confertifolia* (Meyer et al. 1998), to a minimum of 4 years in the case of *Mesembryanthemum nodiflorum* (Gutterman 1993). An annual germination cycle characteristic of many desert plants (Gutterman 1993, Bewley & Black 1994, Hyatt et al. 1999) was not observed in *H. exilis*, despite retrieving and germinating seed at spring and autumn times during the 2.5-year period.

The temporal spacing of germination demonstrated by *H. exilis* will provide multiple opportunities, which, in an unpredictable desert environment, ensures survival of at least some cohorts of any one generation (Rice & Dyer 1994). Heat induced dormancy relief accumulated during summer facilitates germination in cooler seasons when soil

moisture is less limiting. This bet-hedging strategy is in contrast to opportunistic seedling emergence, which is adopted predominantly by annual species that germinate *en masse* even after a single rainfall event (Baskin & Baskin 1998).

The seed burial study recorded a maximum of ca. 30% germination of *H. exilis*, and no decline in viability as a result of soil burial over two years. Consequently, 70% of seed remains viable but dormant in the soil seed bank. The long viability, which is maintained by the dry desert conditions, would contribute to the survival of *H. exilis* under the extreme and unpredictable environment in which the species occurs. The persistent seed bank also functions as 'evolutionary memory' and increases genetic variability (Gutterman 1993, Cabin 1996, Turelli et al. 2001).

Natural soil seed bank

Dry storage of the soil seed bank for 12 months resulted in a 6-fold increase of *H. exilis* seedlings. The low numbers of germinants from the fresh soil seed bank indicate a large proportion of dormant, approximately one year old seed. In contrast, storage reduced numbers of annual seedlings by ca. 1/3, representing the transient soil seed bank. The compression of germination of *H. exilis* from the seed bank into the first few months is contrasted by the protracted germination of harvested seed that was after-ripened at room temperature occurring over more than 5 months (Fig. 1 in Chapter 4). The difference in germination period might be a consequence of the different conditions experienced during after-ripening. In the soil seed bank, seeds experienced high temperatures and fluctuations, which might alleviate dormancy rapidly, whereas seed from the experiment in Chapter 4 was after-ripened at constant room temperature, leading to a gradual release of dormancy. Also, the lack of response to GA_3 by *H. exilis* seeds from both the fresh and stored soil seed bank is in contrast to the germination-enhancing effect observed in the seeds buried for up to 12 months.

Soil samples were taken at the end of summer, after seed dispersal but before the first germination season was completed. Consequently the samples contained several generations of seed produced over the last few years, including the current season's crop. Many seed bank studies have sampled post-seed dispersal/pre-first germination season, an approach that has come under criticism as these samples contain both transient and persistent seed banks (Baskin & Baskin 1998). This criticism is addressed

in this study by comparing the germinable soil seed bank of fresh seed bank samples and samples stored for one year. Differences in seedling numbers between fresh and stored soil seed bank samples quantified the transient seed bank for annuals.

No perennials other than *H. exilis* germinated from the natural soil seed bank, although other species, including several *Acacia, Eremophila, Ptilotus* species and *Scaevola spinescens*, are prominently represented at the field site. Absence of the other species, which in contrast to *H. exilis* produce large or fleshy seeds (except for *Ptilotus*), could be attributed to the lack of dispersal combined with seed predation, to which large seed is more vulnerable than small seed (Hulme 1998, Gutterman 1993, Thompson et al. 2001). Also, in arid environments, the vegetation is generally sparse and larger seeds are rare (Guo et al. 1999). Similarly, there was little congruence between seed bank species composition and standing vegetation of a Saudi Arabian soil seed bank study (Assaeed & al-Doss 2002), while variable patterns were observed in *Lesquerella fendleri* habitats in a New Mexico desert shrubland (Cabin & Marshall 2000).

Most *H. exilis* germinants emerged from samples below the canopy of the mother plant, indicating that the seed is not transported at distance. This topochorous dispersal mechanism is predominant in arid habitats, with 85% of plant species following that strategy (Gutterman 1993). Other studies have similarly shown that the majority of shrub seed in arid ecosystems is found under the protection of the canopy (Marone et al. 1998, Cabin & Marshall 2000, Pugnaire & Lazaro 2000). In contrast, Zammit & Zedler (1994) demonstrated that certain species are associated with shrub canopy, while other species, predominantly opportunists, prefer canopy gaps.

Elaiosome-like features on the ventral side of *H. exilis* seed might encourage myrmecochory, a common dispersal mechanism of Lamiaceae, and frequently found in arid areas of Australia, especially on infertile soils (Baskin & Baskin 1998). Superficially, no ant activity was observed near *H. exilis* bushes during the course of this study. However, the presence or absence of ants would need to be substantiated in a separate study by installing pit fall traps (Anderson & MacMahon 2001). Myrmecochory as an important dispersal factor may only be preliminarily excluded.

This study quantified the germinable soil seed bank, which ranged from 1-180 seeds m^{-2} for perennials and 80-1300 seeds m^{-2} for annuals. These numbers fit within the range of

30 - 5000 seeds m^{-2} calculated by Baskin & Baskin (1998) and Cabin & Marshall (2000), but fall short of the very high estimate by Kigel (1995) of 8000-30000 seeds m^{-2} in warm deserts. The size of the soil seed bank is additionally affected by precipitation such that in wet years it might be 4-6 times greater than in dry years, with annuals contributing most in wet years (Kigel 1995, Gutierrez et al. 2000). Rainfall in the study area in the 12 months before collecting the soil samples amounted to ca. 380 mm, which is above the average of 230 mm (Bureau of Meteorology 2002). The numbers of seeds in this study therefore reflect wetter-than-average conditions experienced during the growing season.

The size of the seed bank in this study represents the "realised" seed bank, and may be an underestimate of the "potential" seed bank (Baskin & Baskin 1998). The underestimate however is minimised as the protracted germination of *Hemigenia exilis* was accommodated during the 10-month recording period. Additionally, only very few germinants were recorded beyond 4 months of observation. *H. exilis* fits the description of a type IV persistent soil seed bank (*sensu* Thompson & Grime 1979), where only a small proportion of seed germinates soon after dispersal, a large reserve of viable seeds remains ungerminated, with most of the germination taking place in autumn. The annuals form transient seed banks of types I or II, which germinate in autumn or spring and leave little seed reserves behind.

In conclusion, this seed burial and natural soil seed bank study demonstrated that dormancy of *Hemigenia exilis* was partially alleviated with time. Since less than 30% of seed germinated after 2.5 years of burial, and since decline in viability was negligible, the species will produce highly persistent soil seed banks, which would enable *H. exilis* to survive in an unpredictable desert environment.

Chapter 6

Comparative ecophysiology of the arid zone endemic *Hemigenia exilis* and widespread sympatric woody species

Abstract

One of the potential explanations of rarity of the long-lived shrub *Hemigenia exilis* may be found in its ecophysiological behaviour, as rare species may have narrower physiological tolerances to environmental stresses than widespread species. To test this hypothesis, pre-dawn xylem potential (Ψ) and aspects of photosynthesis were measured under summer and winter conditions for 13 evergreen species occurring in the same arid zone plant community, including trees, short-lived and long-lived shrubs. In most species tested, xylem Ψ were higher in winter (-1.9 MPa) and lower in summer (<-2.9 MPa), except for the tree *Eucalyptus grasbyi* and the short-lived shrub *Solanum lasiophyllum*. *H. exilis* exhibited seasonal variation comparable to other long-lived shrubs. Photosynthesis followed a similar seasonal pattern, being reduced during summer (4.8 μmol m^{-2} s^{-1}) and high in winter (11.1 μmol m^{-2} s^{-1}), except for *E. grasbyi* and some short-lived shrubs. The same pattern was observed for the 13 species for stomatal conductance, averaging 55.4 mmol m^{-2} s^{-1} in summer and 151.7 mmol m^{-2} s^{-1} in winter. The factors measured failed to explain the rarity of *H. exilis*. Instead they reflected the degree of water stress experienced by the different species, indicating that most species were subject to water stress in summer, except for *E. grasbyi* which probably had access to water at depth thanks to deep tap roots, and some short-lived shrubs which were possibly able to access hydraulically lifted water from nearer the surface. The explanation for rarity of *H. exilis* may be found in other environmental variables, such as geology and mineral nutritional preferences.

Introduction

The distribution and rarity of plant species may by determined by their physiological tolerances to environmental variables, such that rare species have more narrow physiological tolerances than widespread species (Kruckeberg & Rabinowitz 1985). A rare species may be adapted to an uncommon habitat, e.g. it may be an endemic suited particularly to a stressful environment such as the serpentine soils in the arid zone of Western Australia, our study area. Often, endemic species lack the plasticity to expand into less hostile environments, where they are outcompeted by more common, generalist plants with often inherently higher rates of growth and reproduction (Baskauf & Eickmeier 1994).

In the arid zone, water is the most limiting factor determining plant distribution and growth (Evans & Ehleringer 1994). Precipitation in the study area is variable in space and time, with a long-term average of 230 mm (Bureau of Meteorology 2002). In summer, from October to April, hot and dry conditions prevail. These are interrupted occasionally by thunderstorms wetting the surface soil, and by remnant tropical cyclones passing through the area, which may (or may not) recharge the deeper soil layers. The effectiveness of these summer rains is limited by the high evaporation. In winter, a continuous sequence of anticyclones passes through the area from west to east, bringing rain-bearing frontal depressions to the region. This results in effective rains between late May to early August, which recharge the deeper soil profiles. Subsequently, summer conditions gradually become re-established (Pringle et al. 1994). Annual rainfall since 1996 ranged from 240 mm to 470 mm, with 4 years receiving predominantly summer rains and 2 year characterised by winter rains. Abiotic circumstances during extreme years may have a strong bearing on the physiological status of plants (Evans & Ehleringer 1994).

Plants in arid and semi-arid ecosystems have adapted to low water availability by developing a dimorphic root system (Canadell et al. 1996, Lambers et al. 1998). With shallow feeder roots they exploit surface water when available, while with their deep reaching tap roots, they access more reliable ground water in the lower soil profile. Desert plants are able to extract tightly held water from the soil well below the conventional wilting point for agricultural species (-1.5 MPa), and routinely produce plant water potentials ranging from –2.5 to –6.0 MPa (Nobel 1999, Schwinning &

Ehleringer 2001). Different species within a community exhibit a spatial and temporal mosaic with regards to water uptake. Exploitation of different niches, instead of competition for the same resource, enables the species' co-existence (Gebauer & Ehleringer 2000).

The water status of soil has repercussions on the plant's photosynthesis, such that under dry, hot summer conditions, water stress lowers plant water potential, reduces stomatal conductance, thereby reducing transpiration (under extreme vapour pressure deficit (VPD)). This leads to a reduction in carbon assimilation, with an increase in intercellular CO_2 concentration (c_i), thereby conserving water (but also raising temperature beyond the level optimal for photosynthesis). The consequence is an increased water use efficiency (WUE), as expressed by a relatively high ratio of carbon gain to water loss (Sandquist & Ehleringer 1998). The mechanisms triggering stomatal closure under water stress are not fully understood (Lambers et al. 1998, Nobel 1999).

Under cooler, moister winter circumstances, the water potential in the plant is higher, causing opening of the stomata and increasing g_s (under less of a VPD), carbon assimilation, while control of transpiration is less critical. Chemical factors regulating stomatal openings include the accumulation of K^+ in the guard cells via the K^+ channel, a process driven by an electric potential (Nobel 1999); blue light receptors (cryptochromes and photochromes) also mediate stomatal movement (Kinoshita et al. 2001).

The stomata of plants from arid environments have evolved an optimal strategy that maximizes carbon gain and simultaneously minimizes water loss. The correlation between g_s, photosynthesis and water use has long been established (Cowan 1977). This correlation includes synergistic interaction between light, temperature, CO_2, VPD, demand for carbohydrates, and other stimuli.

The roles of stomata have been summarized by Raven (2002) as follows: most importantly, they are responsible for optimizing carbon fixation per unit water lost (=WUE). Secondly, they maintain hydration in the plant when soil water supply is limited and/or evaporative demand is high. To a lesser extent, they prevent embolism when water supply is limited and/or VPD is high. Of minor importance are their cooling function and regulation of nutrient transport in the transpiration stream.

Ecophysiological studies comparing variation in factors associated with photosynthesis have been conducted between endemic and widespread congeners (Walters & Field 1987, Larcher et al. 1991, Baskauf & Eickmeier 1994). These studies focussed on temperate and subtropical species, and the authors found no differences in ecophysiological responses.

More relevant to this study, the seasonality of water stress and photosynthetic activity between different desert species have been analysed by a number of authors (Donovan & Ehleringer 1994, Williams & Ehleringer 1996, 2000, Evans & Ehleringer 1994, Gebauer & Ehleringer 2000, Donovan et al. 2001, Fernandez & Reynolds 2000, Berger et al. 1996, Schwinning & Ehleringer 2001). Global perspectives, including desert biomes, are presented by Reich et al. (1997), and Canadell et al. (1996). These authors generally found that there is a diversity of life forms in a plant community, including evergreen and deciduous trees and shrubs, or salt-tolerant and less tolerant species, together with a wide range in rooting depth. This diversity contributes to temporal and spatial patchwork in water use and water stress, which will affect gas exchange characteristics to variable degrees. Deep-rooted evergreen trees often have access to reliable water at depth, are least affected by water stress and may be photosynthetically active and growing throughout the year. Shallow rooted shrubs may opportunistically use surface water, but during drought reduce activity, while another form of coping with drought is being drought deciduous.

This study adds to the scarce knowledge of ecophysiological behaviour of plants in the Australian arid zone. Here, we compare aspects of plant water relations and photosynthesis of the serpentine endemic *Hemigenia exilis* with sympatric widespread woody species, and we compare different life forms, including short-lived and long-lived shrubs, and trees. Measurements were taken under summer and winter conditions, to illustrate different degrees of water stress and its consequences. It is hypothesised that xylem potential and photosynthetic activity will be lower in summer than in winter, and that the seasonal differences will be less for trees (which are generally deep-rooted) than for shrubs (which generally possess a less deep root system). The endemic *H. exilis* is expected to be more drought affected than similarly long-lived shrubs.

Materials and Methods

The study was conducted at a field site (121°07' east / 28°23' south) 70 km north of Leonora, Western Australia during 3 warm to hot dry days (32-35°C max temperature). in late October 1999 (preceded by a dry warm winter), and during 3 cool days (14-16°C) in early June 2000 (preceded by a wet summer, but no rain in May), thus contrasting summer and winter conditions. The site comprised a diverse range of plant species, and, although remote, was easily accessible. Thirteen key species common at that site were chosen. Broad- and needle-leafed *Acacia aneura* were recorded separately, to evaluate differences associated with the 2 leaf morphologies. Species were assigned to one of 3 life forms, namely short-lived shrubs, long-lived shrubs, and trees (Table 1). All species are evergreen, as opposed to drought deciduous. Only adult plants were examined.

Table 1: List of experimental species, comprising *Hemigenia exilis* and sympatric species, which are divided into three life forms. Letters in brackets are used in Fig. 1.

Trees	Long lived shrubs	Short lived shrubs
Acacia aneura (narrow leafed) (a a n)	*Acacia quadrimarginaea* (a q)	*Sida calyxhymenia* (s c)
Acacia aneura (broad leafed) (a a b)	*Eremophila compacta* (e c)	*Ptilotus obovatus* (p o)
Eucalyptus grasbyi (e g)	*Eremophila latrobei* (e l)	*Solanum lasiophyllum* (s l)
	Eremophila scoparia (e s)	
	Hakea preissii (h p)	
	Hemigenia exilis (h e)	
	Scaevola spinescens (s s)	

Pre-dawn xylem potential was measured on freshly cut stems 2 times per species per day using a stainless steel pressure chamber, to which a pressure gauge limiting maximum pressure to 4.0 MPa was attached. In summer, values exceeding 4.0 MPa were recorded as 4.1 MPa for the analysis. Standard precautionary procedures were followed to minimize experimental errors (Boyer 1995).

Photosynthetic measurements were made on portions of a single leaf in case of large leaved species, an entire single leaf in the case of medium-sized leaves, and several leaves in the case of small-leaved species. All plant material was excised from the plant

for measurement. Measurements were taken using a Li-Cor portable photosynthesis system (LI 6200) with enclosed chamber.

Block temperature was set at 20°C, photosynthetic active radiation at 1500 µmol m^{-2} s^{-1} (PAR), provided by built-in red LED light source (670 nm), and CO_2 levels were set at ambient conditions (ca. 370 µmol/mol) by scrubbing incoming air with lime and mixing in the appropriate amount of pure CO_2 from gas cartridges. Before logging the measurements, leaves were allowed to equilibrate until net assimilation was constant. Measurements were taken from one hour after sunrise for 2-3 hours, until stomata started closing. Measurements of 2 individuals per species were taken ca. 1 hour apart, on 3 consecutive days. Only young, fully expanded and sunlit leaves were used.

The leaves enclosed in the leaf chamber were excised, stored in plastic envelopes and kept cool. Upon return to the lab, leaf surface area was measured with the help of a scanner and Win Rhizo V3.9 software (Regent Instruments, Quebec, Canada), then dried at 70°C for 3 days and weighed, to calculate specific leaf area (SLA). Stomatal conductance (g_S) and intercellular CO_2 concentration (c_i) were calculated from the leaf energy balance model.

To test for significance of differences between species and life forms, data sets for each season were analysed separately by one-way ANOVA. The significance of seasonal differences was calculated by one-way ANOVA analysing the entire data set. Correlations were calculated by simple linear regression.

Additionally, six characters, including area- and mass-based photosynthesis, stomatal conductance, internal CO2 concentration, specific leaf area and pre-dawn xylem potential, were employed in multivariate analysis using Statistica® software. Multidimensional scaling (MDS), resulting from a distance matrix based on single linkage and Euclidian distance, was chosen to illustrate clustering of species and life forms under summer and winter conditions. Of particular interest was the position of *H. exilis* in relation to sympatric long-lived shrubs.

Results

Pre-dawn xylem potential (Ψ)

Most species, except *E. grasbyi* and *S. lasiophyllum*, exhibited significantly lower pre-dawn xylem potentials (Ψ) in summer than in winter (Fig. 1a, Table 2). In summer, Ψ averaged <-2.9 MPa and minimum values for a range of species (marked by *) were beyond the range of the pressure gauge (<-4.0 MPa). The maximum of ca. –1.5 MPa was recorded for the tree *E. grasbyi* and the short-lived shrub *S. lasiophyllum*. In winter, Ψ was less negative and averaged -1.9 MPa. Values ranged from –0.5 to around –2.5 MPa for most species, with only 2 species, *E. scoparia* and *S. calyxhymenia*, reaching ca. –3.5 MPa. Ψ of *H. exilis* were comparable to some other long-lived shrubs, e.g. *Hakea preissii* and *Scaevola spinescens*. These 3 species exhibited higher than average Ψ in both seasons. The range of seasonal differences within a species was as low as 0 MPa (*S. lasiophyllum*) to >2 MPa (*E. compacta*, *E. latrobei*). Overall, Ψ was significantly positively correlated with g_s (r=0.73, $P<0.001$), area-based photosynthesis (r=0.774, $P<0.001$) and mass-based photosynthesis (r=0.643, $P<0.001$).

Specific leaf area (SLA)

SLA varied between species, ranging from as low as 1.1 m^2/kg to over 8 m^2/kg (Fig. 1b). All short-lived shrubs, but less than half of the long-lived shrubs had reduced SLA during summer drought, whereas SLA of the remaining long-lived shrubs and all trees were not affected. *H. exilis* had SLA values of ca. 4 m^2/kg in summer and 5 m^2/kg in winter, which is towards the higher end of values recorded for long-lived species. Overall, SLA was correlated with mass-based photosynthesis (r=0.679, $P<0.0011$), due to an especially strong correlation in winter.

Area-based photosynthesis

Area-based photosynthesis varied between species and seasons for all but 3 species (Fig. 1c, Table 2). In summer it ranged from ca. 1 μmol m^{-2} s^{-1} to 13 μmol m^{-2} s^{-1}, averaging 4.8 μmol m^{-2} s^{-1}. In winter, photosynthetic rate was more than twice as high and less variable, averaging 11.1μmol m^{-2} s^{-1}. *H. exilis* exhibited slightly higher photosynthetic activity but similar seasonal patterns compared to the remaining long-

lived shrubs. Very low or no gas exchange was recorded during anecdotal measurements of shrubs and *A. aneura* around midday, especially in summer. When grouped according to life form, significant seasonal differences were reflected in trees and long-lived shrubs, but not in short-lived shrubs. In winter, area based photosynthesis varied significantly between species but did not vary between the 3 life forms (Table 2, Fig. 1c). There was a consistently strong positive correlation between area-based photosynthesis and g_s (r=0.975, P<0.0001), and Ψ (r=0.775, P<0.001)

Mass-based photosynthesis

Mass-based photosynthesis (Fig. 1d) was lower in summer (15.7 nmol g^{-1} s^{-1}) and 3 times higher in winter (44.8 nmol g^{-1} s^{-1}). Rates were highly variable between all species and seasons, except *E. grasbyi*. When grouped according to life form, seasonal differences were observed in shrubs but not in trees. Increases in mass-based photosynthesis were positively correlated with g_s (r=0.769, P<0.001), SLA (r= 0.679, P<0.001), and negatively correlated with Ψ (r=-0.643, P<0.001)

Stomatal conductance (g_s)

Stomatal conductance for water vapour was significantly higher and more uniform in winter (151.7 mmol m^{-2} s^{-1}) than in summer (55.4 mmol m^{-2} s^{-1}) (Fig. 1e, Table 2). Values in winter were 1.5 to 4 times higher than in summer. Only *E. grasbyi* and *S. lasiophyllum* did not conform to this trend. Values and seasonal patterns for *H exilis* were comparable to other long-lived shrubs. In winter, there were no significant differences in g_s between species nor between life forms; in contrast in summer these differences were significant (Table 2). Stomatal conductance was correlated with area based photosynthesis (r=0.975, P<0.0001), mass-based photosynthesis (r= 0.769, P<0.001), and xylem Ψ (r=0.775, P<0.001), declining to ca. 10 mmol m^{-2} s^{-1} when xylem Ψ was< -4.0 MPa.

Intercellular CO_2 concentration (c_i)

C_i in winter averaged 209 µmol mol^{-1}; in summer, c_i was insignificantly lower at 193 µmol mol^{-1} (Fig. 1f). In winter, values did not vary between species and life forms, whereas in summer these variations were significant (Table 2). There were no

significant differences between seasons. There was no correlation between c_I and g_S. The ratio of ci/ca showed a similar pattern (data not shown). Overall, most species followed the similar seasonal trends. The exceptions were *E. grasbyi* and *S. lasiophyllum*, which were often unaffected by summer drought. The ecophysiological responses of *H. exilis* to drought were comparable to those of most other long-lived shrubs, with values often being slightly above average.

Table 2: Significance of differences in pre-dawn xylem potential(Ψ), stomatal conductance (g_S), area and mass-based photosynthesis (A m^{-2} and A g^{-1}), specific leaf area (SLA) and intercellular CO_2 concentration (c_i), between 13 arid zone species and 3 life forms measured under winter and summer conditions. *=p<0.05, **=p<0.01, ***=p<0.001

	season	Between species	Between life forms
Ψ	winter	<0.001	0.002
	summer	0.0171	0.962
	Seasonal differences	<0.001	0.008
g_S	winter	0.105	0.248
	summer	<0.001	0.018
	Seasonal differences	<0.001	0.011
A m^{-2}	winter	<0.001	0.268
	summer	<0.001	0.159
	Seasonal differences	<0.001	0.036
A g^{-1}	winter	<0.001	<0.001
	summer	<0.001	0.139
	Seasonal differences	<0.001	0.014
SLA	winter	<0.001	>0.05
	summer	<0.001	>0.05
	Seasonal differences	0.048	>0.05
c_i	winter	0.879	0.742
	summer	0.001	0.043
	Seasonal differences	0.155	0.034

Multivariate analysis (Fig. 2) reconfirmed trends inferred from Fig. 1. In summer, all long-lived shrubs formed a tight cluster together with one short-lived shrub and one tree. *Hemigenia exilis* is at the centre of the summer cluster. The remaining trees and short-lived shrubs form a loose separate "cluster". In winter, clustering is less pronounced, and again *H. exilis* is not an excentric species.

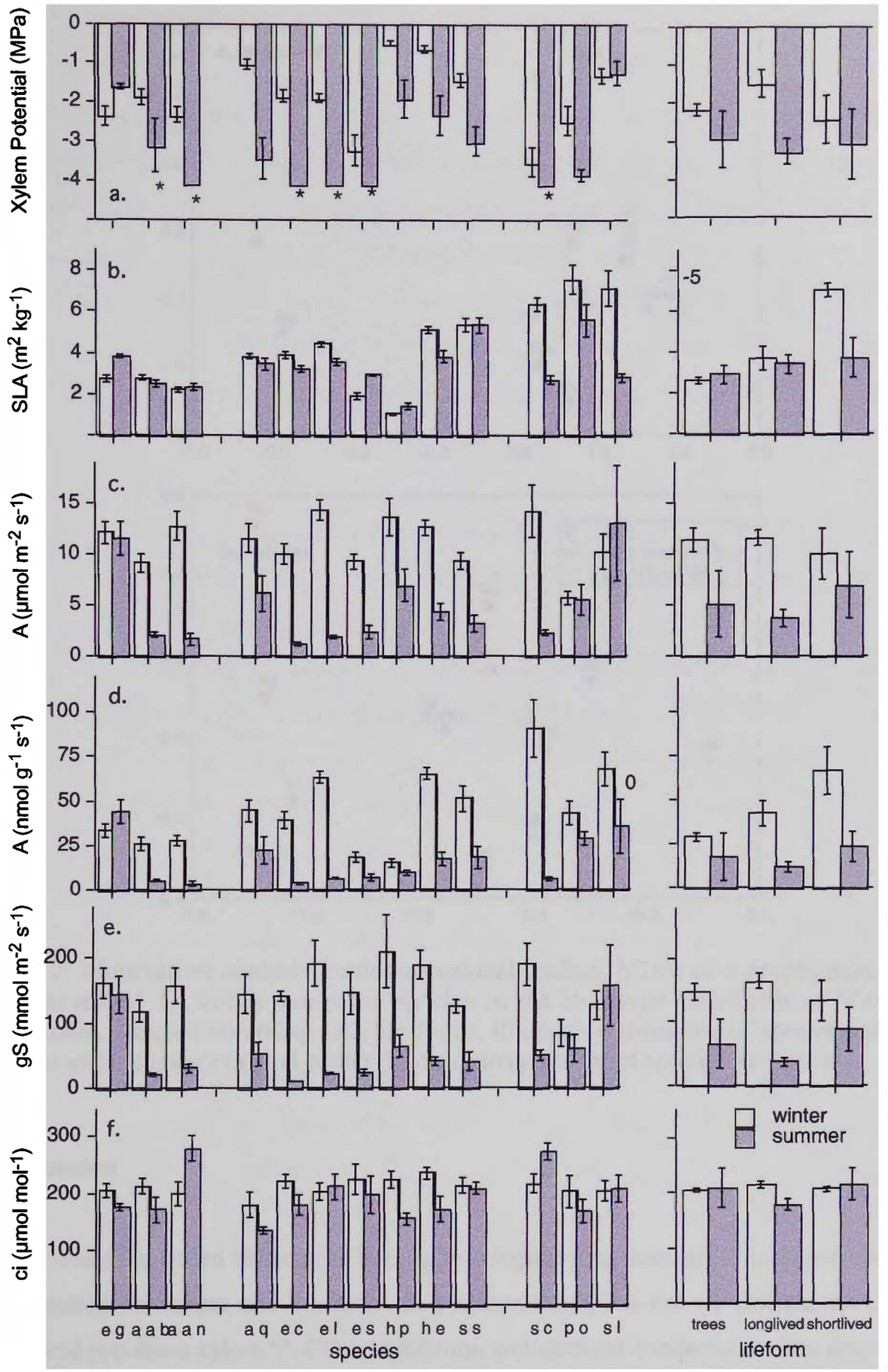

Fig. 1: Xylem potential (a), specific leaf area (b), area-based photosynthesis (c), mass-based photosynthesis (d), stomatal conductance (e) and intercellular CO_2 concentration of 13 species (left column) and their respective life forms (trees, long-lived and short-lived shrubs) (right column) growing in the Northeast Goldfields of Western Australia in winter (white) and summer (shaded). Bars represent standard errors. See Table 1 for details of species.

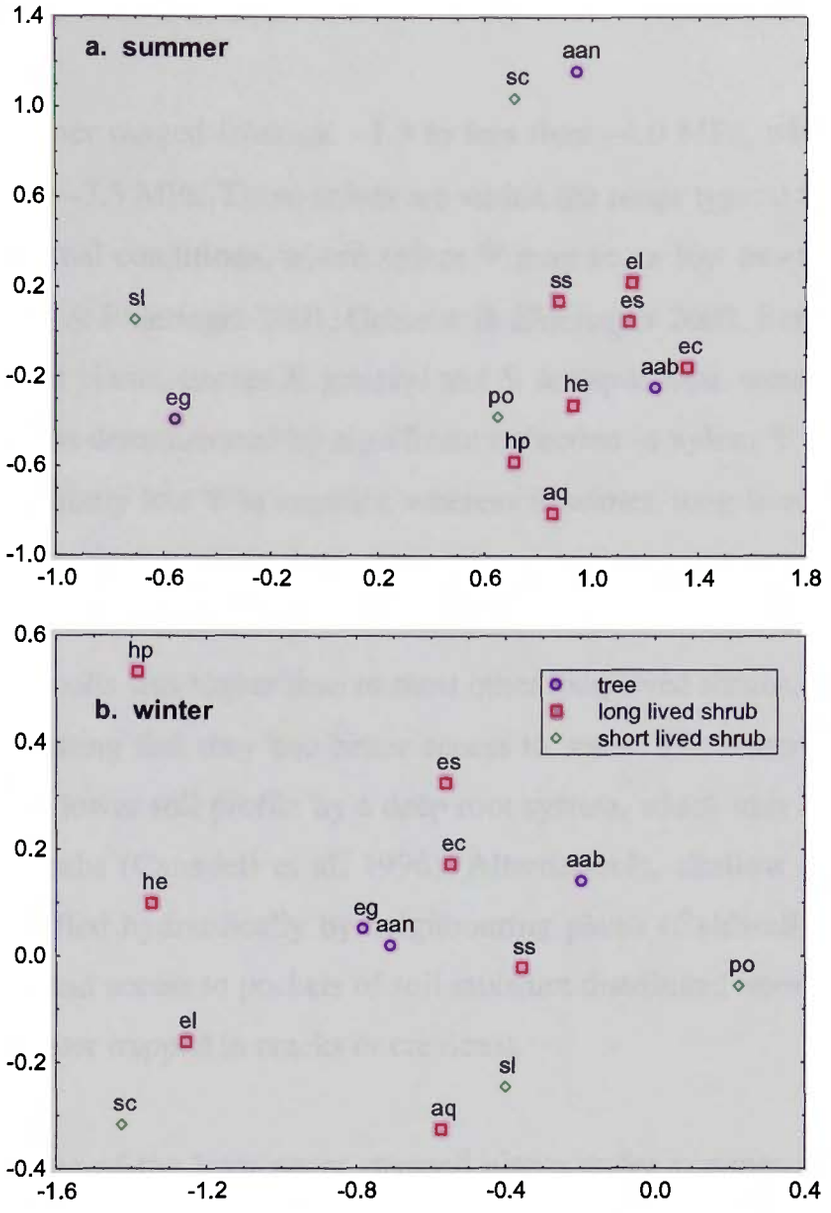

Fig. 2: Multivariate analysis (multidimensional scaling, MDS) of 6 ecophysiological characters of 13 woody sympatric species in the Northeast Goldfields of Western Australia, grouped according to 3 life forms, illustrating clustering of species and life forms under summer (a) and winter (b) conditions. Names of species see Table 1.

Discussion

This study illustrated differences in ecophysiological responses of 13 arid zone species depending on season and life form. In summer, most, but not all species, showed a reduced pre-dawn xylem Ψ, CO_2 assimilation and stomatal conductance. The degree of reduction varied with species and sometimes life form. Summer drought conditions had only little effect on the deep-rooted tree *E. grasbyi* and, unexpectedly, the short-lived shrub *S. lasiophyllum*. The endemic *H. exilis* showed reduction in physiological activity during summer, however levels were often higher than in widespread long-lived shrubs, suggesting that the species was less subjected to water stress.

Pre-dawn xylem potential (Ψ)

Xylem Ψ in summer ranged from ca. –1.3 to less than –4.0 MPa, while the range in winter was –0.5 to –3.5 MPa. These values are within the range typical for plants in arid regions under normal conditions, where xylem Ψ may be as low as –6.0 MPa (Nobel 1999, Schwinning & Ehleringer 2001, Gebauer & Ehleringer 2000, Berger et al. 1996). In this study, most plants, except *E. grasbyi* and *S. lasiophyllum*, were affected by the summer drought, as demonstrated by significant reduction in xylem Ψ in summer. All life forms had similarly low Ψ in summer, whereas in winter, long-lived shrubs had the highest Ψ.

Xylem Ψ for *H. exilis* was higher than in most other long-lived shrubs, both in summer and winter, suggesting that they had better access to water. The water may have been obtained from the lower soil profile by a deep root system, which may extend to below 6 m in desert shrubs (Canadell et al. 1996). Alternatively, shallow roots may have exploited water lifted hydraulically by neighbouring plants (Caldwell et al. 1998), or roots might have had access to pockets of soil moisture distributed unevenly throughout the soil matrix (water trapped in cracks or crevices).

E. grasbyi was one of the least water stressed plants under summer conditions. This strongly suggests that the tree was not limited by water supply and had access to groundwater, due to a deep root system. *Eucalyptus* roots are known to reach as deep as 40 m (Dell et al. 1983, Canadell et al. 1996). Little seasonal changes in xylem Ψ have been reported from other woody desert species with deep roots, e.g. *Chrysothamnus nauseosus* (Evans & Ehleringer 1994) and *Prosopis glandulosa* (de Soyza et al. 1996).

S. lasiophyllum avoids drought stress by dropping its leaves and responds quickly to small amounts of rain (Mitchell & Wilcox 1994). It is not known for a deep root system, but in this study exhibited relatively high xylem Ψ in summer and winter, and no leaf litter was observed under the canopy, despite no rain in the weeks preceding sampling. At both times, xylem Ψ was moderately negative, suggesting that the species, which is characterised by pubescent leaves, might have reduced transpiration and increased WUE, thus maintaining the demand for water from the soil relatively stable (Sandquist & Ehleringer 1998, Lambers et al. 1998). Alternatively, *S. lasiophyllum* might have

benefited from water hydraulically lifted by neighbouring plants (Caldwell et al. 1998, Ryel et al. 2002, Ishikawa & Bledsoe 2002).

Hydraulic lift is the passive movement of water from deep roots into drier superficial soil layers, and is common in arid and semi-arid regions (Caldwell et al. 1998). It can have a profound effect at the functional level on soil and plant processes (Ryel et al. 2002). Redistribution of water takes place when active root systems are spanning a gradient in soil water potential, and when resistance to water loss from roots is low. It may prolong or enhance fine-root activity by keeping the rhizosphere hydrated. Lifted water enhances availability of nutrients, which are usually concentrated towards the soil surface (Caldwell et al. 1998).

Winter xylem Ψ were similar to values measured for desert and chapparal species grown under well watered glasshouse conditions (Donovan et al. 2001). Our values of contrasting seasons were similar to xylem Ψ obtained for 6 woody species from the Sahel zone, with some species exhibiting little (Δ<0.5 MPa), and others exhibiting extreme (Δ=2.0 MPa) seasonal fluctuations to drought (Berger et al. 1996). Similar measurements were obtained studying drought affected shrubs in a cool desert (Gebauer & Ehleringer 2000), were species decreased xylem Ψ by up to 5 MPa to a minimum as low as –6.0 MPa. Summer values were routinely lower than –2.5 MPa, similar to many species in this study. In contrast, xylem Ψ for 3 desert shrubs in a cool, arid woodland with no access to the reliable lower water table were slightly higher at –3.2 MPa, possibly because shrubs lost less water due to lower VPD's typical of cooler environments (Evans & Ehleringer 1994).

The degree of seasonal variation in xylem Ψ may be a reflection of the rooting architecture of arid zone species, which have developed a dimorphic root system (Canadell et al. 1996, Lambers et al. 1998). With shallow feeder roots, plants can access seasonally available soil moisture near the surface. When that reservoir is depleted, plants that rely exclusively on shallower roots reduce their physiological activity as a result of very negative xylem Ψ, and survive drought passively. In contrast, plants that possess both feeder roots and deep-reaching tap have access to deeper soil layers that hold water reserves throughout the year. These plants remain unaffected by drought and can remain active throughout the year (Canadell et al. 1996, Lambers et al. 1998).

The differences between species in the xylem Ψ are most probably a reflection of the variation in the ability to utilize water from different soil depths and of differences in hydraulic conductivity and stomatal conductance. This variability in water relations parameters may allow the coexistence of species in an environment where soil moisture is low (Callaway 1995, Pugnaire et al. 1996, Gebauer & Ehleringer 2000).

Specific leaf area (SLA)

SLA values in this study, in concert with a low net photosynthesis, clustered towards the lower (arid) end of a global study (Reich et al. 1997), and a subsequent study focussing on the Americas (Reich et al. 1999), both encompassing 6 biomes, including deserts. Large seasonal reduction in SLA during summer drought in the present study were observed in all 3 short-lived shrubs, which all have pubescent leaves. A small decrease in SLA in response to drought was observed in only a few long-lived shrubs, including the endemic *H. exilis*. The trees were not affected by summer drought.

Differences in SLA between species may be due to variations in leaf anatomy and chemical composition (Lambers et al. 1998). Low SLA is caused by high numbers of sclerenchymatic cells, small epidermal cells, high numbers of chloroplasts, thick cell walls, high lignin content, and high concentration of secondary plant compounds. The production of dense leaf hairs over summer would also contribute. These features lead to leaves with xeromorphic character, which provide less area for efficient light capture and photosynthetic carbon gain and consequently have lower relative growth rate (RGR) than leaves from more mesic environments (Reich et al. 1997, Lambers et al. 1998, Fernandez & Reynolds 2000).

The seasonal reduction in SLA observed most prominently in the short-lived shrubs may be due to a shrinking or folding of leaves either passively due to drought (leaves may be dying), or actively to decrease the absorbed radiation by producing dense, reflective white leaf hairs, thereby decreasing leaf temperature and leaf transpiration. Alternatively, if photosynthesis still produces some carbon skeletons which however cannot be used by the plant as there is no demand for growth, starch build-up occurs, which leads to an increase in mass (Ehleringer 1995, Lambers et al. 1998).

Stomatal conductance

In this study, g_s in winter ranged from 80 to 210 mmol m^{-2} s^{-1}, whereas in summer it was reduced to 10 –160 mmol m^{-2} s^{-1}. *H. exilis* had g_s and a seasonal response similar to most other species. The values are representative of xerophytic plants with open stomata (Nobel 1999), and the higher values are close to g_{max} for evergreen warm desert shrubs (Körner 1995). Most species were more active during the early morning hours, especially in summer. Later in the morning, a decline in photosynthesis in response to stomatal closure was observed. This pattern is typical of C3 species (Lambers et al. 1998). Overall there was a threefold difference between seasons.

The values of this study are comparable to those obtained for *Juniper* and *Pinus* species from the Southwest of the USA (Williams & Ehleringer 2000), and for an endemic *Prosopis* species in arid Chile (Lehner et al. 2001). Only few warm desert perennials have higher g_s under natural field conditions, e.g. a deciduous *Quercus* species, which was explained by a high leaf specific hydraulic conductance and increase in branchwood at the expense of leaf area (Williams & Ehleringer 2000).

Stomatal conductance in this study continued, albeit at very low levels, at xylem Ψ lower than -4 MPa. In contrast, in evergreen *Juniper* and *Pinus*, stomata closed much earlier, at xylem Ψ ranging from –2.0 to –3.7 MPa (Williams & Ehleringer 2000). Most plants, which are acclimated to water deficit, close their stomata completely at water potential between –1.9 to –5.3 MPa (Raven 2002). The fact that in this study stomata operate under very negative xylem Ψ suggests a high degree of adaptation to this arid environment.

When water potential in the soil is low, the water potential of the leaf and the xylem decreases. Most species respond by closing their stomata to a degree, thereby reducing g_s. This reduced water loss is then in balance again with the low water replenishment from the soil (Ehleringer 1995, Lambers et al. 1998). As stomata control the movement of water out of the plant and CO_2 diffusion into the leaf simultaneously, a decrease in g_s leads to reduced transpiration and photosynthesis (Cowan 1977). This relationship has been substantiated in many recent studies conducted in arid ecosystems (Sandquist & Ehleringer 1998) and generally holds true for this study. Drought tolerant species have a low maximum g_s and low rates of transpiration and photosynthesis. They can withstand

lower water potential before stomata close, due to high solute concentration in live cells and high resistance to cavitation in the xylem (Lambers et al. 1998, Nobel 1999).

Further adaptations to arid conditions may include stomatal closure; stomata that are sunken into the leaf undersurface; open stomata occupying less of the lower leaf surface; and pore depth may be great (Nobel 1999). These factors lead to a reduced g_s, which has increased WUE as a corollary (Lambers et al. 1998), especially when photosynthesis is high (Gebauer & Ehleringer 2000).

Photosynthesis

Photosynthesis of species in this study ranged from 1 – 15 μmol $m^{-2}s^{-1}$ or 5 – 90 nmol $g^{-1}s^{-1}$. *H. exilis* had higher photosynthetic activity than most other long-lived shrubs, indicating that it was less drought affected. Levels of photosynthesis here fall within the lower range in the global context (Reich et al. 1997), and are typical for evergreen arid zone species (Williams & Ehleringer 2000).

All plants, except *E. grasbyi* which probably has access to ground water, had significantly reduced photosynthesis during summer. Low rates of photosynthesis are a result of a range of factors: water stress, high temperature, low demand for carbohydrates for growth, low g_s, plus other factors difficult to isolate under natural conditions (Lambers et al. 1998, Nobel 1999, Williams & Ehleringer 2000). These factors all apply to the plants investigated in this study. Low CO_2 assimilation helps plants in arid ecosystems to persist under water deficit, by not increasing CO_2 diffusion at the risk of lethal water loss (Ehleringer 1995, Reich et al. 1997, Casper et al. 2001).

Photosynthesis is dependent on the demand for carbohydrates within the plant (growth, storage), and the supply of CO_2 via the stomata. Since the internal CO_2 concentration (c_i) did not change over the seasons, the observed reduction of photosynthetic rates in summer in most species were not due to a stomatal limitation of CO_2 supply. Presumably the decreased water supply in summer slowed down or halted plant growth, thereby lowering the demand for carbon, leading to a down regulation of photosynthetic rates.

In conclusion, most species, including the endemic *H. exilis*, exhibited similar seasonal patterns: in summer, during periods of water stress, xylem Ψ became more negative, resulting in a reduced demand for carbohydrates. Consequently photosynthetic rate decreased, and the reduction in g_s helped prevent dehydration of the plants. The only exceptions to this pattern were the tree *E. grasbyi*, which showed little signs of water stress, probably because it had access to water in the lower soil profile, and the short-lived, shallow-rooted shrub *S. lasiophyllum*, which may have had access to water hydraulically lifted. Differences between the three life forms were generally less pronounced, due to the large variation between species. Multivariate analysis confirmed that *H. exilis* is an orthodox plant, similar to other long-lived shrubs. The rarity of *H. exilis* could not be explained by differences in ecophysiological factors chosen for this study, as the species behaved similar to other long-lived shrubs.

Chapter 7

Rehabilitation of *Hemigenia exilis* after mining

Abstract

This study investigated the influence of substrates and seedling pre-conditioning on plant survival and growth for rehabilitation of the serpentine endemic *Hemigenia exilis* after nickel mining. The substrates trialed included waste rock from the ferrugineous zone applied as rock mulch (FZ), topsoil (TS) and a mixture (mix) of both. Seedlings planted into a native *H. exilis* habitat represented the control site. The treatment for seedling pre-conditioning consisted of a drench of 0.1 mM salicylic acid (SA) as an anti-stress agent. Seedlings were planted at the beginning of winter in June 1999 and irrigated during the first summer. SA-treatment did not significantly alter seedling survival, with most of plant deaths occurring within the first 3-6 months after planting. No seedlings survived at the control site after 1 year. Survival after 27 months was highest of seedlings growing in FZ material (69%±6), intermediate when grown in the topsoil (50%±8) and lowest in the mixed substrate (38%±12). Plant height in most treatments reached 100 cm - 110 cm over 2 years, except for pre-treated seedlings in the mix, which attained only 65 cm. The success of the FZ material in promoting plant survival may be attributed to higher soil penetrability allowing water to infiltrate deeper soil layers, concurrent with improved root growth. Additionally, the similarity in chemical properties between native soil and the FZ overburden might have enhanced seedling survival. Further benefits of the FZ treatment included stability against water erosion. The study demonstrated that, if seedlings are irrigated during their first summer, topsoil application may not be necessary to ensure plant establishment and that rock mulch improves rehabilitation efforts in a desert environment.

Introduction

Hemigenia exilis is a rare endemic plant species restricted to the arid northeast Goldfields (NEG) of Western Australia (Western Australian Herbarium, 2002). The plant has been found in at least 6 nickel mining areas associated with serpentine lateritic outcrops, while ca. 50 additional populations occur on serpentine outcrops which are not economically viable for mining. Several populations will be impacted in the course of mining, and it is planned to restore these populations post-mining. While the restoration ecology of common species in the NEG is known, knowledge of the restoration requirements of *H. exilis* particularly after mining and into altered substrates is not known.

Rehabilitation of waste dumps resulting from sulfidic nickel (and gold) mining has been carried out in the NEG since the mid 1980's. In Australia, the mining of lateritic nickel deposits had only become feasible in the late 1990's, as a result of new and improved metallurgical technology. Since the processing of lateritic nickel ore here is only a recent development, rehabilitation of rock waste dumps is in its infancy. Revegetation protocols developed in other countries, which mine lateritic nickel, including subtropical Cuba and New Caledonia, are unlikely to be applicable in the arid Australian context (Boulet 1998).

In the NEG, rehabilitation after mining is generally carried out by direct-return of the topsoil, which contains the native soil seed bank in the top 2-3 cm. More involved methods in rehabilitation consist of broadcasting seeds of a range of species that have been successful in colonising waste dumps as pioneer species. Often, these communities include species not native to the area (eg. *Rumex acetosa*, *Atriplex* from the more mesic south of Western Australia or overseas), and locally native species that recruit easily from seed. The range of local perennial plants typically used in rehab in the NEG is limited to commonly occurring *Acacia*, *Atriplex*, *Maireana*, *Senna* and *Eremophila* species, and some annuals (Richmond & Chinnock 1994, Mitchell & Wilcox 1994).

Rehabilitation after mining using seedlings is rarely undertaken in the arid zone, due to the high costs involved and difficulties associated with coordinating plantings with unreliable rainfall events. For rare species with limited seed resources, however, it may

be a viable alternative to directly plant greenstock, provided environmental conditions are optimised to ensure establishment of the transplants.

Successful re-introduction requires identification of suitable plant material, knowledge of a plant's preferred soil properties (chemical, physical and in the case of symbiotic plants, biological), correct preparation of the transplanting site, appropriate timing of seeding or planting, and the protection of transplant populations (Frankel et al. 1995). Generally, seedlings raised from local seed survive better than seedlings raised from non-local provenances (Knapp & Rice 1994, Meyer et al. 1995, Montalvo & Ellstrand 2000, Humphrey & Schupp 2002). Re-introduction using seedlings, rather than seed, is a high cost, high risk activity. This is especially true in arid environments with low, variable and unpredictable rainfall. Here, lack of water is considered the single most limiting resource for plant growth (Boyer 1985, Grantz et al. 1998)

Establishing seedlings in arid areas is enhanced by appropriate irrigation, but is dependent on the type of delivery, frequency and amount (Iverson & Wali, 1992, Bainbridge et al. 1995, Padgett et al. 2000). For transplanted seedlings, drip irrigation appears to be the most suitable method, as it delivers water at a controllable, slow and steady rate to the rooting zone of the target plant (Bainbridge et al. 1995, Walker & Powell 2001). In contrast, overhead sprinklers deliver water to all plants indiscriminately, and has been shown to encourage weeds. Irrigation during the first dry period is often, but not always, sufficient to ensure high rates of plant survival in subsequent years (Walker & Powell 2001). Year-round irrigation may lead to a monoculture, a the expense of a natural vegetation structure (Padgett et al. 2000). For the purpose of this chapter, drip irrigation is taken as a given.

Irrigation brings further benefits by leaching toxic substances, such as soluble compounds of heavy metal residues, away from the root zone of the young plant. Chemical properties of mining waste may be inhibitory to plants. The toxic effects of residual quantities of target metals, especially when their solubility is high under acidic pH, inhibits shoot and root growth which leads to increased drought susceptibility. Plant growth is further impacted by the low nutrient status of the materials, particularly N and P, and organic carbon (Bainbridge et al. 1995, Tongway & Ludwig 1996).

The soil moisture regime may further be ameliorated by creating furrows to trap water, ripping along the contour of waste dumps to reduce run-off and erosion, mulching with organic materials (but with the risk of introducing weeds), or by rock mulching, which creates niches for seed to be trapped and may promote condensation (Biggins et al 1985). Waste rock applied as rock mulch may used when topsoil is in short supply. The reasons for short supply may be because the topsoil exists only as a thin veneer in the natural landscape (which is frequently the situation in the NEG), or may be due to poor topsoil management. Plant establishment in a rocky environment has been variable, ranging from poor in a South African trial (Holmes 2001) to being better than topsoil in the Mojave Desert (Walker & Powell 2001).

Seedling survival may further be enhanced by treating the young plants with compounds that induce stress tolerance caused by drought. With agricultural and forestry species, this has been achieved with triazoles (Fletcher et al. 2000) and salicylic acid (Senaratna et al. 2000). In theory the physiological and biochemical mechanisms triggering stress tolerance should operate when transplanting into a particularly stressful environment.

The aim of this study was to evaluate the optimal substrate and seedling treatment prior to planting nursery grown seedlings of the rare *Hemigenia exilis*. Three types of substrate were tested, namely rock mulch from the ferrugineous zone overburden (FZ) excavated at a depth of 0–6 m, topsoil, and a 1:1 mix of both. As seedling pre-treatment, salicylic acid, a known anti-stress agent, was tested.

Materials and Methods

The east facing slope of the waste rock dump was shaped to an angle of 20° in early 1999 and prepared for rehabilitation in May 1999, after the materials had dried sufficiently from summer rainfall (125 mm in February and March 1999). Most of the slope was covered with 30 cm of dry topsoil, which had been stored for 1 year (TS treatment). A strip ca. 20 m wide was left uncovered, such that the waste rock overburden (2-20 cm diameter in size), including a small proportion of saprolite clays, from the FZ remained exposed, simulating rock mulch (FZ treatment). Another strip of same width was covered with a thin veneer of topsoil, which was incorporated into the rocky surface (TS:FZ mix treatment). All topsoil originated from low mulga shrubland

that did not support *H. exilis* in its understorey. The slope was ripped along the contours by a single tyne attached to a D9 bulldozer.

At the nursery in Kings Park Botanic Gardens, Perth, seedlings of *Hemigenia exilis* were grown in pots measuring 5 cm x 5 cm 13 cm. Seeds were picked in November 1998 from 3 populations within the southern genetic provenance, thus maintaining genetic integrity of the rehabilitated population (see Chapter 2). Seedling production was limited by the scarcity of viable seed, as optimal harvesting time was nearly missed. Seeds were germinated in early 1999. A total of 190 seedlings were produced, which reached a height of 3-5 cm until planting in June 1999 at the mine site. Three days prior to planting one half of the seedlings were drenched with 0.1 mM salicylic acid (Senaratna et al. 2000). This treatment was expected to reduce transplant stress and provide drought tolerance. The remaining half of the seedlings received untreated, potable water.

The experiment consisted of a factorial design, including four substrates (three on the rock waste dump, namely FZ, TS, mix, and one control substrate (native) in soil within the nearest native *H. exilis* population, 30 km southwest of the mine site), and two seedling pre-treatments (+/- 0.1 mM salicylic acid). There were three replicates of seven seedlings each, totalling 168 plants.

Seedlings were planted ca. 1.5 m apart and were watered with 0.5 L of potable water at the time of planting. Plants were protected from potential grazing by rabbits and kangaroos with wire mesh cages, which were removed after 9 months, as there was no evidence of these animals on the site. On the waste dump, drip-irrigation was installed within 1 month of planting and maintained over the first 12 months, to promote plant survival in the absence of rain. At the control site, seedlings were hand watered. During the first year, all plants received 1L irrigation once per week or fortnight, depending on natural rain. A control treatment without irrigation was omitted, as each seedling was deemed too valuable to be allowed to perish in case of drought. Drip-irrigation during the first dry period has been shown to improve seedling survival over non-irrigation.

Plant survival and plant height were scored after 3, 6, 10, 14 and 27 months. Flowering events were also noted, as was presence of seedlings recruiting from the soil seed bank and erosion.

At all sites, four soil samples were taken from the top 25 cm for soil chemical analysis. Samples were analysed for ammonium and nitrate, phosphorus and potassium, extractable sulphur, organic carbon reactive iron, conductivity and pH (Rayment & Higginson 1992). Nickel and Cobalt were extracted with aqua regia digest and analysed using AAS (McGrath & Cunliffe 1985). To quantify physical soil properties, *in situ* soil compaction was measured with a cone penetrometer (Rimik CP 20 Cone Penetrometer, manufactured by Rimik Agricultural Electronics) with a tip length of 23 mm and a base diameter of 12 mm. At all four sites, recordings of 10 data triplets were taken at 20 mm intervals to a potential maximum depth of 60 cm. At the time of measurements, soils were dry.

Results

Seedling survival (Fig. 1a) after 27 months ranged from 0% to 76%, depending on substrate and pre-treatment. Most of the plant deaths occurred within the first 6 months of planting. Seedlings at the control site did not survive the first summer. Thus the analysis of plant survival and growth after 27 months focuses on seedlings planted onto the waste dump at the mine site.

The type of substrate had a significant (P= 0.036) impact on survival of seedlings, which was highest at 76% in the FZ –SA treatment, and was reduced to 19% in the mix +SA treatment. Although SA appeared to influence survival, the effect was statistically not significant (P=0.28). Survival was not dependent on the position of the seedlings along the slope.

Plant height (Fig. 1b) after 27 months ranged from 65 cm to 109 cm and was significantly affected by soil type (P=0.008) and SA (P=0.016). Most of the effects were due to the reduced plant height (65 cm) in the mix +SA treatment, compared to 101 cm to 109 cm in the remaining treatments.

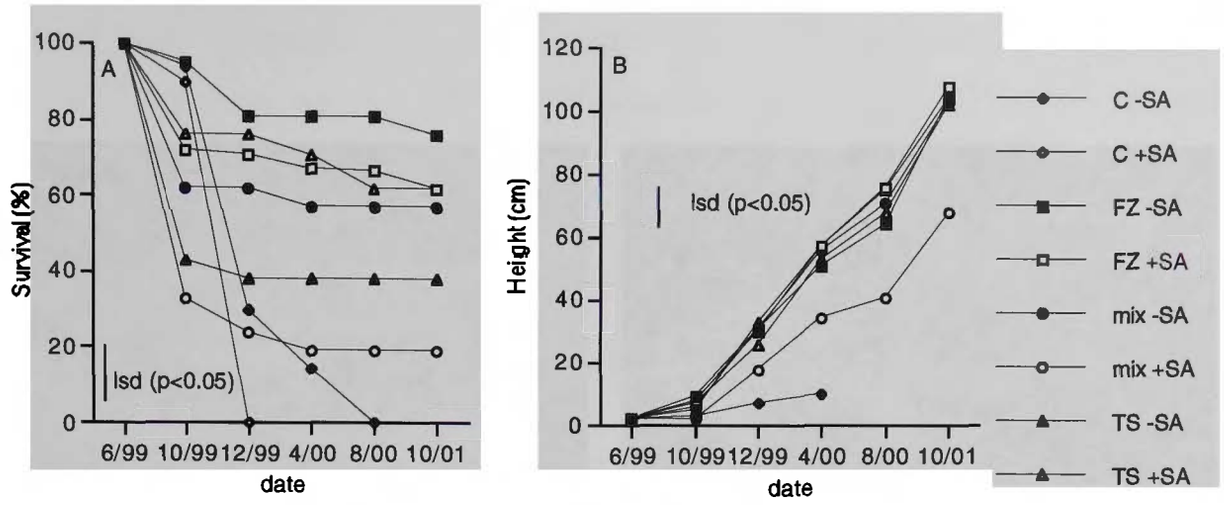

Fig. 1: Seedling survival (a) and growth (b) of *Hemigenia exilis*, planted at a control site in the native habitat (C) and on 3 substrates on a waste rock dump (FZ, mix, TS), with and without salicylic acid (+/- SA) over a 27 month period.

Heavy rains in early 2000 (100 mm in January, 80 mm in February and 185 mm in March, exceeding the mean annual rainfall of 230 mm) caused erosion, which was significantly more severe as the amount of topsoil in the trial plots increased (Table 1, Fig. 2) ($r=0.995$, $P<0.01$).

Table 1: Number and depth of erosion gullies on a lateritic nickel waste dump covered with 3 surface treatments (±se)

	FZ	Mix	TS
Number of gullies	1 (±0)	2.7 (±0.3)	4 (±0.58)
Depth of gullies (cm)	11.7 (±1.7)	38.3 (±4.4)	53.3 ± (3.3)

On the rock waste dump, seedlings of *Dysphania*, *Erodium*, *Myriocephalum*, *Velleia*, everlastings and *Atriplex* (annual species only), *Ptilotus* and *Acacia aneura* were observed in the first year, recruiting from the soil seed bank. Some *H. exilis* seedlings reached reproductive stage within one year, and most had produced seed by the second year. After 2 years there were five self-recruited *H. exilis* seedlings, which must have resulted from the seeding event, since no *H. exilis* seed would have been present in the topsoil that originated from habitats without this species.

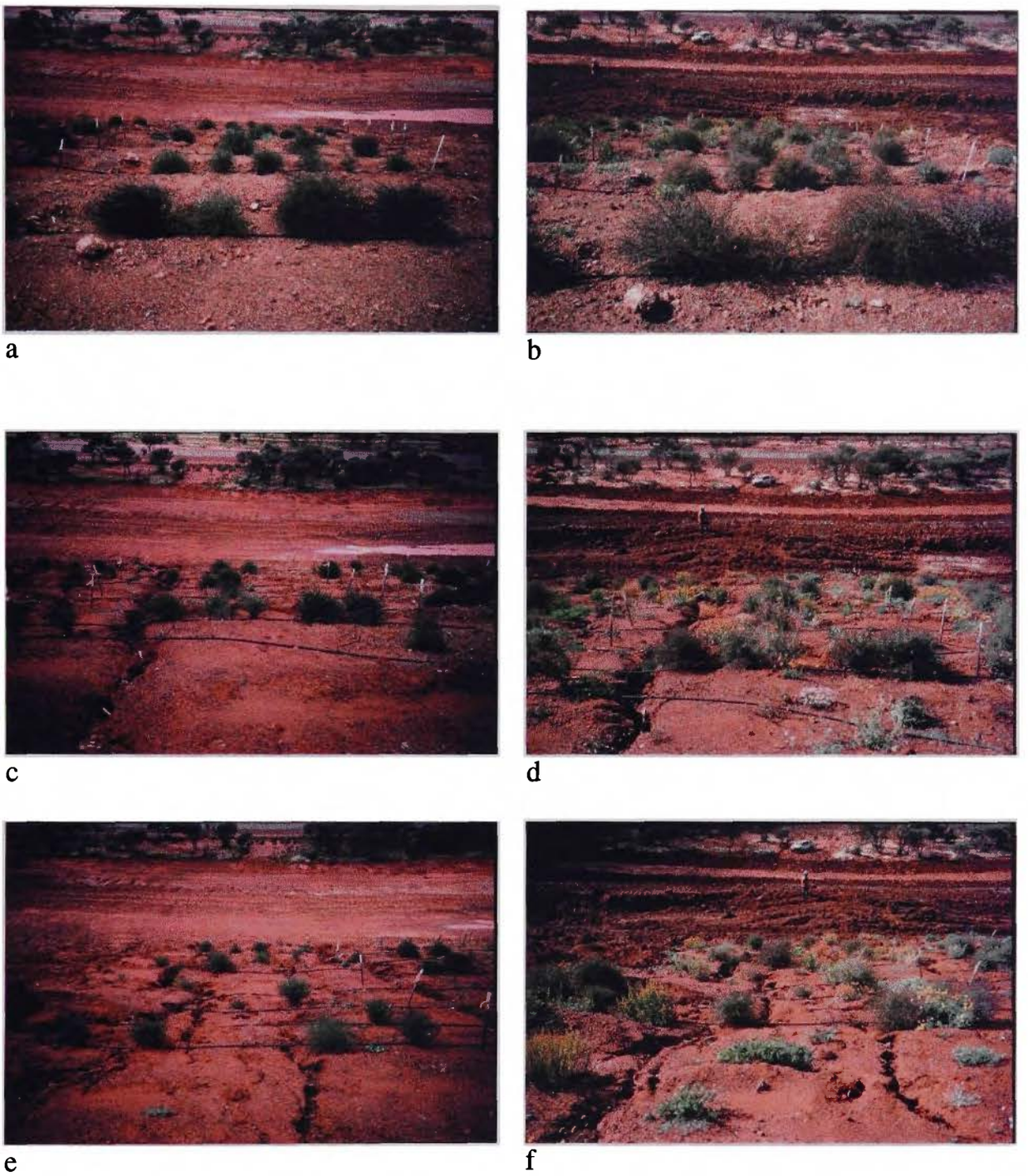

Fig 2: Rehabilitation of a rock waste dump with *Hemigenia exilis*, comparing 3 substrates FZ, (a,b); mix (c,d), TS, (e,f), in April 2000 (left column) and August 2000 (right column). This series illustrates the stability of the FZ treatment with very little erosion, compared to the mix treatment with intermediate erosion, and the TS treatment showing signs of severe erosion.

The substrates varied significantly in several chemical soil properties (Fig. 3). Amounts of Ni in FZ material were similar to those found in the native habitat, and were 4 times higher than in the mix and 10 times higher than in the topsoil (Fig. 3a). Trends were similar with regard to Co and K (Fig. 3b and e). Although FZ contained only 1/2, 1/3 to 1/10 of the amounts of nitrate and ammonium compared to the mix and topsoil, these differences were not significant (Fig. 3c). In contrast, FZ contained significantly less reactive Fe (Fig. 3g) and organic carbon (Fig. 3h). Conductivity was lower in the FZ and mix, and 3 times higher in the topsoil (Fig. 3i). The pH of the FZ was neutral to slightly alkaline, whereas in the mix and topsoil, it was slightly acidic. Despite the significant differences in several chemical characteristics between substrates, survival and plant growth were not affected by these properties ($P>0.05$).

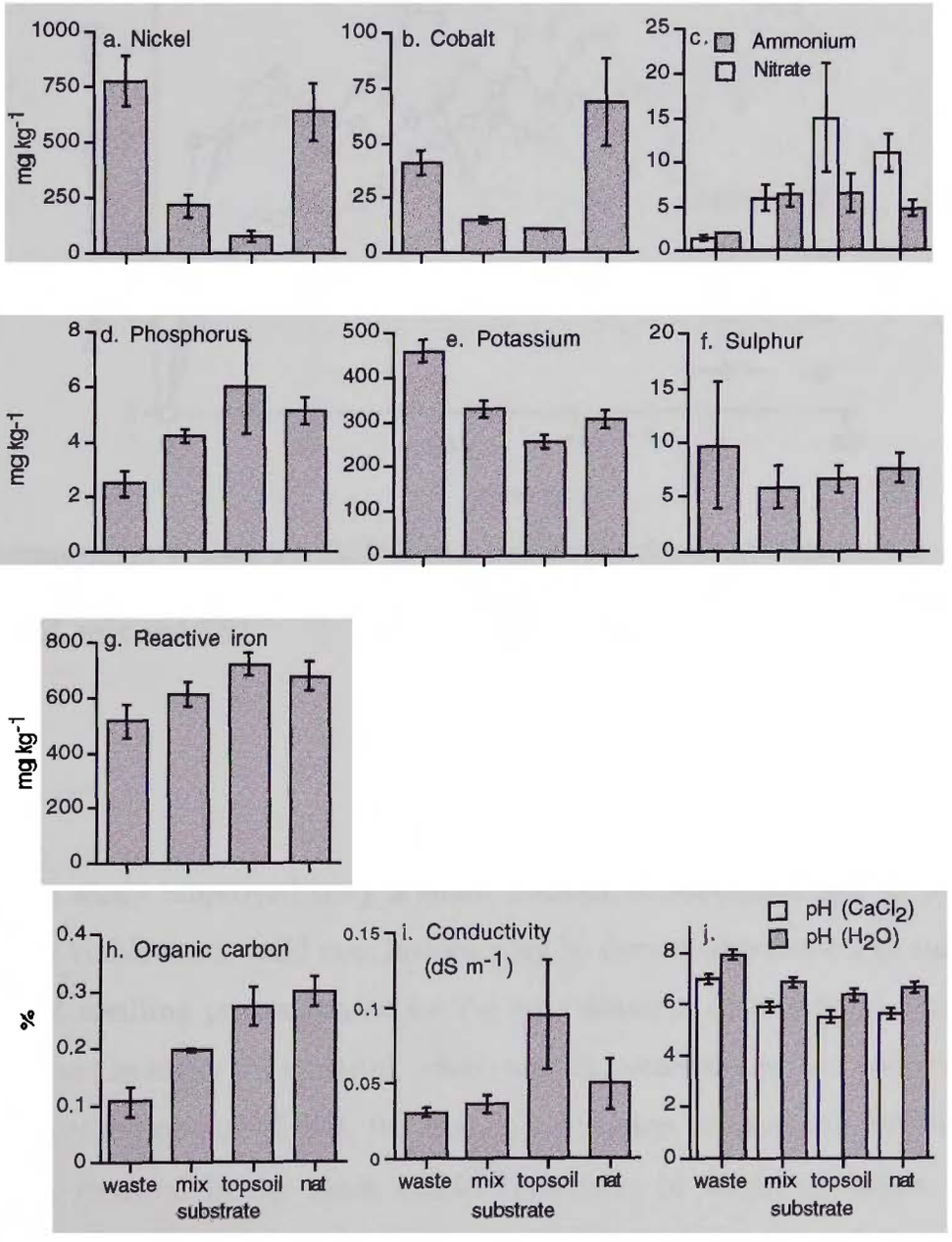

Fig. 3: Chemical soil analysis of the 3 substrates on the waste rock dump (waste, mix and topsoil) and soil in the native habitat (nat) (±s.e.).

Soil compaction (Fig. 4) varied significantly between sites ($P<0.01$). Throughout the profile, pressures were lowest in the FZ sites at 2.8 MPa (±0.2 MPa), and maximum penetrability was consistently higher, up to 48 cm. Soils from the native habitat exhibited high resistance even near the surface with an average of 3.3 MPa (±0.3 MPa) throughout the short profile), and had the lowest depth of penetrability of only 22 cm maximum. The mix and TS sites on the waste rock dump exhibited intermediate penetrability (3.13 MPa ± 0.2 MPa, 30 cm for mix; and 3.14 MPa ± 0.2 MPa, 32 cm for TS). Survival was negatively correlated with amount of soil compaction throughout the profile ($r=-0.914$, $P<0.05$) and positively correlated with maximum depth of penetration ($r=0.916$, $P<0.05$).

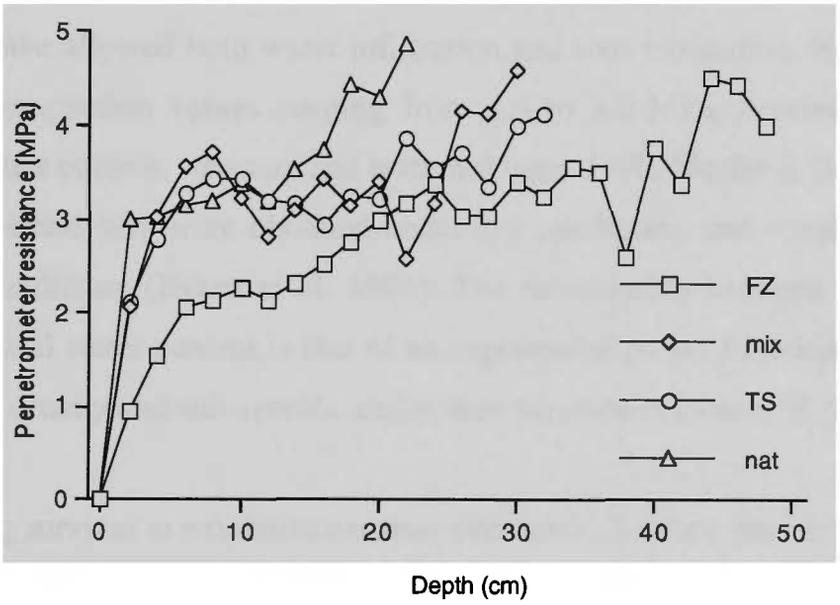

Fig. 4: Penetrometer resistance (MPa) and maximum depth of penetration (cm) in a native *Hemigenia exilis* habitat (nat), and on the rock waste dump in the 3 trial substrates (FZ, mix and TS).

Discussion

Although this study employed only a small number of seedlings, due to the limited availability of viable seed, valid conclusions may be drawn with respect to suitable soil substrates and seedling pre-treatment for the rehabilitation of *Hemigenia exilis*. Plant survival was best in rocky FZ material, which was characterised by low soil compaction and high concentrations of Nickel, but low concentration of most macronutrients. FZ material also resulted in the most stable landforms in terms of degree of gully suppression. Treating seedlings with an anti-stress compound produced no appreciable benefit and may not be required for species naturally adapted to arid conditions.

The relatively high plant survival on the waste rock dump (>50% on average) in contrast to the 100% mortality of seedlings on the control site may primarily be due to unfavourable soil physical conditions, represented by high soil compaction. In the native habitat, depth of penetrability was 22 cm at most due to the naturally occurring cemented hardpan. Values were as high as 5.0 MPa, which is not only inimical to root growth by itself (Unger 1996, Moffat & Boswell 1997), but may also have reduced hydraulic conductivity (K_{sat}) leading to low soil moisture content (Shafik et al. 1994), and causing seedling deaths.

In contrast, the significantly lower soil compaction in the top 20 cm on the rock waste dump would have allowed both water infiltration and root elongation. Root growth is restricted at compaction values ranging from 1.5 to 3.0 MPa, depending on plant species, soil water content, structure and texture (Unger 1996, Moffat & Boswell 1997). The values reported here were obtained under dry conditions, and would be reduced under moist conditions (Evans et al. 1996). The relationship between penetrometer resistance and soil water content is that of an exponential power function and depends on soil particle density and soil-specific calibration parameters (Vaz et al. 2001).

Higher seedling survival in rehabilitation sites over native habitats has been observed in mine sites, including sand mines, in a seasonally arid environment and was attributed to low impedance in the early stages of seedling establishment (Rokich 1999), and on gypsum mines in a arid environment, where it was attributed to right species selection and conducive soil properties (Rao & Tarafdar 1998).

Within the rock waste dump, the high survival of seedlings on the FZ material may be attributed to physical soil properties, as FZ had the lowest soil impedance. Alternatively, root growth in the FZ material may have been improved in the early stages of plant establishment by the heterogeneity of the substrate, allowing roots to establish in fissures and crevices. The loss of seedlings in the soil types containing topsoil indicated that soil chemical properties may also play a role. Some chemical properties of the FZ material were more similar to the native habitat than the remaining two substrates on the rock waste dump, which might be beneficial for survival and growth of *H. exilis*. The amounts of macronutrients contained in the FZ material generally lower than in the topsoiled treatments, but were similar to those found in the

natural habitat. Since higher nutrient levels in the topsoiled treatments appear to have no beneficial effect on the survival and growth of *H. exilis*, this suggests that the species may be well adapted to the nutrient poor soils typical of its habitat.

Surface rock mulch led to an increase in soil water retention on overburden dumps in the Mojave Desert (Walker & Powell 2001). Rock mulch has been shown to promote condensation, reduce water loss and promote germination. Analogous to mulch provided by tree branches (Tongway & Ludwig 1996), rock mulch increases the surface roughness and soil topography, reduces erosive sheet water flow, provides niches for litter and seed, thus creating fertile patches and resulting in a more stable land surface (Iverson & Wali 1992, Walker & Powell 2001, Chambers 2000).

On the rock waste dump, the growth rate of seedlings under drip irrigation for the first year was surprisingly high, growing from 5 cm at the time of planting to over 100 cm within 2 years. This is in contrast to the slow growth observed of the very few seedlings recruiting in two natural populations, which reached ca. 20 cm height within the same period. Similarly, small plants in the natural habitats did not produce shoot growth comparable to irrigated plants on the waste rock dump, despite 2 years of above average rainfall. These results and observations support the findings of many rehabilitation studies in the arid zone that water is the most limiting factor of plant growth and development (Sandell et al. 1986, Bainbridge et al. 1995, Grantz et al. 1998, Walker & Powell 2001).

The survival of seedlings in this study is higher than reported in comparable trials (Grantz et al. 1998, Padgett et al. 2000). The success of plant establishment on the waste rock dump may be attributed to drip irrigation (Bainbridge et al. 1995, Pelaez et al 1996, Montalvo & Ellstrand 2000, Walker & Powell 2001, Villalobos & Pelaez 2001), choice of the appropriate genotypes (Knapp & Rice 1994, Frankel et al. 1995, Meyer et al. 1995, Montalvo & Ellstrand 2000, Humphrey & Schupp 2002), innate ability to grow even at low soil water potential (Villalobos & Pelaez 2001), and absence of weeds and other seedlings competing for the same resources (Distel et al. 1996, Bertiller et al. 1996, Whitford et al. 2001, Ewing 2002). Furthermore, the second summer, when seedlings were no longer irrigated, was wetter than average, with 170 mm rain in February 2001.

Plant growth probably benefited from irrigation. Substrates with higher nutrient content did not produce bigger plants. This suggests that *Hemigenia exilis* is adapted to low nutrient levels and would not benefit from addition of mineral fertiliser. The self-recruitment of seedlings of *H. exilis*, and the establishment of other perennials including *Acacia aneura* which dominates the surrounding vegetation, may be a first indicator of a very early stage of a self-sustaining population. The rate of natural seedling recruitment might have been accelerated as a result of the two years post-rehabilitation being wetter than average.

This study demonstrated that seedlings of rare species can be established at post-mining rehabilitation sites in the arid zone with the help of irrigation, and rock mulching, if topsoil is in short supply. This outcome is particularly relevant to the NEG where topsoil is often present only as a thin veneer over bedrock and hardpan.

Chapter 8

General Discussion

This thesis presents the first comprehensive study concerning the conservation biology of an endemic long-lived shrub associated with serpentine outcrops in the arid zone of Western Australia. The findings of these investigations, in the areas of conservation genetics, germination ecology, ecophysiology and rehabilitation after mining, are considered relevant to other species with similar life forms and growing in an arid environment, and under extreme edaphic conditions.

The major findings of the study include:

1. *Hemigenia exilis* exhibits higher levels of genetic diversity than found for other endemic, long-lived perennials, and is differentiated into a northern and southern genetic provenance.
2. Phylogenetically, *H. exilis* is situated at the base of a clade comprising *Hemigenia*, *Hemiandra* and some *Westringia*, suggesting that additional analyses, both with respect to number of species and characters, are required to clarify unresolved relationships within that group.
3. The seeds of *H. exilis* are characterised by a combination of mechanical and physical dormancy, which is alleviated by removal of the seed coat - endosperm complex, resulting in 100% germination, or addition of GA_3, resulting in 41% germination.
4. Seeds benefited from soil burial, achieving 20%-30% germination after 30 months, depending on genetic provenance. Seeds responded to an after-ripening period of 12 months of dry storage, resulting in a 6-fold increase in germination performance.
5. As a consequence of protracted dormancy and long-term viability, *H. exilis* forms a persistent soil seed bank.
6. Attempts to explain the rarity of *H. exilis* by a potentially narrow ecophysiological tolerance were not conclusive, as the species exhibited seasonal patterns in xylem potential and photosynthesis similar to other sympatric long-lived shrubs.

7. After solving issues associated with genetic provenance and seed dormancy, the rehabilitation of *H. exilis* on waste rock dumps was successful, provided seedlings were irrigated during the first summer. Survival was highest in waste rock mulch from the ferrugineous zone, and was reduced by the presence of topsoil. This outcome is particularly relevant to situations when waste rock material can not be covered with topsoil due to low supply.

During the course of this study, *Hemigenia exilis* was re-classified by the Department of Conservation and Land Management from being "presumed extinct" ("taxa which have not been collected ... over the past 50 years despite thorough searching ...") to "rare" ("taxa which are considered to have been adequately surveyed and which, whilst being rare in Australia, are not currently being threatened"), as a result of extensive flora surveys since the late 1990's targeting serpentine / ultramafic outcrops. This geobotanical approach, assisted by exploration geologists with a strong botanical interest, has lead to the location of over 50 populations of *H. exilis*, ranging in size from a few individuals to >4000 plants. In the case of *H. exilis*, the conservation status was dependent on the intensity of flora surveys, which is relatively low in the expansive remote areas of arid Australia.

The importance of population genetics in the management and rehabilitation of rare species

The maintenance of genetic diversity has become a prominent focus of conservation biology. Genetic diversity encapsulates the evolutionary potential and ensures that populations can adapt to continuous environmental changes. One of the first steps in the conservation of rare species is to delineate evolutionary significant units within the species. These units are represented as genetically differentiated populations which, reflecting adaptive differences, require separate management. Knowledge of the genetic diversity and structure of a rare species will assist in managing fragmented populations, minimising loss of genetic diversity and inbreeding depression. Integration of genetic knowledge is imperative when devising a rehabilitation plan, particularly where populations are impacted by land disturbance.

This study demonstrated that with a carefully devised sampling strategy, genetic diversity can be quantified and, importantly, patterns of diversity can be detected. It also

illustrated the power of RAPDs, a relatively simple and cheap molecular technique in comparison with AFLP, to provide answers to conservation genetic questions.

The high levels of genetic diversity found in this study contradict the rule, that rare species, typically existing only small populations, are genetically impoverished. Genetic diversity in *H. exilis* was unexpectedly high. While the tenet "rare plant = small population = low diversity" generally applies (Frankham 2002), there is an increasing body of work being published, to which this study contributes, illustrating exceptions to this general rule.

The high diversity recorded for this species may be directly related to polymorphisms for seed aborting recessive equivalents, which are common in Australian arid zone species (James 2000). This genetic feature also may explain the low seed set found in *H. exilis*. These high levels of diversity may ultimately be attributable to the stressful environment exerting strong selection pressures (Linhart & Grant 1996). Population genetic studies of other *Hemigenia* species, both from the arid zone and mediterranean or temperate environments, and other Lamiaceae genera from contrasting environments, will place the high genetic diversity of *H. exilis* into context (Gitzendanner & Soltis 2000).

Genetic differentiation into a northern and southern provenance observed in *H. exilis* was significant, but weak. Analysis of additional populations, especially those situated towards the periphery of the species' distribution, may strengthen that trend, or may even reveal further differentiation. New populations are still being discovered by targeting prospective nickel-bearing serpentine outcrops.

Genetic differentiation of the northern and southern populations may warrant a phylogenetic approach to determine whether re-classification into two subspecies is valid. In contrast to the phylogenetic study at the subfamily level using the slowly evolving gene *rbcL*, elucidation of intra-specific relationships could be accomplished by sequencing a relatively fast evolving gene, e.g. chloroplast DNA *matK* or the *atpB-rbcL* intergenic region, or nuclear DNA including *adh-1*, *gapA* or *Pgi* (Soltis et al. 1999). This should be undertaken using a few individuals representing each differentiated group. At the same time, the suggestion by de Queiroz & Gauthier (1994) that

exhaustive subsidiary taxa be abandoned to construct a stable nomenclature and efficient classification, should be followed.

Phylogenetic analyses involving conspecific populations may reveal multiple lineages that would warrant protection as evolutionary distinct units. Thus phylogenetic information might assist in setting conservation priorities.

The phylogenetic relationship of *Hemigenia exilis* within the Australian subfamily Chloanthoideae (Lamiaceae)

The value of phylogenetic studies lies in the elucidation of evolutionary relationships between taxa, which subsequently help in establishing a phylogeny-based classification. Phylogenetic trees can be constructed from molecular markers or from morphological analysis. The statistical methods used in constructing phylogenetic trees yield reliable trees, provided there is sufficient information, that is, number of loci, nucleotides or amino acids, or morphological characters, or preferably a combination of these (Nei 1996).

This study is the most comprehensive phylogenetic investigation of the subfamily Chloanthoideae based on DNA sequence information to date. *H. exilis* is embedded within a *Hemigenia – Hemiandra – Microcorys – Westringia* (HHMW) clade, within which several genera are polyphyletic. The polyphyletic relationships in the HHMW clade have been established in morphometric analyses and a molecular analysis using a small number of taxa, and are reflected in this study. Similarly, relationships within *Pityrodia* remain unresolved. Comparing previous studies to the present one, topologies within unresolved groups differ only in minor detail. Also, the Australian *Teucrium racemosum* is not related to the Mediterranean species where the centre of diversity now lies for this genus (Cantino 1992).

The ambiguities expressed in the phylogenetic tree in this study support the need for further investigation. The gene chosen for this investigation, *rbcL*, is suitable at the family and species level. To resolve taxonomic relationships within unresolved groups, such as the HHMW complex, or the genus *Pityrodia*, *rbcL* or faster evolving genes (cpDNA sequences of *matK*, *trnL* intron, *trnL-trnF* spacer, *atpB-rbcL* intergenic region, or nuclear DNA including ITS and 5S rDNA) would be suited. The gene *rbcL* would be

appropriate for a study encompassing more genera, including *Chloanthes*, genera with very few species, for example *Hemiphora* and *Mallophora*, and the monotypic taxa *Wrixonia* and *Eichlerago*. Ideally, the combination of independent data sets will result in trees with strong bootstrap support, giving guidance to potential re-classification. It is worth noting that the re-classification proposed by Junell (1934), which was based on careful measurements of morphological and sometimes ecological characters, but which went when it was published, is very similar to the molecular phylogenetic arrangements of today.

Systematics using phylogenetic information, especially when based on a combination of independent data sets, can contribute to both conservation theory and practice, and should be integrated in the establishment of conservation priorities.

The role of seed germination ecology in the conservation of rare species

Knowledge of the germination requirements of rare species is fundamental to developing effective conservation strategies. This information contributes to the understanding of plant recruitment in the natural habitat, and to devising propagation protocols for rehabilitation. Prior to this study, knowledge of the germination ecology of *H. exilis* was restricted to information pertaining to low viability of seed and the presence of some form of innate seed dormancy. No information was available regarding the nature of the dormancy mechanisms, the underlying causes, or methods to fully release dormancy. In addition there was little known of the dynamics of the soil seed bank.

At the beginning of this study, before seed became available, propagation by tissue culture was attempted, with success as far as shoot elongation, but little success in induction of root formation. Propagation from shoot cuttings also yielded low numbers of rooted plants, despite the use of root-inducing hormones.

In germination ecology, determination of viability and identification of dormancy mechanisms and their release are the first steps in promoting germination. Arid zone species are generally characterised by physical and physiological types of dormancy (Baskin & Baskin 1998). Consequently, the presence of physical dormancy was tested first, and was excluded, as fresh and aged seed were both equally able to imbibe.

Physiological dormancy was investigated next, and found to inhibit germination. Addition of GA_3 resulted in 40% germination, leaving 60% of seed still dormant (and viable). Chemical inhibitors could not be isolated, and consequently chemical dormancy was excluded.

In a subsequent exhaustive study involving embryo extraction, successive removal of embryo covering structures identified mechanical restrictions to germination, which, when removed, yielded 100% germination, thus identifying mechanical dormancy as the major cause of dormancy. Seedlings produced by seed coat and endosperm removal developed normally, thus excluding morphophysiological dormancy. The success with alleviating dormancy completely is significant when dealing with rare species with low viable seed production, such as *H. exilis*.

Consequently, *H. exilis* possesses multiple dormancy traits, with a dominance of mechanical dormancy. Although mechanical dormancy is generally not a major component in arid zone species on a global scale (Baskin & Baskin 1998), it is prevalent in a range of species in the Australian arid zone (Richmond and Ghisalberti 1994, Bell 1999), which co-occur with *H. exilis*.

Hemigenia exilis fits into the category of "deeply dormant" seed (Tieu et al. 2001). Under natural conditions, the seed responded to prolonged soil burial by partially releasing the physiological (endogenous) component of dormancy, and gradually reducing mechanical (exogenous) dormancy as a result of the degradation of the seed coat. Total release of physiological dormancy might have been achieved by conditions not tested in this study, such as alternating day / night temperatures, or intermediate high temperatures (40°C to 70°C) as dormancy breaking treatments, or after soil burial periods exceeding 2.5 years. Seeds might also have responded to germination temperature regimes not assessed here.

This study was unable to differentiate between physiological dormancy release as a result of after-ripening under room conditions (dry, shelf storage under room conditions) or as a result of the environmental variables to which the seed was exposed during burial in the native habitat. The soil-seed interactions taking place during burial include daily and seasonal temperature fluctuations, wetting / drying cycles and biotic activity of the soil in the form of microorganisms and enzymatic activity. These

multiple cues may, or may not, be required to act on endogenous and exogenous components releasing dormancy of *H. exilis* seed. Changes in the seed coat as a result of burial were both visible and measurable, resulting in exfoliation of the seed coat and reduction of seed coat strength. Over 2.5 years, dormancy release was only partial and over protracted periods, with virtually no loss of viability. Full dormancy release may be achieved after longer burial, or under conditions not experienced by the seed during the study period, for example severe frost.

Only a small proportion of the seed bank germinated during this investigation. Viability remained high over that period, and dormancy release was gradual and protracted over many months. These characters demonstrate a bet-hedging strategy by the plant, which can be considered as an adaptive feature to the unpredictability of the arid environment (Gutterman 1993). As a consequence, a persistent soil seed bank will ensure survival of the species in the native habitat through a wide range of environmental perturbations.

This study documented a difference in soil burial responsiveness exhibited in the two genetic provenances. The respective seed harvesting and burial sites probably differ slightly in environmental conditions, with the northern provenance experiencing a slight predominance of summer rainfall and the southern provenance being exposed to slightly more winter rainfall. The two provenances presumably encode for this difference in rainfall regime, and the different responses exemplify the plasticity of the species.

Mechanical and physiological dormancy emerged as the two mechanisms governing germination of *H. exilis*. Together with a protracted seed dormancy (equating with extended viability), these factors produce persistent soil seed banks and help ensure the persistence of the species in their native habitat.

Ecophysiological approaches as a means to explain rarity

The parameters of photosynthesis and water relations tested in this study did not conclusively explain the rarity of *H. exilis*. The ecophysiological approach showed that the species resembles most sympatric long-lived shrubs. In the arid zone, water stress is the overriding factor dictating plant functioning in summer (Ehleringer 1994), leading to a halt in plant growth, which reduces the demand for carbon and leads to a reduction of photosynthesis.

There were two exceptions where plants remain active during the summer drought. These include the tree *Eucalyptus grasbyi*, a species which presumably has access to water at depth due to a deep root system; and, unexpectedly, the short-lived shrub *Solanum lasiophyllum* (Mitchell & Wilcox 1994), which may have accessed water held in cracks and crevices in the heterogeneous soil matrix, or may have been protected by the pubescent nature of its leaves (Lambers et al. 1998).

The source of plant available water was not investigated in this study. This question may be addressed in future with the help of stable isotopes, taking advantage of the difference in "signatures" of ground water and rain. Similarly, water use efficiency (WUE) could be investigated by carbon isotope ($\delta^{13}C$) studies.

Other aspects influencing physiological performance in areas not investigated here relate to soil preferences and mineral nutrition. In contrast to the known nickel hyperaccumulator *Hybanthus floribundus* (Cole 1973), *H. exilis* does not accumulate nickel to appreciable levels (E. Mattiske pers. comm. citing the late R. Brooks). Similar to *H. floribundus*, the presence of *H. exilis* indicates high levels of nickel in the soil and rocks on which it grows. A study comparing plant nutrition of *H. exilis* with co-occurring species (most of which occur equally in serpentine and non-serpentine soils), or with *Hybanthus floribundus*, may furnish more conclusive answers as to the rarity of *H. exilis*.

Strategies to define successful methods for the rehabilitation of the species after mining

Several *H. exilis* populations are threatened due to expansion of nickel mining, and will be re-established in the post-mining rehabilitation phase. First, genetic aspects were addressed, in order to re-establish populations with maximum genetic diversity and implicit genetic fitness. Subsequently, successful propagation methods were established to maximise reproduction. Now, sites and edaphic conditions, which give the plants best chances for survival, need to be identified.

As the species is closely associated with nickelferous outcrops, it seemed logical to trial revegetation of low grade nickel waste rock dumps. *Hemigenia exilis* established

surprisingly quickly, aided by drip irrigation over the first summer. All plants reached reproductive maturity within 2 years, which for a long-lived, slow-growing species is relatively fast. Seedling recruitment was also observed.

The findings of this study suggest the following 5-step plan for rehabilitation of *Hemigenia exilis*:

1. Delineate suitable genetic sources from which to collect plant material. This may be achieved by application of a range of genetic markers established during this study.
2. Establish reliable propagation methods and seed treatments. In the case of *H. exilis*, once germination requirements had been identified, propagation from seed is more successful than propagation by tissue culture or cuttings.
3. Trial planting of seedlings, or broadcasting of seed in selected areas and soil types to identify the most suitable environment.
4. If planting seedlings of rare species, drip irrigation over the first summer appears highly advisable in rehabilitation projects conducted in the arid zone.
5. Monitor the new populations for several years to observe plant turn-over and reproductive processes, and to gain an understanding of long-term demographic processes of the species.

With appropriate modifications, this approach is likely to be suited to a wide range of species in arid ecosystems.

Survival of the species

Unlike the mediterranean and semi-arid areas of Australia, which have suffered from broad scale clearing for agriculture, the arid zone has been subjected to less disruption. However, the impact of extensive grazing of sheep and cattle by the pastoral industry has resulted in widespread changes to the composition of native vegetation and some habitat loss. The mining industry, while having a profound impact, has restricted impact to comparatively small and well-defined areas. *Hemigenia exilis* has withstood the impacts of grazing and mining within its range for over 100 years, and the anthropogenic pressures on the species and its habitats have not intensified.

Overall, the species is at variance with the general behaviour of rare plants in arid habitats, in terms of high genetic diversity, types of dormancy mechanisms, and ease of rehabilitation and self-recruitment. In other aspects, such as stress tolerance, *H. exilis* is indistinguishable from species with similar life forms. The combination of these and other characteristics might have enabled the species to survive by exploiting certain niches in the environment. The persistence of native populations of *H. exilis* is probably assured, and is reflected in the recent change of its official conservation status from "presumed extinct" to "rare but not threatened".

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Appendix

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Conservation genetics and implications for restoration of *Hemigenia exilis* (Lamiaceae), a serpentine endemic from Western Australia

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Abstract

Hemigenia exilis (Lamiaceae) is a rare plant endemic to serpentine soils of the Goldfields of Western Australia. The species was presumed extinct until 1995, when it was re-discovered on a nickel ore deposit. To delineate the origin and extent of seed collection for rehabilitation after mining, and to identify the impact of removing one population due to mining, we assessed level and partitioning of genetic variation and differentiation. Twelve populations were sampled for DNA fingerprinting using the random amplified polymorphic DNA (RAPD) technique. Ten primers produced 89 bands, 97% being polymorphic. Genetic diversity within populations ranged from 0.197 to 0.409, averaging 0.38 at the species level, which is high compared with most other endemic species. Heterozygosity within populations ranged from 0.355 to 0.431, averaging 0.27 over the species. AMOVA partitioned over 80% of the total variation within populations. Multidimensional scaling revealed weak but significant differentiation into a northern and southern provenance. Despite selective sampling, the genetic data provided useful information for the management of *Hemigenia exilis*. For restoration, seed should be collected from a range of habitats of several populations, while keeping the two provenances separate. This strategy is likely to maintain high genetic diversity and locally adapted populations.

Keywords: Genetic diversity; RAPD; Provenance; Arid zone

1. Introduction

The Goldfields of Western Australia are part of the Yilgarn Block, a granitoid craton nearly 3 billion years old and one of the oldest continental nuclei of the world. The land has not been affected by glaciation since the Permian, and the surface has been stable since the Miocene. Consequently, Western Australia possesses one of the most ancient and eroded landscapes in the world. The modern Australian flora has developed in isolation over the last 45 million years, resulting in a rate of species endemism ranging from 30 to 80% (Hopper et al., 1996). Evolution during this long period is likely to have lead to high genetic diversity and genetic differentiation.

Hemigenia exilis S. Moore (Lamiaceae), a formerly presumed extinct species, was re-discovered in 1995 during a botanical survey around a lateritic nickel deposit, 800 km northeast of Perth. Subsequent searches, specifically targeting serpentine outcrops, have located over 50 populations. The species is now listed as "rare but not threatened" (Western Australian Herbarium, 1998). Populations have been recorded only in the northeast Goldfields, a hot and arid region comprising ca. 90 000 km^2 (Fig. 1).

Most *Hemigenia exilis* populations are associated with Archaean outcropping serpentine rock, which is rich in iron, magnesium, nickel, cobalt and chromium, but low in calcium and major plant nutrients (Brooks, 1987). Serpentine soils often support vegetation specifically adapted to the high levels of metals and low levels of macronutrients. It was first observed in the sixteenth century that serpentine outcrops support distinctive

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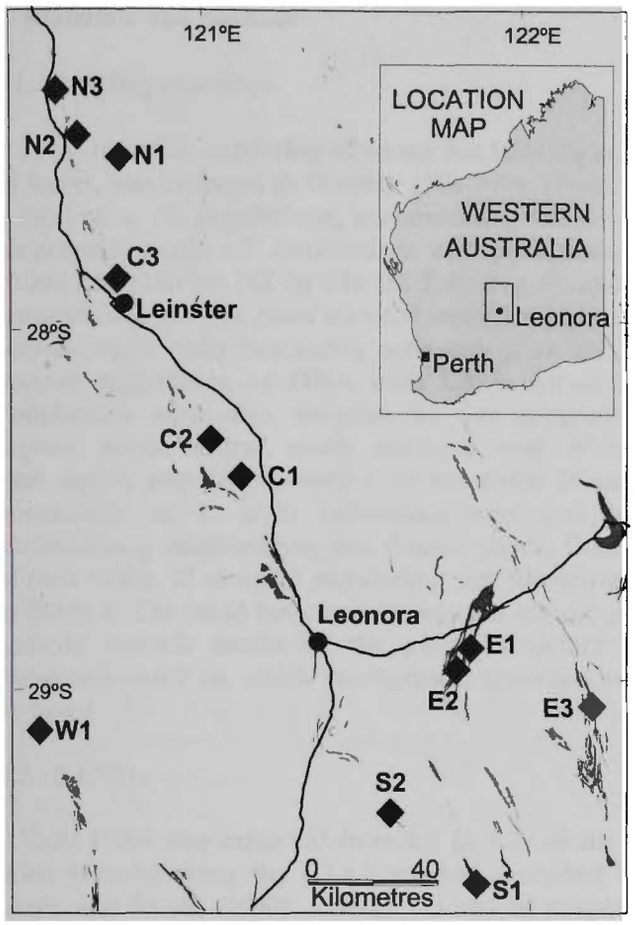

Fig. 1. Geographic distribution of sampling sites for *Hemigenia exilis*, including presence of serpentine geology (shaded).

vegetation. This relationship has been studied in increasing detail in terms of plant ecology, geobotany, biogeochemistry (Brooks, 1987; Kruckeberg, 1992), and lately genetics (Furnier and Adams, 1986; Mayer et al., 1994; Westerbergh and Saura, 1994; Bachmann and Hombergen, 1997; Williamson et al., 1997; Mengoni et al., 2001; Oline et al., 2000; Wolf, 2001). Many plant species worldwide have now been recognised as serpentine endemics. *Hemigenia exilis* co-occurs in many populations with the known serpentine endemic *Hybanthus floribundus* (Cole, 1973), supporting the idea *Hemigenia exilis* could be assigned a serpentine-endemic status.

Hemigenia exilis grows in a range of habitats, for example along rocky, exposed ridges, on slopes, and along creek lines where the soil is deeper and moister than in the other habitats. It grows as an understorey species in the shade and shelter of *Acacia aneura* trees, or as a (co)dominant species in low shrubland receiving full sun all day. The species appears to be slow growing and long lived. Flowering plants range in size from 30 to 250 cm in height. Plants flower profusely in spring, attracting butterflies as potential pollinators. Although seed set is high, only 20–30% of seed contains embryonic tissue (Cochrane et al., 1999). Despite several years of above average rainfall during the 1990s, few seedlings have been found (personal observation). Field observations indicate that *Hemigenia exilis* may be clonal. Many young plants appear to be connected by a shared root system, and damaged plants resprout readily from the base of the stem. By definition, vigorous vegetative and poor sexual reproduction are characteristic traits of clonal plants.

Mining will impact conservation of one of the *Hemigenia exilis* populations located on a commercially viable nickel ore deposit. Additionally, the species is targeted for rehabilitation after mining. For conservation purposes, it was important to firstly quantify the degree and pattern of genetic diversity of the species in general and of the impacted population in particular. The species was expected to exhibit high levels of genetic diversity as a result of high pressures from a diverse and arid environment (Linhart and Grant, 1996; James, 2000). As most Lamiaceae are outcrossing (Judd et al., 1999), most of the genetic diversity of *Hemigenia exilis* was expected to be partitioned within populations (Hamrick and Godt, 1996).

Secondly, as most serpentine outcrops produce differentiated plant populations (Linhart and Grant, 1996), genetic differentiation resulting in provenances was expected. A provenance is a local genotype, resulting from adaptive variation, which is suited to a particular environment (Frankel et al., 1995). Generally, plants recruited from local seed sources possess increased fitness over non-local genotypes. Mixing of provenances may lead to reduced fitness, weak hybrids and outbreeding depression, thus compromising the long-term viability of local populations. Only when populations are genetically impoverished or sterile does mixing of provenances have a beneficial effect (De Mauro, 1993; Frankel et al., 1995). The genetic integrity of rehabilitated populations of *Hemigenia exilis* should be maintained by delineating provenances.

To elucidate population genetics aspects of *Hemigenia exilis*, we have chosen the random amplified polymorphic DNA method (RAPD; Welsh and McCleland, 1990; Williams et al., 1990). This PCR based technique produces a large amount of dominant multilocus markers and has been used widely in population and phylogenetic studies of plants, fungi, bacteria and animals (Haig, 1998).

The present study is one of the few population genetic investigations of Australian arid zone plants (Coates and Hnatiuk, 1990; Sampson et al., 1995; Li, 2000, all investigating taxa belonging to the Myrtaceae), and the first one focussing on a species of Lamiaceae, and on a rare Australian serpentine endemic. We discuss the consequences that removal of a population will have on the genetic structure of the species, and develop recommendations for restoration after mining.

2. Materials and methods

2.1. Sampling procedure

Shoot material, consisting of young but fully expanded leaves, was collected in October 1998 from 12 out of a total of c. 50 populations, encompassing the entire geographic range of distribution of approximately 90 000 km^2 (350 km NS by 250 km EW; Fig. 1), and a range of habitats. The plant material was dried in sealed polycarbonate vials containing activated silica gel to prevent degradation of DNA until DNA extraction. Populations were also assigned to five geographic regions: north, central, south, east and west. Within each region, populations were 1–30 km apart. In each population, up to eight individuals were sampled, encompassing neighbouring and distant plants. Details of each of the 12 sampled populations are summarized in Table 1. The small but carefully selected sample size aims to provide results of the genetic structure of *Hemigenia exilis* on which management strategies can be based.

2.2. RAPDs

Total DNA was extracted from 0.2 to 0.3g of silica-dried material using the CTAB method described by Doyle and Doyle (1990). One μl (15 ng) of template DNA was subjected to polymerase chain reaction (PCR) amplification using 12.5 μl RAPD reaction volume containing 50 mM KCl, 10 mM Tris–HCl (pH 9.0), 0.1% Triton X-100, 2.0 mM $MgCl_2$, 0.2 mM of each dNTP, 0.5 units of Taq polymerase, 50 ng of primer, and 7 μl of sterile water. Amplifications were carried out in a Hybaid Omnigene Thermocycler with heated lid programmed for an initial step at 94 °C for 5 min to denature DNA, followed by 35 cycles of 94 °C for 15 s, 34 °C for 45 s, 72 °C for 60 s, and a final extension step at 72 °C for 10 mm, 37 °C for 1 min and 4 °C hold temperature. Five μl of amplification product, including negative control, with 2 μl of loading buffer, and size marker, were loaded onto a 1.5% agarose gel (60 wells) and electrophoresed for 2.5–3 h at 70 V in 1×TBE buffer. Gels were stained in a 0.5 μg/ml ethidium bromide bath for 20 min and bands were visualized under UV. Banding patterns were captured digitally, and presence/absence of bands was scored manually.

A number of 9- and 10-mer random primers (OPA1-OPA15 from Operon Technologies kit A, and a–f from Perkin and Elmer, and R1-R10 from Bresatec) were screened for producing a high proportion of polymorphic and reproducible banding patterns. Ten primers (Table 2) fulfilled this criterion.

2.3. Data analyses

The RAPD technique produces dominant multilocus markers. Statistical methods developed for co-dominant markers have been modified for use with RAPD or amplified fragment length polymorphism (AFLP) markers. For the purpose of this study we assumed absence of non-homologous co-migrating fragments. To avoid distortion of results due to problems regarding reproduciblity (Williams et al., 1990; Jones et al., 1997), only reproducible patterns were included in the data analysis, resulting in 57 samples.

Genetic diversity was calculated on the presence/absence (1/0) of bands, choosing the Nei and Li (1979) similarity index (S), where $S=2m_{xy}/(m_x+m_y)$, m_{xy} being the number of shared markers between two

Table 1
Details of populations sampled for measuring genetic diversity of *Hemigenia exilis*

Name of population	Geographic region	Longitude (E)/Latitude (S)	Approximate size of population	No. of samples included in RAPDs
N1	North	120°43.2′/27°31.7′	100	3
N2	North	120°34.2′/27°27.8′	250	6
N3	North	120°33.6′/27°23.7′	350	6
C1	Central	121°07.2′/28°22.7′	350	6
C2	Central	121°00.0′/28°20.5′	600	8
C3	Central	120°43.8′/27°52.6′	150	3
E1	East	121°47.4′/28°57.0′	300	6
E2	East	121°46.8′/28°58.9′	500	8
E3	East	122°11.4′/29°03.7′	200	4
S1	South	121°49.2′/29°32.5′	3	3
S2	South	121°34.2′/29°22.7′	1	1
W1	West	120°27.6′/29°07.2′	150	3

Table 2
Summary of *Hemigenia exilis* RAPD bands obtained with 10 primers

ID of primer bases of extension in ()	GC content (%)	Total no. of bands	Average no. of bands/plant	Range of bands/plant	Approximate band size range (bp)
R1 (CCC ACC AAC)	66	10	3.9	1–7	300–1100
R2 (CCC TCC TTC)	66	11	3.5	1–7	200–1600
R3 (GGG TTG TGG)	66	8	4.0	1–7	300–1400
R5 (GGG TGG TTG)	66	9	2.8	1–5	450–1600
R7 (GGG TGG TGG)	77	10	4.1	2–6	300–1300
opa1 (CAG GCC CTC C)	70	8	2.7	1–6	520–1750
opa2 (TGC CGA GCT G)	70	6	2.6	1–4	750–1900
opa3 (TGC CGA GCT G)	60	8	2.9	1–6	600–1500
opal3 (CAG CAC CCA C)	60	11	3.4	2–7	600–1500
d (CAG GCG CAC A)	70	8	4.7	1–8	650–1800
Total		89	34.6	20–44	

samples, while m_x and m_y are the numbers of markers for each sample. The estimate of diversity was then calculated as $D=1-S$. We estimated heterozygosity, averaged over all loci at the population and species level using the Bayesian approach developed by Zhivotovsky (1999). With dominant markers, bands derived from heterozygotes can not be distinguished from bands resulting from homozygotes. This poses statistical problems when estimating allele frequencies. Lynch and Milligan (1994) developed a procedure to reduce bias in parameter estimates. However, this may lead to severely biased estimates under some circumstances (Isabel et al., 1999). Zhivotovsky (1999) developed a Bayesian approach for dominant markers, resulting nearly unbiased estimates. Also, his approach is less sensitive to sample size, while in outcrossing species departure from Hardy–Weinberg equilibrium is negligible. Bayesian methods have been applied successfully in a number of genetics studies (Krauss, 2000).

Correlation between population size, sample size and % of population sampled with measures of genetic diversity was calculated by simple linear regression in Statview®.

2.4. Partitioning of genetic diversity

To partition diversity within and among populations, as well as among geographic regions, analysis of molecular variance (AMOVA) was performed using the Arlequin software (Schneider et al., 1997), which was based on the original AMOVA adapted for dominant data (Stewart and Excoffier, 1996). AMOVA converted the 57×57 inter-individual Euclidean distance matrix into an equivalent analysis of variance. The relevant variance components were extracted, and significance levels were computed. AMOVA was additionally applied a posteriori, partitioning diversity among the two provenances.

2.5. Genetic differentiation and geography

The ordination technique multidimensional scaling (MDS) was employed to visualize the overall genetic similarities of populations and regions. MDS is an iterative process that rearranges objects in an n-dimensional space (here, $n=3$), to arrive at a configuration that best approximates their original genetic distances. This is achieved mathematically by a steepest descent algorithm, which reduces the 'stress' of the final configuration (Kruskal, 1964). For MDS, a matrix based on clustering, unweighted pair group method with arithmetic averages (UPGMA) and squared Euclidian distance was employed using Statistica®. Fisher's exact test of population differentiation, developed for diploid/dominant marker data (Raymond and Rousset, 1995), was performed to statistically test the significance of genetic differentiation. Correlation between genetic and geographic distance was calculated in Statview®.

3. Results

The 10 primers produced 89 bands, ranging in size from 200 to 1900 basepairs. Nine-mer primers produced an average of 9.6 bands, while decamers produced 8.2 bands on average (Table 2). Diagnostic bands for a single population were not observed, while one band was present only in the two populations (E1, E2) occurring on the Murrin Murrin South nickel ore body. Five bands were diagnostic for the South and East regions, and two bands were present only in North and Central region.

3.1. Levels of genetic diversity

Of bands 97.7% (87/89) were polymorphic over the entire species. Six bands were >90% monomorphic (present in 52–56 individuals/57). Polymorphism on a

population basis ranged from 45.5% for the smallest and completely sampled population to 91.7% for one of the larger populations, averaging 72.0%. Populations encompassed 52–81% of all bands, averaging 66% (Table 3). Levels of polymorphism increased significantly as the number of samples per population increased ($r^2=0.884$, $P<0.0001$); they increased only weakly with larger populations ($r^2=0.701$, $P=0.0013$), while decrease in polymorphism with higher sampling% was also weak ($r^2=0.372$, $P=0.046$).

Nei and Li (1979) genetic diversity within populations ranged from 0.197 to 0.409 and averaged 0.378 over the entire species (Table 3). Diversity within populations was not significantly affected by population size ($r^2=0.421$, $P=0.031$), % of population sampled ($r^2=0.409$, $P=0.0341$) or number of samples ($r^2=0.532$, $P=0.011$). No clonality was detected, as each plant sampled was genetically distinct, even when growing within 30 cm of each other (as in population S1).

Total species heterozygosity (H_T) averaged 0.267 over all 89 bands (Table 3). At the population level, it varied between 0.355 (C2) and 0.417 (N1) and averaged 0.389. No significant relationship was detected between heterozygosity and population size ($r^2=0.613$, $P=0.004$),% of population sampled ($r^2=0.156$, $P=0.229$), but was significantly reduced as number of samples increased ($r^2=0.902$,$P<0.0001$). Levels of diversity of the population impacted by mining (E1) were similar or lower than in the neighbouring population.

Table 3
Genetic diversity of *Hemigenia exilis* calculated at the population and species level

Name of population	% Polymorphic bands	Nei & Li Genetic diversity	Bayesian heterozygosity (no. of bands)
N1	60.4	0.300	0.417±0.005 (48)
N2	81.7	0.363	0.372±0.011 (60)
N3[a]	82.5	0.357	0.369±0.012 (57)
C1	78.7	0.321	0.379±0.011 (60)
C2	86.8	0.325	0.355±0.013 (68)
C3	57.7	0.270	0.409±0.004 (53)
E1[b]	80.3	0.329	0.364±0.010 (65)
E2	91.7	0.409	0.369±0.012 (72)
E3[a]	64.1	0.237	0.399±0.008 (57)
S1[a]	45.5	0.197	0.415±0.005 (54)
S2 (solitary plant)			
W1[a]	63.0	0.317	0.413±0.007 (46)
Average for population	71.5	0.311	0.389±0.007 (58)
Average for species	97.7	0.378	0.267 (89)

[a] Denotes populations at the margin of the range
[b] Denotes population impacted by mining.

3.2. Genetic differentiation and geography

MDS of the 57 individuals revealed that *Hemigenia exilis* separates into two genetic provenances: a northern one comprising the North and Central regions, and a southern one comprising the South and East regions. (Fig. 2). The provenances are not completely distinct, rather they form a continuum. The exact test (Table 4) showed that the differentiation was weak but significant. The designation of the West region is intermediate, possibly because only a few individuals of one population were sampled. There was no relationship between genetic and geographic distance ($r^2=0.086$).

3.3. Partitioning genetic diversity

AMOVA partitioned 83.9% of the total variation within populations, 8.5% were partitioned among populations within the five regions and 7.6% among regions (Table 5). AMOVA applied a posteriori to partition genetic variation among the two provenances showed a similar pattern with 77.4% of the total variation partitioned within populations, 14.1% among populations within the two provenances and 8.5% among the two provenances.

4. Discussion

In this study we addressed genetic aspects of conservation and restoration issues of the rare serpentine endemic *Hemigenia exilis*. With moderate and selective

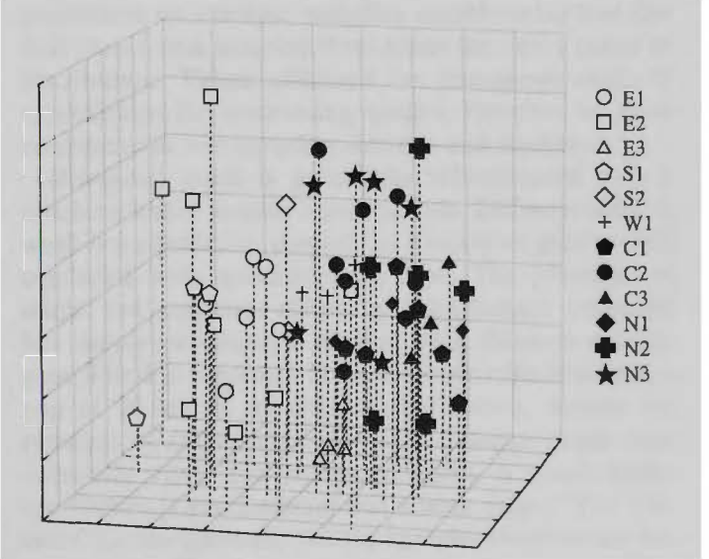

Fig. 2. Multidimensional scaling (MDS) plot of the genetic relationship among 57 individuals of *Hemigenia exilis*, resolving the two provenances.

Table 4
Fisher's exact test of population differentiation into the five geographic regions

	North	Central	West	South	East
North	*				
Central	0.7679	*			
West	1.0000	1.0000	*		
South	0.2715	0.4576	1.0000	*	
East	0.0001	0.0000	1.0000	0.9998	*

Results over loci: X-sq = 113.65, d.f.: 178, overall $P = 1.0$

Table 5
Analysis of molecular variance (AMOVA) using 89 RAPD loci, partitioning genetic variability within and among populations as well as among five regions (North, Central, South, West, East)

Source of variation	df	SS	Variance component	% Of variation
Among regions	4	107.65	1.01	7.6
Among populations within regions	7	115.96	1.13	8.5
Within populations	45	503.29	11.18	83.9
Total	56	726.90	13.32	

sampling it was possible to firstly determine that the removal of one population due to mining would not compromise the genetic structure of the species, and secondly to define provenances for rehabilitation after mining. The small and carefully selected sample size provided the information required for developing management strategies. This outcome is particularly relevant to conservation studies that have to rely on limited resources. If the results had contradicted our expectations in terms of high species diversity, high diversity within populations and at least some degree of differentiation, then we would have had to sample more intensively.

Genetic diversity was high, as evidenced by a species polymorphism of 97%, a mean genetic diversity of 0.378 and a heterozygosity of 0.267. Diversity of the population impacted by mining was similar to the non-impacted population nearby. About 80% of the total variation was partitioned within populations, and, importantly, genetic differentiation into a northern and a southern provenance was detected. Contrary to our expectation, the species was not clonal.

4.1. Population genetics of *Hemigenia exilis*

Total genetic diversity is generally higher in widespread than in endemic species (Hamrick and Godt, 1996), however no differences have been found when comparing rare and widespread congeners (Gitzendanner and Soltis, 2000). In the absence of congeneric data, comparison of *Hemigenia exilis* diversity to RAPD and allozyme surveys (Bussell, 1999; Hamrick and Godt, 1996) of plants with similar life traits indicated that diversity and heterozygosity of *Hemigenia exilis* are high. This comparison should be treated cautiously, as allozymes and RAPDs may (Aagaard et al., 1998) or may not be (Fritsch and Rieseberg, 1996) similarly sensitive. Furthermore, heterozygosity is highly correlated within a genus (Gitzendanner and Soltis, 2000). Heterozygosity of *Hemigenia exilis* populations is generally high, suggesting that the Bayesian estimates are robust. Serpentine specialists often have higher genetic diversity than generalist species, as a result of small-scale heterogeneity typical of serpentine soils (Linhart and Grant, 1996). Other factors, including climate, seed aborting lethal polymorphisms (James, 2000), high innate diversity of the genus (Gitzendanner and Soltis, 2000), and a diverse, long-lived seed bank (Baskin and Baskin, 1998) may also contribute.

Partitioning of diversity is influenced by breeding system, such that selfing species partition about 50%, whereas outcrossing species partition on average 20% among populations (Hamrick and Godt, 1996; Bussell, 1999). About 16% of the variation of *Hemigenia exilis* is contained among populations, suggesting an outcrossing mating system. Outcrossing is further suggested by field observations of flowering plants attracting butterflies, which may be involved in pollination. Outcrossing and insect pollination are common in Lamiaceae (Judd et al., 1999). The high degree of diversity partitioned within populations of *Hemigenia exilis* may also be an indication of gene flow, despite the naturally fragmented distribution and the large distances between populations. These distances appear to be overcome by the pollinator. To avoid distorted estimates of partitioning caused by uneven sampling (Hamrick and Godt, 1996), we sampled five plants per population on average, including neighbouring and distant plants, and sampled throughout the entire range of distribution. Values obtained for *Hemigenia exilis* fit expectations for outcrossing species, therefore we have confidence in our sampling strategy and analysis.

Hemigenia exilis is genetically differentiated into a northern and a southern provenance. Differentiation is weak but significant, possibly as a result of genetic drift combined with sporadic gene flow. The provenances might not represent diverging evolutionary processes but simply populations divided by a distance making gene flow less common. Differentiation coincides with a gap of 90 km in the species distribution, despite the presence of serpentine outcrops, a distance larger than occurring within the two provenances. A major drainage feature (Lake Raeside) and a wide ridge ("The Terraces") in the generally flat topography characterize this area where *Hemigenia exilis* is absent, and possibly constitute additional obstacles to gene flow. Serpentine outcrops commonly produce strongly differentiated

plant populations (Linhart and Grant, 1996; Kruckeberg, 1992). Strong differentiation has been observed in many serpentine endemics (Mayer et al., 1994, Rajakaruna and Bohm, 1999; Westerbergh and Saura, 1994; Williamson et al., 1997; Wolf, 2001; Oline et al., 2000). While genetic differentiation is often proportional to the distance between populations, breeding system, genetic drift, and environmental heterogeneity, which affect phenological traits may also contribute (Linhart and Grant, 1996). For example, variability in soil moisture leads to differential flowering, which in turn poses barriers to gene flow and enhances genetic differentiation, especially when populations are isolated. These phenological differences have been observed in populations of *Hemigenia exilis*.

4.2. Managing Hemigenia exilis for conservation and mine site restoration

The aims of conservation and restoration programs are to establish and maintain long-term viable populations, to restore ecosystem functions and processes, and, more recently, to preserve and restore the evolutionary potential of the species by maintaining or recovering natural levels and patterns of genetic diversity (Frankel et al., 1995; Lesica and Allendorf, 1999). Populations with high diversity are given higher priority than genetically depauperate ones. Differentiated populations, or provenances, need to be protected as separate entities (Montalvo and Ellstrand, 2000).

Conservation aims to protect as many diverse populations as possible. However, there may be overriding reasons that lead to the loss of plants. In our case, at least one population, El, is threatened by mining, being situated on a commercially viable nickel deposit. A similar scenario might apply to C1. At both sites, there are other populations nearby, which contain similar levels and patterns of diversity, thus mitigating the loss of genetic diversity.

In restoration, provenance, resulting from intraspecific genetic differentiation, has become increasingly an issue. Provenance has a long history in forestry (see review by Langlet, 1971). As early as the eighteenth century, foresters noticed differences in growth rate, disease resistance and frost tolerance in tree plantations originating from a range of seed sources. In the mid 1800s, the Swedish government cautioned against the use of foreign seed for timber plantations, while in the 1930s the US government recommended local seed collected from specified "seed collection zones" be used for reforestation (Montalvo and Ellstrand, 2000). The importance of provenance in restoration and rehabilitation of natural ecosystems has only recently been recognized (Millar and Libby, 1991; Knapp and Rice, 1994). Increasingly, ecologists advocate genetic surveys to delineate provenances before embarking on seed collection. The aim is to maintain the genetic integrity of reinforced and restored plant communities, with beneficial consequences for population fitness (Lesica and Allendorf, 1999, Frankel et al., 1995).

Provenances represent genetic adaptations to a local environment. Co-adapted gene complexes make local plants uniquely suited to a specific environment. This adaptation is increasingly significant as the environment becomes more stressful. Serpentine outcrops are one example of extreme environments, and serpentine endemics appear to be particularly well adapted to or even dependent on conditions at a very local scale (Linhart and Grant, 1996; Wolf, 2001). The "home site advantage" as illustrated by increase fitness has been demonstrated in trees from temperate (Tibbits and Hedge, 1998), tropical (Khurana and Singh, 2001) and arid (Roupsard et al., 1998) environments, in grasses (Knapp and Rice, 1994), shrubs (Montalvo and Ellstrand, 2000) and herbaceous annuals and perennials (Wolf, 2001; Keller et al., 2000). Mixing of provenances could lead to gene flow with a number of detrimental consequences. These include outbreeding depression, concomitant with loss of fitness due to lack of local adaptation. The introduction of non-local provenances could swamp the local provenances through gene exchange, and may result in hybrids with reduced vigour (Lesica and Allendorf, 1999). Only in genetically depauperate or sterile populations may a foreign genotype increase genetic diversity and fitness (De Mauro, 1993).

Based on conventional population genetics theory, restoration of *Hemigenia exilis* should aim at encompassing maximum genetic variation and maintaining local adaptation. Consequently, seed should be collected from as many habitats as possible of several populations within a provenance. Just a few populations may encompass a very high proportion of adaptive variation coding for topographic, biotic and edaphic heterogeneity (Lesica and Allendorf, 1999). The two provenances need to be kept separate as local genotypes. They may be genetically adapted to different local environments, and therefore are generally more successful than non-local genotypes in terms of fitness. This seed collecting strategy would ensure that genotypes adapted to a range of soil types, soil moisture regimes and temperatures are represented in the new populations. Adaptability to a wide range of soil conditions is particularly important, as soil profiles constructed on rehabilitated mine dumps will be dissimilar to the native habitat, and will pose a novel and potentially stressful edaphic environment.

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