Population Genetics of *Hakea carinata* F. Muell. ex Meissner (Proteaceae)

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

Doctor of Philosophy

from

The University of Adelaide

by

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October 2001

THE UNIVERSITY OF ADELAIDE
AUSTRALIA

Declaration

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Abstract

This study is an examination of the population genetic structure and gene flow in a sclerophyllous plant species, *Hakea carinata*, that is endemic to South Australia. The species has a naturally fragmented geographic distribution. It survives in very small populations by using a predominantly selfing mating system, however this is combined with very low levels of gene flow and substantial differentiation between populations.

The distribution of genetic diversity in *H. carinata* was represented by a sample of 30 populations covering the range of the species. The level of genetic diversity in the species is concomitant with expectations for similar woody perennial shrubs, but populations contain little variation and most is represented as between population differences. Overall gene flow between populations is insufficient to prevent further population differentiation. Outcrossing is very low and unexpected for widely distributed species in the Proteaceae.

Geographical clustering of populations coincides with genetic similarity. Isolation by distance can be used to explain the greater differentiation between furthest populations. Populations that are relatively close still appear to be exchanging too few genes to prevent differentiation.

Clustering of related individuals occurs within populations, with neighbourhoods comprising approximately five individuals. Little if any gene flow occurs between individuals more than 100 m apart in continuous stands. Isolation by distance is detectable within populations down to 10 m between plants.

Ecological observations show that autogamy may be the main source of seed set, as insect visitors to flowers appear not to effect significant levels of fertilisation. The behaviour of insect visitors equates with the low rate of outcrossing.

Hakea carinata demonstrates a high degree of isolation by distance. The investigations in this study provide important information on an evolutionary process that may become more prevalent as the effects of habitat fragmentation increase worldwide.

Acknowledgments

I extend my appreciation to the many people who assisted me to undertake this work.

I thank Dr. Susan Carthew, my supervisor, for the way in which she shared her experience with me and enabled me to see and correct the flaws in my work. Valuable assistance was received from Drew Tyre, Ben Sparrow, Ian Overton, Frazer McGregor, Jo Chivell, and Michelle Lorimer, most of whom were fellow students having similar experiences.

Mark Adams of the Evolutionary Biology Unit, SA Museum taught me how to operate a genetics lab and offered advice on the analysis of genetic data. His book was my bible.

Paul O. Lewis of the University of New Mexico provided advice in using the power of his computer program GDA (Genetic Data Analysis), after we had worked out the bugs.

Bryan K. Epperson of the Department of Forestry, Michigan State University advised on adjusting predicted values from his papers on spatial autocorrelation so that they reflected my sampling scheme.

Bill Barker of the SA Herbarium assisted with the selection of subject species and encouraged and complimented my work whenever we met.

David Ayre of the University of Wollongong provided valuable comments on a draft of Chapter 3.

Seed was collected under permits from SA Department of Environment and Natural resources No. Q23476 and SA Department of Primary Industry: Forestry Nos. 06920 and 10988.

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CHAPTER 1. INTRODUCTION

Evolution is the change in genetic composition of a population over successive generations. Individuals are descended from the previous generation but are not identical to them, and these changes accumulate over time as generations succeed each other. Individuals do not change over their lifetime, apart from the slow accumulation of mutations. Populations, however, do change over time. The genetic composition of a population is the sum of the genetic composition of all the individuals in the population. Differential survival and reproductive success of individuals result in changes to the population genetic composition.

Population genetics and the evolutionary forces that result in changes to population genetic structure have been the subject of enthusiastic study since the 1920's and 1930's, including work by Wright (1931) and Dobzhansky (1937). The study of population genetics seeks to understand how evolutionary processes change the genetic composition of populations over time. Foremost of the evolutionary processes studied are selection and migration, otherwise known as gene flow. Selection is the differential survival and reproductive success of some genotypes over others, resulting in a greater representation of these genes in the next generation. Gene flow is the movement of genes within and between populations, resulting in changed population genetic composition between generations. These forces, together with other random processes such as the sampling of genes from the parent population that is the consequence of sexual reproduction, combine to cause populations to evolve over time.

These evolutionary forces are mostly invisible and as such cannot be viewed directly. To enable them to be studied it is necessary for a whole repertoire of scientific and statistical tools to be used to detect and differentiate between them. Primary among these is to observe present day population genetic composition. Study of the amount and distribution of genetic variation results in a picture at a point in time of the genetic structure of populations. These observed patterns of genetic variation in populations can be used to elucidate past and present evolutionary processes. Furthermore, an

understanding of past and present evolutionary processes can suggest the evolutionary future of populations and entire species. These speculations on the evolutionary future of species are a central component of conservation biology, as we seek to understand the process of extinction and how to conserve what remains.

Conservation biology seeks to maintain, as much as possible, the evolutionary processes within species in the face of increasing environmental changes brought about by human activity. Species extinction rates have dramatically increased in the last four hundred years (Holsinger 1996). Solutions are needed if we are to minimise extinction rates as much as possible. Much has been made of the importance of maintaining levels of genetic variation in species as a major goal of conservation biology (Ellstrand 1992, Lande 1988, Levin and Kerster 1974, Ouborg *et al.* 1999). The loss of genetic variation in species as a result of human activity, usually caused by reduction in population sizes or a change in mating systems, makes species more vulnerable to extinction (Ellstrand and Elam 1993, Holsinger 1993). The connections between levels of genetic variation, population genetic structure, and the probability of extinction still require study if we are to properly understand them.

The term "Genetic variation" refers to the range of genetic material of which species are composed. The variation is differences between individuals in DNA sequence at gene loci. A specific gene locus will perform the same function in every individual, but minor differences in DNA sequence between them may lead to slightly different properties of the protein produced by the gene. These genetic variants may have future selective advantages or disadvantages as a result of their properties, but in most cases genetic variants appear functionally equivalent and co-exist within populations. The advantage for a species in having a high level of genetic variation is that in the event of changed environmental circumstances, selective differences between variants may become apparent. A large amount of genetic variation increases the probability that the species will have a variant selectively suited to the new environment, and ensure the continued survival of the species. Low genetic variation in the event of environmental change may increase the likelihood of extinction.

The amount of genetic variation varies markedly between species, with some containing relatively large amounts of genetic variation and others having virtually no detectable variation. In plant species the amount of genetic variation appears to be correlated with the geographic range of the species, with widespread species generally having greater genetic variation than more narrowly distributed species (Hamrick and Godt 1989). The amount of genetic variation *per se* is not an indication of the likelihood of extinction of a species. However greater genetic variation provides species with more raw material for adaptation to changing environmental circumstances. Of concern from a conservation perspective is when genetic variation declines in a species, either from a rapid reduction in number of individuals or a slow decline due to random drift.

The amount of genetic variation in a species is not a constant over time. It may vary due to dynamic processes both within and external to the species. The pattern of mating within the species will affect the amount of genetic variation, with inbreeding reducing heterozygosity and the number of alleles maintained, and outcrossing having the effect of maintaining heterozygosity and alleles. Associated with this is the effect population size has in maintaining genetic variation. Smaller populations are less able to avoid inbreeding. Loss of genetic variation through drift is also faster in smaller populations. Migration between populations can replace genetic variation lost through drift. Migration can however be sensitive to distances between populations, with greater distances effectively reducing or eliminating movement between populations.

One perspective of the genetics of a population is its genetic structure. Genetic structure refers to the spatial arrangement of genetic variation. Spatial variation can occur between geographically subdivided populations as well as within continuous populations (Heywood 1991). The way in which genetic variation is distributed among populations of a species is affected by much the same factors that influence species level genetic variation. In plants, those species that are outcrossed and wind pollinated tend to have less than 10% of their genetic variation distributed between populations, meaning that most of the species level genetic variation exists within any population (Hamrick and Godt 1989). In contrast, selfing species generally have more than 50% of their total variation distributed between populations (Hamrick and Godt 1989). This

difference shows that the mating system can be an important factor in influencing the distribution of genetic variation between populations of a species.

A related factor is patterns of dispersal for individuals and propagules. With outcrossing wind-pollinated plants there tends to be greater dispersal of pollen between plants and more mixing of the genetic variation than with selfing species. The ability of plant propagules to disperse is a function of the adaptations for dispersal mechanisms and mating systems in the species. Substantial spatial structure occurs in most populations, in part due to limits to the dispersal distances of individuals and propagules (Epperson and Li 1997). Violations of the Hardy-Weinberg assumption of complete random mating occur in nearly all populations. Whether dependant on directions of wind current or the behaviour of animals carrying pollen or seed, the successful arrival of pollen on a flower or seed in a place suitable for germination is not totally random. Wind usually prevails from one direction. Animals fly or walk in particular directions and for distances determined by many factors such as topography or availability of resources. Restrictions to wide dispersal result in individuals being more related to others nearby and less related to those at greater distances.

Dispersal is important therefore to the dynamics and evolution of populations. Where dispersal is limited there will be matings between proximate individuals and a build up of genetic isolation by distance (Wright 1943). Wright showed that the effect of restricted dispersal between geographically subdivided populations can be described in terms of the number of migrants between populations, or the gene flow between them.

Gene flow is an important influence on population structure. The amount of gene flow between populations determines the extent to which populations evolve together. A large amount of gene flow between populations will mean that they evolve together and little gene flow will result in populations evolving almost independently. Wright (1931) addressed the question of how much gene flow is necessary to prevent independent evolution in populations. In his island model, there are many local populations each randomly exchanging genes. The amount of gene flow necessary to prevent local differentiation must exceed the strength of genetic drift. This is closely related to population size as drift is stronger in smaller populations. Wright showed

that gene flow is sufficient to overcome the effects of drift and prevent fixation of alternative alleles when it exceeds $N_M = 1$. N is the effective population size and M is the proportion of the population that migrates. There is therefore a balance between gene flow and genetic drift that determines the evolutionary connectedness of populations.

A number of conditions are assumed for the above to hold true. One is that selection is not favouring different genotypes in different populations. This would cause populations to differentiate. Alternatively, selection that favours the same allele in different populations would prevent differentiation regardless of the amount of gene flow (Slatkin 1994). Strong selection would therefore disrupt the effects of gene flow on population differentiation at those loci at which it operates. The one-migrant-per-generation rule has recently been critically evaluated (Mills and Allendorf 1996) and the meaning shown to be more complex than this simple representation, nevertheless the principles of genetic connectedness remain stable for the analysis of evolutionary change as studied here.

Inferences about the role of gene flow in population differentiation also require there to be an equilibrium between genetic drift and gene flow. This can be achieved where a large number of populations remain constant in size and there is no population extinction. This may not be the case in many ecosystems due to the expanding human use of the environment. The amount of habitat available to species is being reduced and that which remains is becoming increasingly fragmented. This results in smaller, more isolated populations that are surrounded by different, human-altered environments.

There are a variety of effects of habitat fragmentation on population genetics. Reductions in population size may result from disturbance and land clearance. Genetic drift operates more strongly to reduce genetic variation when population sizes are small (Ellstrand and Elam 1993). Drift affects genetic variation within populations through the loss of heterozygosity and the fixation of alleles, and increases the differentiation between populations. Continued small population size also increases the rate of inbreeding which further increases the erosion of genetic variation. Habitat

fragmentation can affect dispersal and gene flow between populations by increasing the distances between them and changing the surrounding matrix. Substantial fragmentation can result in populations becoming completely isolated from each other and therefore allowing genetic drift to shape genetic structure in the absence of gene flow.

Not all species however suffer from declining genetic variation through having small population sizes, unless such small size is the result of a sudden or catastrophic process. Many species are naturally patchy in their distribution, due to restricted areas of suitable habitat, and barriers or other limitations to dispersal. Those species that have survived for very long times despite small numbers have adapted to the constraints of a restricted distribution. Plant species with continuous distributions across large geographic areas are possibly more likely to suffer the deleterious effects of habitat fragmentation (Holsinger 1993) than those that are naturally patchily distributed. An important focus of study in conservation genetics is to compare the population genetic structure and patterns of gene flow in a variety of species with different natural distributions. There are also important comparisons to be made between naturally fragmented landscapes and those that are fragmented due to human activity.

One such species that shows adaptation to a naturally fragmented landscape has been investigated by Templeton *et al.* (1990). The North American salamander, *Cryptobranchus alleganiensis*, is totally aquatic and incapable of terrestrial dispersal. They occur in rivers in the Ozark Mountains that, due to topography, form two separate and distinct drainages, one to the north and one to the south. Templeton and co-workers found no shared haplotypes between these drainages. There were even sufficient differences between populations from nearby rivers to indicate that they were genetically isolated from each other. The apparent absence of gene flow in this species was taken to suggest that there was no opportunity for recolonisation in the event of any local population extinction. This uncommon example of isolation in a naturally fragmented landscape shows the possible consequences from human induced fragmentation in what are now continuous populations and species.

Plants also demonstrate genetic differences between isolated populations over time. These differences may be due to changes to the reproductive processes within smaller populations. The effects on plant reproductive output of recent forest fragmentation in Argentina were shown to be related to fragment size and isolation (Aizen and Feinsinger 1994). These effects have become manifest in plant species that are typically found in continuous forest. The clearance of dry Chaco Serrano forest in Argentina has left a fragmented landscape of forest isolates and peninsulas in a matrix of pastures and crops. The agricultural zone is still surrounded by nearly continuous forest. Aizen and Feinsinger found that plant pollination, fruit set and seed set were significantly lower in small (<1 ha) forest fragments than in large (>2 ha) fragments. Both were lower than in the surrounding continuous forest.

The changes to population genetic structure caused by continuous spatial distributions becoming fragmented has a deleterious effect on plant reproductive output (Holsinger 1993). Assessing the changes to genetic processes as a result of fragmentation requires an historical perspective. The difficulty arises in detecting the changes to population genetic structure from events in the last few hundred years, given the few generations encompassed by that time. Historical rates of gene flow may not match contemporary rates if fragmentation is recent, but gene flow and drift will not be in equilibrium and population genetic structure will reflect historical rates of gene flow (Sork *et al.* 1999).

A greater understanding of the potential effects of habitat fragmentation will come from studies of naturally fragmented species and the effects of isolation by distance on population genetic structure. In species where gene flow is restricted by distance, genetic differentiation between populations should increase as a function of the distance between them (Slatkin and Maddison 1990). Conversely gene flow should reduce at greater distances. The isolation by distance hypothesis predicts a negative correlation between gene flow and distance, and allows a validation of the gene flow estimates derived from population genetic differentiation (Ouborg *et al.* 1999). It also provides a test of the equilibrium assumption as isolation by distance will only be detectable at equilibrium (Slatkin 1993). This can be used to infer if recent habitat fragmentation has changed population distributions in such a way as to disturb dispersal and gene flow.

Selection of subject species

The subject species selected was *Hakea carinata*. The intention of this study was to select a plant species in the Proteaceae, because of the prominent position of this plant family in the Australian flora. Several Proteaceous genera have been well studied in terms of their genetic variation and relations with pollinators (Goldingay and Carthew 1998). These genera include *Banksia*, *Grevillea*, and *Persoonia*. The family is characterised by showy, colourful flowers, a high degree of outcrossing and self-incompatibility, and prominent pollen presenters in the floral morphology. They are mostly bird or animal pollinated. Within this family, *Hakea* has attracted little attention, perhaps because of its lack of large, showy, colourful flowers and hence little horticultural interest.

Many of the plant species in the Proteaceae have potentially complex mating systems as they attract a wide variety of floral visitors (Collins and Rebelo 1987). Despite observations of pollinator movement suggesting that most pollen transfer occurs within plants or between neighbouring plants (eg. Carthew 1993, Vaughton and Carthew 1993) many species display almost obligate outcrossing (Carthew *et al.* 1998). Some species of *Grevillea* have been reported to have variation in mating system between populations of the same species (Ayre *et al.* 1994, Hermanutz *et al.* 1998), perhaps in response to very small population sizes. These species can be self compatible but display a preference for outcrossed progeny (Vaughton 1996). Several *Grevillea* species display variation in self compatibility with insect pollinated species displaying stronger self incompatibility than bird pollinated species (Hermanutz *et al.* 1998, Richardson *et al.* 2000). In contrast, *H. carinata* shows a high degree of self compatibility, including autogamy, whilst being insect pollinated (Loram 1994). This level of self compatibility is unusual in the Proteaceae and warrants the investigation of mating system parameters in this species.

H. carinata is a common species, endemic to South Australia. This makes the entire range of the species highly accessible as well as providing a large number of populations over a range of habitats. Previous work (Loram 1994) had suggested that

H. carinata was capable of self fertilisation and hence atypical of the Proteaceae. It is also visited by insects only as the flowers and inflorescences are too small for birds and mammals to visit. The scattered population distribution is consistent with the patchiness of the remnant vegetation in South Australia, suggesting that it may have been affected by land clearance in the time since European settlement. Herbarium records also pointed to an extensive presence in remnant vegetation throughout the Mt Lofty Ranges.

Aims

This study aims to address issues of the extent of gene flow in a plant species with a naturally patchy distribution. This is to determine the evolutionary consequences of patchiness in this species. The effects of low gene flow and isolation by distance are hypothesised to be a high degree of genetic differentiation between populations and a low level of genetic diversity in small populations. Furthermore, this methodology is to determine if the present distribution of populations shows evidence of recent disturbance and fragmentation, perhaps as a result of human activity in clearing land for agriculture.

The specific objectives of the study are:

- Survey genetic variation in *H. carinata* to determine the level and distribution of genetic variation in comparison to other plant species with similar life history traits. Method: sample plant material from a sample of populations over the geographic range of the species. Determine isozyme systems suitable for assay, and which plant material is best to resolve them. An explanation for the use of isozymes in preference to other genetic techniques follows these aims.
- Determine species level gene flow between populations, and examine if there is a relationship between geographic distance and genetic distance. Method: assess gene flow between populations in a hierarchical manner from species wide to local clustering of populations. Use a variety of techniques including UPGMA (Unweighted Pair Group Means with Arithmetic averages) to assess geographic clustering of genetic variation.

- Investigate isolation by distance between populations across the geographic range of the species. Method: use regression techniques to assess the size of genetic neighbourhoods within which plants are exchanging genes through mating, and beyond which there is insufficient gene flow to prevent genetic differentiation.
- Survey spatial distribution of genetic variation in one large population of *H. carinata* to assess the scale of isolation by distance within population. Method: assay 400 plants and map their spatial arrangement in a population covering approximate half a square kilometre. Use spatial autocorrelation and related statistics to determine the scale of clustering of related individuals and infer the primary evolutionary forces responsible for the pattern of genetic variation within population.
- Observe the ecological relations between *H. carinata* and its insect floral visitors to assess the extent of pollination services provided and the implications for seed set and gene flow. Method: observe floral phenology over a flowering season in several populations, and the floral visiting behaviour of insects. Quantify flower to fruit ratio and the impact of possible pollen dispersal on genetic variation.

Genetic assay methodology

The genetic data in this study were gathered as isozymes in preference over alternative genetic techniques, for several reasons. Isozymes maintain a role in genetic analysis despite the emergence of a large array of more sophisticated genetic techniques (Brown 1990). Large scale surveys of genetic variation in species, regardless of final technique used, are well advised to use an initial survey of isozyme variation. This enables the establishment of a broad estimate of genetic variation in the species that will influence sampling methodology and the appropriate scale for the final sampling when other techniques are used.

A substantial amount of genetic polymorphism exists at the level of the species. Hamrick and Godt (1989) found that about half of isozyme loci are polymorphic within a single plant species, and about a third are polymorphic within the average

population. This usually provides enough detectable genetic variation to comparatively measure the genetic variation within a population, and measure the divergence between two populations. These are some of the aims of the present study.

DNA based molecular data are more powerful and flexible in that they provide much more detailed data to enable differentiation between individuals, but such detail is not necessary to establish systematic relationships at the level of the population or species. The additional effort and expense to obtain genetic data using DNA based methodologies is not warranted for this reason. Isozyme analysis is cheaper, easier, and faster than molecular analysis. The samples are usually crude extracts that do not require purification, and large samples such as required by species level analyses can be run comparatively quickly and economically. The much larger samples made economically feasible by using isozymes are well suited to monitoring microevolutionary processes such as mating system (Brown 1989), migration, variation in paternal parentage (Ellstrand 1984), and local differentiation (Heywood 1986).

Thesis outline

Following this introduction, Chapter 2 presents a survey of species-wide genetic diversity and estimates of genetic differentiation between populations. Overall species level gene flow was estimated from these, and the analysis included outcrossing rates and the clustering of genetic relatedness of populations by geographic distance. In Chapter 3 the patterns of distribution of genetic variation from the previous chapter are stratified by smaller geographic regions and gene flow is investigated by a regression analysis of all pairwise combinations of populations. Chapter 4 presents a dataset of genetic variation of a large sample of geographically mapped plants from a single large population. Spatial autocorrelation is used to investigate clustering of related individuals, and statistical techniques taken from simulation studies used to infer the evolutionary forces that have most strongly influenced the observed patterns. Chapter 5 presents the ecological observations of insect behaviour and flowering in the species, and the correlates between the observations and genetic data discussed.

CHAPTER 2. A SURVEY OF GENETIC VARIATION AT THE LEVEL OF THE SPECIES [§]

2.1 Introduction

The availability of genetic variation in a species enables evolutionary change (Hamrick and Godt 1989). An important aspect of the study of evolution is therefore the assessment of levels of genetic variation maintained by species. Also of interest is the way the genetic variation is distributed within and between populations. Indications are that small populations maintain lower levels of genetic variation than large populations (van Treuren *et al.* 1991, Ellstrand and Elam 1993), and that isolation of populations leads to greater genetic differentiation (Wright 1943).

Habitat fragmentation has led to the reduction in size and isolation of populations with the potential to cause loss of genetic variation. However the deleterious effects of fragmentation are more severe in some species than others. For example, those plant species which naturally have a patchy distribution are not expected to be adversely affected by fragmentation (Holsinger 1993) as they should have adapted to their discontinuous distributions. Investigations of the degree of population differentiation in patchily distributed plant species can highlight the importance of gene flow in the evolution of isolated populations. High levels of gene flow between populations enable them to evolve together. Gene flow of less than approximately one migrant per generation is insufficient to counteract the effects of drift, and populations are expected to evolve almost independently (Slatkin 1994).

Hakea carinata is a sclerophyllous Proteaceous shrub (Haegi and Barker 1985) that has a patchy distribution in woodland scrub. It is endemic to South Australia and well represented in many small and large patches of remnant vegetation. It occurs in dense

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[§] This chapter is based on a previously published paper (Starr and Carthew 1998).

clusters and as scattered individuals but never dominates the understorey vegetation. Land clearance has extensively fragmented areas where it is thought to have been common yet there are some populations that appear to be naturally isolated.

This study investigated the level and distribution of genetic variation in *H. carinata* as a way of assessing whether habitat fragmentation has altered evolutionary process such as genetic drift and gene flow in the species.

Although work on population genetics has been done on other genera in the Proteaceae such as *Banksia* (Carthew *et al.* 1988, Coates and Sokolowski 1992, Carthew 1993) and *Grevillea* (Ayre *et al.* 1994), *Hakea* is poorly understood. Proteaceous species are commonly considered to be primarily outcrossing and many, including *H. carinata*, have protandrous flowers that should enhance outcrossing. *Hakea carinata* has been shown, however, to be capable of self fertilisation (Loram 1994) and I investigated the outcrossing rate in the species, and implications of the breeding system for the maintenance of genetic variation. The species is widespread and not considered endangered, but there is evidence that extinction of small populations has occurred in the recent past.

2.2 Methods

2.2.1 Species Survey

Hakea carinata is distributed over a range of approximately 500 km through the southern Flinders Ranges and Mt Lofty Ranges and into the south-east of South Australia (Figure 2.1). It occurs in small forest reserves and conservation parks generally on poorer soils and steep aspects (mainly because these sites are not suitable for clearance of native vegetation) and also along roadsides. Individuals are patchily distributed even within large expanses of suitable habitat. *Hakea carinata* is an erect sclerophyllous shrub to 3 m with narrow spiky leaves. It flowers profusely and retains the two-seeded fruit on the plant for many years until the branch dies when the winged seeds are released. Flowers are small and white and presented in inflorescences of up to 20 flowers measuring approximately 1 cm wide overall. Flowers are visited by

many different species of flying insects from native bees and honeybees to flies and beetles.

Seeds were collected from 30 populations across the range of *H. carinata* (Table 2.1). Nearly all populations located were sampled, provided there were fruit available. Because some populations numbered only a few plants, numbers of plants sampled per population varied from two to 16. Plants were randomly chosen along transects across the widest dimension of each population. The sampling scheme therefore represents a hierarchical plan with a random sample of seeds taken within plants, and a random sample of plants taken within populations. The data analysis reflects this hierarchical arrangement.

2.2.2 Electrophoresis

Initially plant leaf tissue was sampled for isozyme assay, but it was not found possible to detect any activity. The leaves are narrow, dry, and hard, and cell contents are difficult to extract. Leaves were ground up using either of two methods, liquid nitrogen or ground glass. The techniques of Wendel and Weeden (1989) were used in respect of grinding buffers and extraction procedures. Following the failure of using leaf tissue, germinated seeds were used. A trial run of 42 enzyme systems was undertaken from which the enzymes that displayed reliable and repeatable resolution were used.

Between seven and 98 seedlings were genetically assayed per population, using cellulose acetate gel electrophoresis. Seeds were germinated on moist filter paper. Fully expanded cotyledons were ground in a few drops of a 0.1 M Tris-HCl pH 7.4 buffer containing 20 mg/mL polyvinylpyrrolidone (MW 40000), 4 μL/mL 2-mercaptoethanol and 1 mg/mL dithiothreitol. Gels were run at 200 v for 2 hr 15 min on either a TM buffer (0.05 M Tris-maleate pH 7.8 comprising 50 mM Tris and 20 mM maleic acid) or a Phos buffer (0.02 M phosphate pH 7.0 comprising 11.6 mM Na_2HPO_4 and 8.4 mM NaH_2PO_4). Enzymes run on the TM buffer were peptidase-D (PEP-D, EC 3.4.13.9 using substrate Phe-Pro), shikimate 5-dehydrogenase (SKDH, EC 1.1.1.25), and diaphorase or dihydrolipoamide dehydrogenase (DIA, EC 1.8.1.4), and those run on the Phos buffer were phosphoglycerate kinase (PGK, EC 2.7.2.3),

and glucose-6-phosphate isomerase (GPI, EC 5.3.1.9). Enzyme assays were as described in Richardson *et al.* (1986) with the exception of Shikimic acid dehydrogenase (SKDH). This used 10 mg Shikimic acid, 2 mL 0.1 M Tris-HCl pH 8.0 buffer, 0.2 mL 25 mM NADP, 0.2 M 14.5 mM MTT, 0.2 mL 6.5 mM phenazine methosulphate, 0.2 mL 0.45 mM pyruvate and 0.2 mL 0.73 mM pyrazole all combined and corrected to pH 8.0 with 1 M NaOH (M. Adams personal communication).

Six zones of activity were scored and each was assumed to represent a single locus. Genetic interpretation of allozyme banding patterns was based on segregation patterns of open pollinated progeny arrays from the same maternal parent.

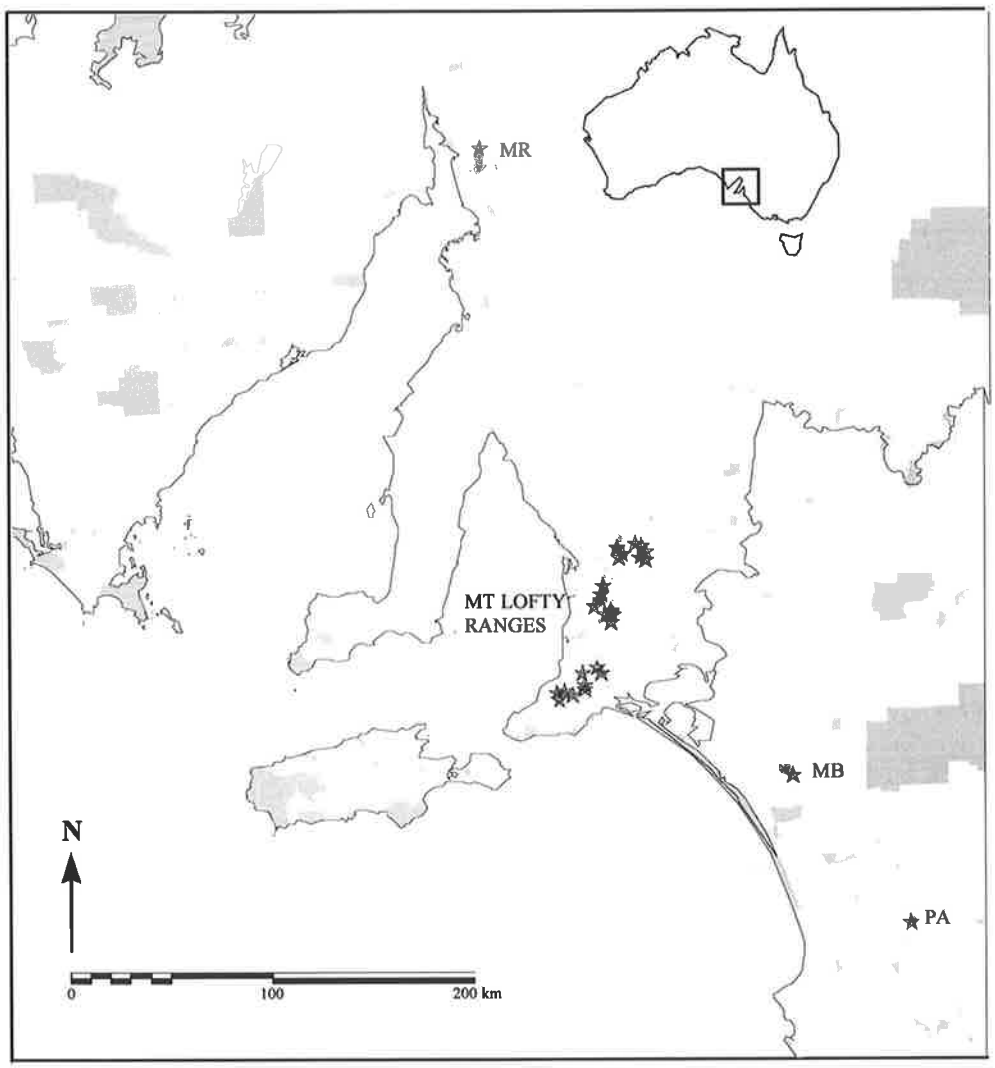


Figure 2.1 Location of 30 sampled populations in the Mid-North, Mt Lofty Ranges, and south-east of South Australia. Shaded areas are reserves

Population	Lat. S	Long. E	# Plants (seeds) sampled	Population type	Size
Mt. Remarkable (MR)	32°43.3′	138°04.1′	16 (98)	national park	large
Warren (WA)	34°42.1′	138°55.1′	5 (19)	reservoir	small
Jenkins Scrub (JS)	34°42.7′	138°57.6′	12 (46)	forest reserve	medium
Para Wirra (RT)	34°43.0′	138°48.7′	10 (47)	recreation park	medium
Humbug Scrub (HS)	34°43.2′	138°48.8′	10 (49)	recreation park	large
Cricks Mill (CM)	34°44.5′	138°58.7′	10 (39)	roadside	small
Goods Gully (GO)	34°44.8′	138°50.5′	9 (24)	forest reserve	medium
Bundy (BU)	34°46.0′	138°56.7′	10 (30)	forest reserve	medium
Brewers Corner (BC)	34°46.3′	138°49.2′	10 (49)	roadside	small
Cromer (CR)	34°46.8′	138°58.6′	10 (47)	cons. park	medium
Morialta (MO)	34°54.1′	138°43.5′	10 (39)	cons. park	medium
Horsnell Gully (HO)	34°56.7′	138°42.4′	10 (20)	cons. park	medium
Cleland (CL)	34°58.2′	138°42.1′	8 (19)	cons. park	large
Belair (SH)	35°00.3′	138°39.8′	5 (13)	roadside	small
Vimy Ridge (VR)	35°01.3′	138°45.8′	39 (39)	cons. park	medium
Mylor #2 (M2)	35°02.0′	138°46.2′	10 (49)	recreation park	medium
Mylor #1 (M1)	35°02.6′	138°46.3′	10 (42)	roadside	small
Longwood (LW)	35°02.8′	138°43.9′	10 (44)	roadside	small
Kuitpo (OD)	35°05.0′	138°45.9′	10 (48)	mine reserve	large
Mt. Magnificent (MM)	35°18.5′	138°40.5′	10 (45)	cons. park	large
Mt. Compass (MC)	35°19.7′	138°35.0′	10 (51)	roadside	small
Cox's Scrub (CS)	35°20.0′	138°41.9′	10 (47)	cons. park	large
Wood Cone (WC)	35°23.3′	138°36.0′	2 (7)	roadside	small
Sth. Wood Cone (WS)	35°24.9′	138°35.8′	10 (35)	roadside	small
Yulte (YU)	35°25.0′	138°28.4′	8 (30)	cons. park	medium
Nixon-Skinner (NS)	35°25.4′	138°25.9′	10 (38)	cons. park	medium
Spring Mount (SM)	35°26.4′	138°31.5′	10 (48)	cons. park	medium
Myponga (MY)	35°27.5′	138°26.5′	10 (38)	cons. park	medium
Mt. Boothby (MB)	35°51.3′	139°49.2′	12 (49)	cons. park	large
Padthaway (PA)	36°35.1′	140°31.0′	16 (48)	cons. park	medium

Table 2.1 Population designation and location listed from north to south, number of plants and seeds sampled, population type and size. Cons. Park = conservation park. Approximate size categories; small = < 300 plants, medium = < 1000 plants, large = > 1000 plants

2.2.3 Statistical Analysis

Gene diversity was calculated as unbiased expected heterozygosity (Nei 1978) within populations (H_S) and in the total population (H_T). The degree of gene differentiation between populations (G_{ST}) was calculated as $G_{ST} = (H_T - H_S) / H_T$. To minimise the bias of unequal sample sizes, gene frequencies for these diversity statistics were meaned across all seeds for each adult plant. Gene frequencies and plant sample sizes are given in the appendix.

Genetic diversity statistics calculated for each population were percentage of loci polymorphic (P), mean number of alleles per locus (A), mean number of alleles per polymorphic locus (A_P), observed (H_o) and expected (H_e) heterozygosity under Hardy-Weinberg.

Deviations of genotypic frequencies from Hardy-Weinberg expectations were tested using exact tests with the alternative hypothesis of heterozygote deficiency. This is a more powerful test than the usual two-tailed probability test (Rousset and Raymond 1995).

Genetic structure within and between populations was estimated using F-statistics which measure departures from expected levels of heterozygosity. Adult plants within populations were treated as subpopulations. The several seeds that were genotyped per plant are the individuals in the statistical analysis. This additional hierarchical level was necessary as seeds from predominantly selfing plants are expected to be highly related and cannot be treated as individuals within populations (M. Adams personal communication). The formulae and notations are those of Weir and Cockerham (1984) and Weir (1990). The F-statistics were used to partition total inbreeding F (Wright's F_{IT}) into components resulting from inbreeding within subpopulations f (F_{IS}) and subdivisions between subpopulations θ_S (F_{SP}) and between populations θ_P (F_{PT}). Two indirect estimates of gene flow (N_M) were calculated, one using population subdivision ($\theta_P = F_{ST}$) as

$$N_M = \tfrac{1}{4}\left(1/F_{ST} - 1\right) \quad \text{(Wright 1951)}$$

and the other using the frequency of private alleles $\bar{p}(1)$ as

$$\log_{10}\left[\bar{p}(1)\right] \approx a \log_{10}(N_M) + b$$

where a and b depend on the number of individuals sampled from each population (Slatkin 1985, Barton and Slatkin 1986). Multilocus outcrossing rate (t) was estimated from progeny arrays in each population following the maximum likelihood model of Ritland and Jain (1981) and using the program MLTR (Ritland 1990).

Genetic distances between populations were calculated using D_A distance (Nei *et al.* 1983) which gives a better topology for dendrograms than the commonly used D. Grouping of populations based on genetic distance was performed with an UPGMA dendrogram.

Observed and unbiased expected heterozygosities were calculated using BIOSYS-1 (Swofford and Selander 1981). Exact tests for heterozygote deficiency and the estimate of N_M using private alleles (corrected for sample size) were performed using GENEPOP v1.2 (Raymond and Rousset 1995). F-statistics were calculated using GDA (Lewis and Zaykin 1997). Genetic distances were calculated using DISPAN (Ota 1993), and the resultant UPGMA dendrogram was constructed using SYSTAT (Wilkinson 1990).

2.3 Results

The total gene diversity for *H. carinata* was $H_T = 0.317$ which is commensurate with the mean value for 406 plant species of $H_T = 0.310$ found by Hamrick and Godt (1989). This value is also similar to those from the same review for geographic range (regional $H_T = 0.308$) and breeding system (mixed - animal $H_T = 0.304$). In contrast the gene diversity within populations of *H. carinata* was $H_S = 0.168$ and the coefficient of gene differentiation was $G_{ST} = 0.469$, indicating that the diversity held within populations is low and a substantial amount of diversity is held between populations.

All loci that were scoreable showed some polymorphism (Table 2.2). The lack of monomorphic loci may overestimate the following statistics but mean polymorphism is a modest $P = 50.5\%$ and the mean number of alleles per locus is a low $A = 1.53$. The mean number of alleles per polymorphic locus is $A_P = 2.05$, scarcely above the minimum possible value of 2.00.

Observed heterozygosity was very low at a mean of $H_o = 0.031$ with six out of 28 polymorphic populations showing no heterozygosity at all. *Hakea carinata* has a predominantly selfing breeding system as evidenced by the mean estimated outcrossing rate of $t = 0.111$. The deficits of observed heterozygosity against Hardy-Weinberg expectations were significant in 81 of 87 cases (93%), with 58 of those (72%) significant at $P < 0.001$ (Table 2.3).

Inbreeding is high in *H. carinata* as evidenced by the high mean *F*-statistics (Table 2.4). All 99% confidence intervals of jackknifed *F*-statistic means are positive and do not include zero. The mean estimate of among population differentiation ($\theta_P = 0.481$) resulted in a gene flow estimate of $N_M = 0.270$ migrants per generation. The estimate from private alleles produced $N_M = 0.129$ migrants.

Population	P	A	A_P	H_o	H_e	t
BC	33.3	1.3	2.0	0	0.143	0
BU	50.0	1.5	2.0	0.023	0.147	0.075
CL	50.0	1.7	2.3	0.029	0.142	0.008
CM	66.7	1.7	2.0	0.045	0.202	0.169
CR	50.0	1.5	2.0	0.028	0.151	0
CS	83.3	2.2	2.4	0.070	0.324	0.256
GO	50.0	1.5	2.0	0.007	0.117	0.121
HO	33.3	1.3	2.0	0	0.088	0
HS	83.3	1.8	2.0	0.091	0.219	0.200
JS	33.3	1.3	2.0	0.028	0.078	0.170
LW	50.0	1.5	2.0	0.060	0.206	0.210
M1	50.0	1.5	2.0	0.037	0.115	0.166
M2	50.0	1.5	2.0	0.045	0.172	0.069
MB	66.7	1.7	2.0	0.004	0.217	0.035
MC	83.3	1.8	2.0	0.037	0.270	0.040
MM	83.3	2.0	2.2	0.030	0.247	0.177
MO	16.7	1.2	2.0	0.041	0.083	0.156
MR	0	1.0	-	0	0	-
MY	83.3	1.8	2.0	0.047	0.180	0.258
NS	66.7	1.7	2.0	0.066	0.258	0.321
OD	33.3	1.3	2.0	0.025	0.083	0.061
PA	33.3	1.3	2.0	0	0.060	0
RT	33.3	1.3	2.0	0	0.102	0
SH	33.3	1.5	2.5	0	0.183	0
SM	83.3	1.8	2.0	0.019	0.167	0.123
VR	33.3	1.3	2.0	0.072	0.160	-
WA	0	1.0	-	0	0	-
WC	16.7	1.2	2.0	0	0.059	0
WS	83.3	1.8	2.0	0.074	0.298	0.241
YU	83.3	1.8	2.0	0.042	0.346	0.139
Mean	50.5	1.53	2.05	0.031	0.161	0.111
Standard deviation	25.7	0.29	0.13	0.027	0.088	0.098

Table 2.2 Genetic diversity statistics for each *Hakea carinata* population. P = the percent polymorphic loci, A = the mean number of alleles per locus, A_P = the mean number of alleles per polymorphic locus, H_o = the observed heterozygosity, H_e = the unbiased heterozygosity expected under Hardy-Weinberg, t = outcrossing rate

	Locus					
Population	PepD	Skdh-2	Skdh-1	Dia	Pgk	Gpi
BC	-	-	1.000***	-	1.000***	-
BU	0.796***	0.638***	-	-	1.000***	-
CL	-	-	-	0.618*	-	1.000***
CM	0.776***	0.916***	-	-	0.715***	-
CR	0.833***	0.751***	-	-	1.000*	-
CS	0.634*	0.582***	-	0.915***	0.874***	0.994***
GO	0.839**	1.000*	1.000*	-	-	-
HO	-	1.000***	-	1.000**	-	-
HS	0.409*	0.662*	0.402**	0.546*	0.943***	-
JS	-	0.689***	-	-	-	-
LW	-	0.507**	-	0.711***	-	1.000***
M1	1.000***	0.490**	-	0.760***	-	-
M2	-	0.753***	-	0.703***	-	1.000*
MB	1.000***	-	0.935***	1.000***	-	1.000***
MC	0.959***	0.736***	0.000	-	1.000***	1.000***
MM	0.882***	0.889***	-	0.842***	0.785**	1.000***
MO	-	0.518**	-	-	-	-
MR	-	-	-	-	-	-
MY	-	0.609**	0.000	0.000	1.000***	1.000***
NS	-	0.889***	0.277	1.000***	0.918***	-
OD	-	0.376	-	0.793***	-	-
PA	1.000*	-	-	-	-	1.000***
RT	-	-	1.000***	-	1.000***	-
SH	-	1.000***	-	-	-	1.000***
SM	-	0.777***	1.000*	0.899***	0.951***	-
VR	-	0.553**	-	0.548**	-	-
WA	-	-	-	-	-	-
WC	-	-	-	-	-	1.000
WS	1.000***	0.538**	-	0.598***	1.000*	1.000***
YU	0.892***	0.806***	-	0.741***	1.000***	0.929***

Table 2.3 Fixation indices (*f*) for each polymorphic locus. Results of exact tests for heterozygote deficiency; (*) $P < 0.05$, (**) $P < 0.01$, (***) $P < 0.001$

Locus withheld	F	θ_P	θ_S	f
Pep-D	0.893	0.436	0.822	0.397
Skdh-2	0.921	0.476	0.827	0.542
Skdh-1	0.911	0.484	0.835	0.460
Dia	0.905	0.469	0.812	0.493
Pgk	0.888	0.480	0.841	0.295
Gpi	0.896	0.506	0.840	0.349
Mean	0.901	0.481	0.833	0.427
Standard deviation	0.025	0.047	0.024	0.189
99% confidence int.	0.847, 0.957	0.299, 0.578	0.749, 0.878	0.012, 0.792

Table 2.4 Wright's *F*-statistics calculated by jackknifing over loci. Confidence intervals obtained by bootstrapping over loci with 5000 replicates. $F = F_{IT}$ (within total), $\theta_P = F_{PT}$ (among populations), $\theta_S = F_{SP}$ (among subpopulations), $f = F_{IS}$ (within subpopulations)

Genetic distances cluster populations similarly to geographic clustering (Figure 2.2) with few exceptions. The geographically distant south-eastern populations (MB and PA) fall together at a greater genetic distance from all other populations. The Mt Lofty Ranges distribution clusters fairly well into three distinct groups that correspond to the northern, central, and southern divisions of the range. The most northerly isolated population (MR) revealed no genetic polymorphism at all, as did one other population (WA), and they are not distinguishable from the main distribution.

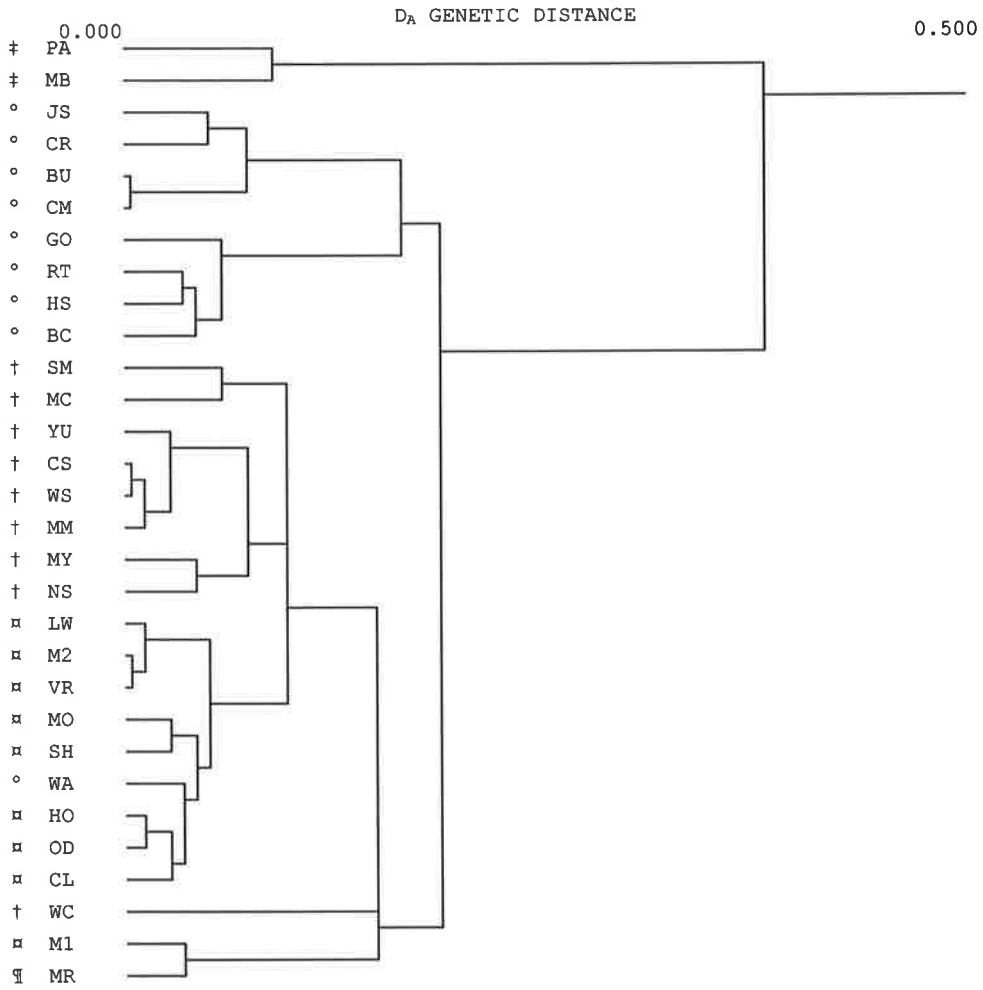


Figure 2.2 UPGMA dendrogram of *H. carinata* populations based on D_A genetic distance. Populations with the same symbols occur in the same geographic groups; from north to south, (¶) Mt Remarkable, (°) northern Mt Lofty Ranges, (¤) central Mt Lofty Ranges, (†) southern Mt Lofty Ranges, (‡) south-eastern SA

2.4 Discussion

Hakea carinata populations usually occur as small and often dense groups of individuals. These are often associated with limited habitat space such as roadside verges, small reserves by pine plantations, and creekbeds in steep gorges. They also occur in similarly small groups in large expanses of relatively uniform suitable habitat, suggesting that small populations are the natural distribution of the species rather than solely a result of any habitat modification or fragmentation. Preliminary observations suggest that both pollen and seed dispersal are low (G. J. Starr, unpublished data). A lack of seed dispersal may explain the low occupancy within suitable habitat.

The genetic data indicate a uniform high level of inbreeding in populations of *H. carinata*. Outcrossing rates in *Hakea* have not been reported heretofore. Previous work suggests that the Proteaceae are highly outcrossed and that examples of inbreeding are rare but this may be due to a bias toward *Banksia* in publications. Most of these *Banksia* species are highly outcrossed (eg. *Banksia spinulosa* and *B. paludosa*, Carthew *et al.* 1988, *B. ericifolia*, Carthew *et al.* 1996). The remaining species demonstrate a mixed mating system yet outcrossing rates are never lower than 0.65 (Coates and Sokolowski 1992, Sampson *et al.* 1994). *Grevillea* and *Persoonia* are less well represented in the literature. *Persoonia mollis* is highly outcrossed (Krauss 1994) and *Grevillea barklyana* exhibits mixed mating with some populations highly selfed, although these populations are all of fewer than 50 plants (Ayre *et al.* 1994). All of the Proteaceous species that have previously exhibited mixed mating are rare in contrast with the abundance of *H. carinata*. They do have in common however a distribution of small, isolated populations.

Total genetic diversity in *H. carinata* is comparable with plant species sharing similar characteristics (eg. regional geographic range, animal pollinated mixed-mating, Hamrick and Godt 1989) but little of that variation is held within populations. More than twice as much variation is accounted for between populations of *H. carinata* (G_{ST} = 0.469 against G_{ST} = 0.216 for species above). Other Proteaceous species demonstrate more typical levels of population subdivision, eg. *Banksia cuneata*, G_{ST} = 0.227 (Coates and Sokolowski 1992), *Persoonia mollis*, G_{ST} = 0.217 (Krauss

1997). Indications are that populations of *H. carinata* are genetically substantially isolated from each other and display symptoms of long term small population size. High levels of inbreeding, as demonstrated in this study, combined with strong genetic drift would account for the low levels of genetic variation held within populations, although drift need not be strong if population isolation occurred a substantial time ago.

Isolation due to land clearance and consequent fragmentation of native vegetation in South Australia has occurred in only the last 160 years. If the lifespan of individuals is greater than 25 years as appears to be the case (G. J. Starr, personal observation), then this is insufficient time for the species to have lost substantial diversity through increased inbreeding and drift caused by recent reductions in population size. The assumption of long term isolation of populations of *H. carinata* is supported by the high values of the *F*-statistics and the hierarchical clustering of genetic distances. The estimates of gene flow over all populations vary and range between $N_M = 0.129$ and $N_M = 0.270$ yet they are both much less than the minimum of one migrant per generation necessary to prevent independent evolution in these populations (Slatkin 1994).

Populations therefore appear to be acting as independent evolutionary units. There also appears to be a relationship between geographic distance and genetic differentiation. Populations at the periphery of the distribution could be expected to have become isolated from the main range longer ago and therefore be more divergent genetically. This appears to be the case with the distant south-eastern populations MB and PA. Similarly, populations that are geographically clustered into the northern, central, and southern parts of the main range should have been in genetic contact more recently than with other regions and thus be at lower genetic distances. The UPGMA dendrogram supports this conclusion.

This study has shown that breeding systems and population genetic structure in the Proteaceae are more diverse than previously thought. *Hakea* is poorly represented in studies to date, and would benefit from more investigation into the differences

between this genus and others in the family. Additional work on the scale of genetic subdivision in *H. carinata* is presented in the following chapters.

Hakea carinata deserves consideration for conservation for, while common, it is not locally abundant and populations may become extinct. At least three populations recorded in the Adelaide Herbarium appear to have become extinct in the last 25 years (G. J. Starr, personal observation). Populations may however be more at risk from demographic rather than genetic stochasticity (Lande 1988) as the species appears to have adapted well to small population size and high levels of inbreeding. Nevertheless, such a substantial part of the genetic diversity of *H. carinata* is accounted for between populations that a large number of populations will need to be protected if genetic diversity in this species is not to erode.

CHAPTER 3. GENE FLOW BETWEEN POPULATIONS

3.1 Introduction

Gene flow is a significant evolutionary force in the way it counters the effects of genetic drift and can therefore minimise the development of genetic differentiation between populations of a species. The amount of gene flow between populations determines whether the populations of a species evolve together or independently (Slatkin 1994). Gene flow of one or more migrants per generation is required to overcome independent evolution through genetic drift (Wright 1931). There are several reasons why gene flow in a species may be insufficient to homogenise genetic variation between populations, including low dispersal ability and physical barriers to dispersal. In species where dispersal is limited, gene flow is expected to decline with increasing geographic distance and this relationship was termed "isolation by distance" by Wright (1943). Although this was originally proposed for continuous populations there is expected to be a similar relationship in the stepping-stone model of geographically structured populations (Slatkin and Maddison 1990) where gene flow is between immediately adjacent populations.

Recent studies have sought to describe the mathematical relationship between gene flow and geographic distance in terms of regression analyses, using several different methodologies eg. Slatkin (1993), Rousset (1997). These methodologies enable estimates to be made of neighbourhood size ie. the number of individuals in the area from which parents of an individual can be drawn (Slatkin and Barton 1989), as well as giving indications of the geographical scale of gene flow between populations. A few empirical studies have addressed the geographic scale of gene flow in species using a regression methodology eg. Hellberg (1994), Peterson (1996), but this has rarely been done for plants. Studies of the scale of genetic differentiation in plant species have usually applied an hierarchical analysis of *F*-statistics eg. Williams and Guries (1994), McCue *et al.* (1996), which provides estimates of gene flow at discrete rather than continuous scales. The hierarchical method is a powerful analysis of

population genetic structure and is well established. On the other hand, the detection of isolation by distance using regression has yet to receive widespread use. This is despite its suitability for use for sampling at any geographic scale. The combination of hierarchical and regression analyses provides more complete information on the scale of dispersal.

Both the aforementioned statistical methodologies were applied to *H. carinata*. The previous chapter showed that there is genetic evidence of highly restricted gene flow in this species. The species is highly self-fertilising with outcrossing rates within 30 sampled populations varying between 0 and 32%. The level of genetic variation in the species is equivalent to other woody perennials (Hamrick and Godt 1989) but the proportion of diversity distributed between populations is very high (G_{ST} = 0.469). Overall gene flow (N_M = 0.28) derived from this estimate suggests that these populations are genetically isolated and are evolving independently.

I first conducted an hierarchical analysis of genetic differentiation at two scales. I then compared these findings with a regression analysis to provide an empirical example of this method and a comparison with the results of the hierarchical analysis.

3.2 Materials and Methods

3.2.1 Sampled Populations

The 30 populations of *H. carinata* sampled in the previous chapter cover a range of 500 km although most of these (27) are situated within 100 km of each other in the Mt Lofty Ranges. The Mt Lofty Ranges are the central and most populous part of the distribution of *H. carinata*. Land clearance in the Mt Lofty Ranges has been fairly extensive and the remnant vegetation is quite fragmented. Perhaps as a result of this, *H. carinata* occurs in the Mt Lofty Ranges in three relatively distinct geographic groups (Figure 3.1). Nine populations were sampled in each of these three groups. This analysis is confined to the genetic and geographical relationships between these 27 populations and three groups.

Samples for genetic assay were taken from transects through the widest dimension of each population and six seeds were collected from each of 10-12 plants. Sample sizes per population are described in Table 3.1. Some populations had fewer seed bearing plants, due either to bird predation (CL, YU) or small population size (GO, SH, WA, WC). Population VR had only a few fruits per plant so the number of plants sampled was increased to 39. Seed germination was less than 100% which meant that sample sizes were uneven; corrections for this are accounted for in the *F*-statistic analysis (Weir and Cockerham 1984).

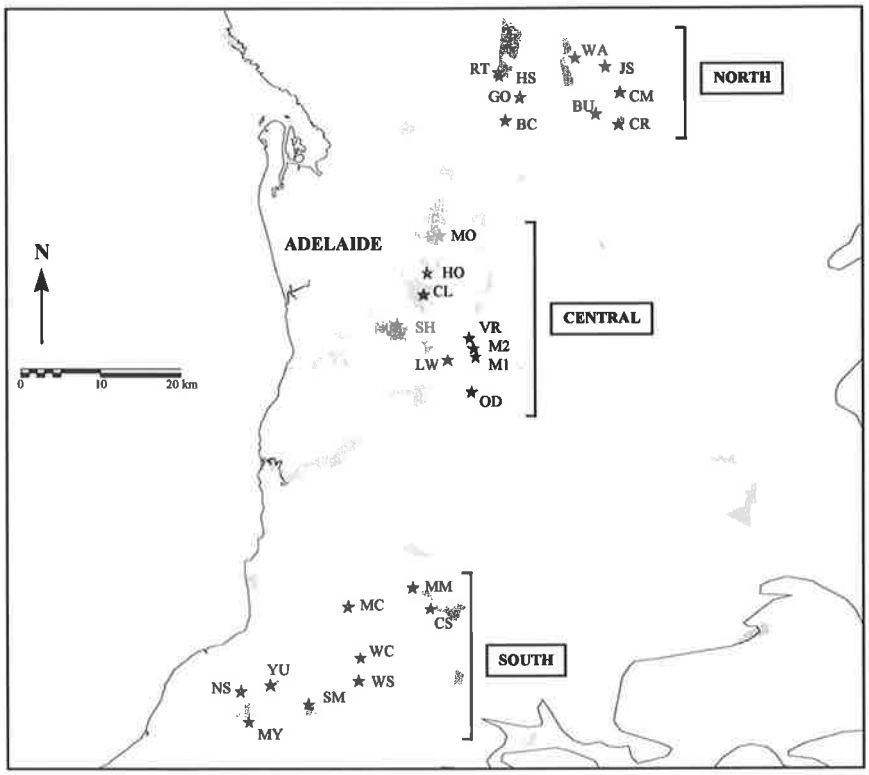


Figure 3.1 Distribution of 27 populations sampled from the Mt Lofty Ranges for genetic analysis. Groups (North, Central and South) correspond to an intermediate level of hierarchical structure used in *F*-statistic analysis. Shaded areas are remnant vegetation in parks

Site	Code	N_P	N_S
North			
Brewers Corner	BC	10	49
Bundy	BU	10	29
Cricks Mill	CM	10	37
Cromer	CR	10	47
Goods Gully	GO	9	24
Humbug Scrub	HS	10	48
Jenkins Scrub	JS	12	46
Para Wirra	RT	10	47
Warren	WA	5	19
Central			
Cleland	CL	8	19
Horsnell Gully	HO	10	20
Longwood	LW	10	44
Mylor #1	M1	10	41
Mylor #2	M2	10	49
Morialta	MO	10	39
Kuitpo	OD	10	48
Sheoak Rd, Belair	SH	5	13
Vimy Ridge	VR	39	39
South			
Cox's Scrub	CS	10	47
Mt. Compass	MC	10	51
Mt. Magnificent	MM	10	45
Myponga	MY	10	38
Nixon-Skinner	NS	10	37
Spring Mount	SM	10	47
Wood Cone	WC	2	7
Sth. Wood Cone	WS	10	31
Yulte	YU	8	30

Table 3.1 Populations sampled from the Mt Lofty region for estimates of gene flow. N_P, number of plants sampled per population; N_S, number of seeds sampled per population

3.2.2 Data Analysis

Genetic variation in *H. carinata* was assessed using the genetic data from gel electrophoresis as described in Chapter 2. Populations were defined as continuous stands of plants clearly separated by at least 500 m from the next nearest stand. Previous work had indicated that sufficient genetic distance occurs between stands at this geographic scale to identify them as distinct populations (Chapter 2).

Total genetic variation was divided into variation within populations as defined above, as well as into three groups representing the distinct regional clustering of populations in the Mt Lofty Ranges (Figure 3.1). The definition of the regional groups was originally based on the geographical clustering of populations that was evident from sampling all readily identifiable populations of *H. carinata* in the Mt Lofty Ranges, but was supported by the results of the cluster analysis in Chapter 2. This showed that genetic relatedness appeared to bear a clear relationship with the geographical grouping. As a result, this enabled the analysis in a nested hierarchy of population genetic structure. Genetic differentiation between components was assessed at two scales; between populations, and between groups of populations. Each of the geographical groups at the highest scale were composed of nine sampled populations.

Genetic differentiation at each level of the hierarchy was quantified using Wright's *F*-statistics. Hierarchical *F*-statistics were calculated following the procedures of Weir (1990) and using the program GDA (Lewis and Zaykin 1997). Total inbreeding (F) was divided into inbreeding within populations (f), and the proportion of inbreeding distributed between populations within groups (θ_P), and between groups within the total (θ_G). Means and standard deviations of *F*-statistics were obtained by jackknifing over loci. The significance levels of the multilocus *F*-statistic estimates were expressed as 99% confidence intervals, calculated from 5000 bootstrap replicates. Separate *F*-statistics were calculated without hierarchical clustering for each geographical group. Population differentiation was expressed as θ and jackknifed means and standard deviations and confidence intervals were calculated as before.

Gene flow was assessed in two ways, firstly as numbers of migrants, N_M, between the geographically defined units (populations, groups) in the island model, and secondly as neighbourhood size, N_b, in the lattice or continuum model. The first method is related to the commonly used method of reporting a single N_M value for an entire species. It also provides additional information on the scale at which gene flow exceeds unity and at which geographically defined units may be evolutionally linked. The second method provides additional information on the scale of gene flow without being limited by the sampling scale, and takes into consideration the kurtosis of dispersal distributions that is expected to occur in natural populations (Rousset 1997).

Gene flow in an island model is quantified as N_M, the number of gametes or individuals that migrate between populations or subdivisions. Gene flow was calculated for both levels of the hierarchy using the relationship,

$$N_{Mx} = \tfrac{1}{4}\left(1/\theta_x - 1\right) \quad \text{(Wright 1951)}$$

where x is the level of the hierarchy and $\theta = F_{ST}$ (Weir and Cockerham 1984). Gene flow was similarly calculated for each geographical group; North, Central, and South.

Gene flow in the lattice or continuum model it is given as N_b, the neighbourhood size (Wright 1969). The neighbourhood size is approximately the number of individuals who together form the genetic pool from which the parents of an individual would have come. It has been shown that for a self-compatible species $N_b = 4D\pi\sigma^2$ (Wright 1969, Slatkin and Barton 1989), where D is the density of individuals and σ is the standard deviation of the distance moved by each gamete in any one direction. The calculation of N_b proposed by Rousset (1997) involves the least squares linear regression,

$$\theta/(1-\theta) = a + b\ln(\text{distance})$$

where $\theta = F_{ST}$ (Weir and Cockerham 1984) and the inverse of the slope b gives an estimate of N_b.

The least squares regression was done using all (351) pairwise combinations of populations. An assumption of this methodology is that statistically significant estimates of genetic differentiation between populations are evenly distributed among the pairwise comparisons and are not due to only one or a few populations (Bossart and Prowell 1998). To test if this assumption was violated, each pairwise estimate of θ was tested using permutation procedures to see if it was significantly greater than zero. This involved permuting multi-locus genotypes among samples using the program FSTAT (Goudet 1995). For each significance test a total of 5000 permutations was used. Overall, 98% of estimates of θ between pairwise combinations of populations were significantly greater than zero, and no population had more than two non-significant values. It was taken that the assumption of the regression analysis was satisfied.

3.3 Results

The hierarchical *F*-statistics showed that the scale of the greatest mean genetic difference was between populations within groups rather than between groups (Table 3.2). Values of θ_P were high at all loci with the jackknifed mean being 0.407. This translated to an estimated gene flow of $N_{MP} = 0.31$ migrants between populations. Somewhat incongruously, at the higher level in the hierarchy the genetic differences between divisions were less, with $\theta_G = 0.150$ between groups. The gene flow estimate derived from this results was $N_{MG} = 1.42$.

Overall fixation was high ($F = 0.878$) and all *F*-statistics were significantly positive as indicated by the 99% confidence intervals being greater than zero (Table 3.2).

	F-statistics				
Locus	*F*	*f*	θ_G	θ_P	θ_S
Pep-D	0.872	0.372	0.134	0.371	0.796
Skdh-2	0.903	0.481	0.170	0.413	0.813
Skdh-1	0.889	0.394	0.157	0.402	0.817
Dia	0.881	0.430	0.126	0.378	0.791
Pgk	0.867	0.204	0.153	0.432	0.833
Gpi	0.871	0.295	0.156	0.428	0.818
Mean	**0.878**	**0.366**	**0.150**	**0.407**	**0.814**
Std.dev.	0.028	0.203	0.033	0.052	0.032
99% CI	0.824, 0.945	0.017, 0.778	0.083, 0.216	0.256, 0.515	0.724, 0.875

Table 3.2 Population genetic statistics for each polymorphic locus for 27 populations in the Mt Lofty region. Means and standard deviations obtained by jackknifing over loci. Confidence intervals obtained by bootstrapping over loci with 5000 replicates. Wright's *F*-statistics; *F* = total inbreeding, *f* = inbreeding within sub-populations, θ_G = subdivisions between groups within the total, θ_P = populations within groups, θ_S = individuals within populations

Group	Maximum pairwise population distance (km)	θ_P (99% confidence interval)	N_M
North	16.6	0.507 (0.225-0.617)	0.24
Central	20.6	0.260 (0.020-0.273)	0.71
South	27.2	0.254 (0.116-0.423)	0.73

Table 3.3 Jackknifed means and bootstrapped confidence intervals

Jackknifed mean estimates of θ_P for each region were similarly significantly greater than zero. The estimates of gene flow (N_M) calculated from θ for populations within groups were less than one for each group; North, Central, and South (Table 3.3). These were for population groups separated by maximum distances of between 16.6 km and 27.2 km.

The regression analysis using all pairwise combinations of 27 populations indicated that the level of gene flow was influenced by the geographic separation between them. The regression produced the equation $\theta/(1-\theta) = 0.104 + 0.188$ ln distance (Figure 3.2). The 'slope' estimate of neighbourhood size using this methodology gave $N_b = 1/0.188 \approx 5$ plants. The low R^2 of 0.03 is unimportant as the relationship is used only to predict neighbourhood size. Of the 351 pairwise population comparisons, 282 (80%) produced estimates of N_M of less than one. Similar regression analyses for within regions using all pairwise combinations of nine populations produced non-significant equations. The range of distances between populations within groups was small, and as the regression used the natural log of distance there remained very little variation to provide power to the analysis.

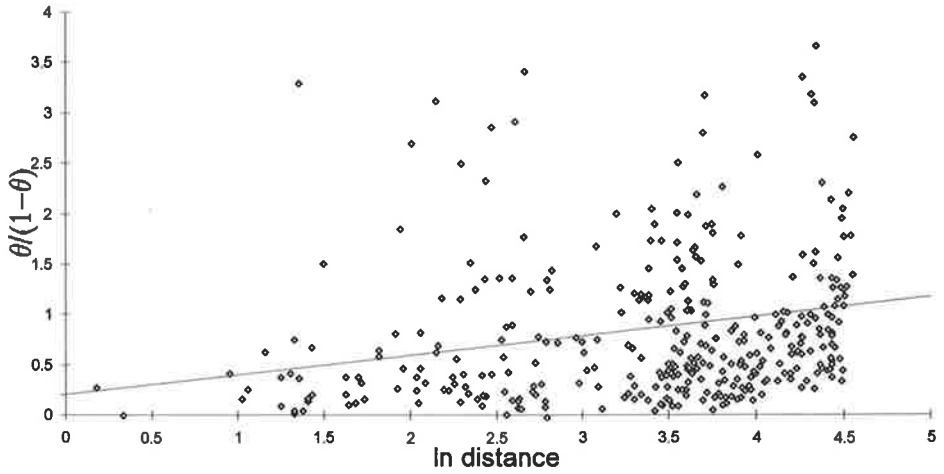


Figure 3.2 Regression of $\theta/(1-\theta)$ between pairs of populations (using θ_P) against log of geographic distance (km) for all pairwise combinations of populations.
Regression line equation: $\theta/(1-\theta) = 0.104 + 0.188 \ln \text{distance}$.

3.4 Discussion

The previous analysis of genetic variation in *H. carinata* (Chapter 2) demonstrated that average gene flow over the distribution of the species (30 sampled populations from a range of 500 km) was much less than one migrant per generation, and therefore insufficient to prevent independent evolution of populations. This chapter indicates that the scale of this isolation is between populations at the smallest scale. This level of isolation between reproductive units is atypical of species in the family Proteaceae. Proteaceous species usually exhibit outcrossed reproduction and high gene flow at geographical scales similar to this study eg. Coates and Sokolowski (1992), Carthew (1993), Harwood *et al.* (1997), Krauss (1997), and the few exceptions are rare or highly restricted species eg. Ayre *et al.* (1994). Plant species in general usually exhibit sufficient gene flow to homogenise genetic variation between populations and only those with very high selfing rates are likely to have highly restricted gene flow (Hamrick and Godt 1989, Ellstrand 1992), however the scale of isolation was not addressed in these studies.

The pattern of increasing genetic differences with distance is expected if gene flow occurs only between neighbouring populations, assuming that gene flow and drift have reached equilibrium (Slatkin 1993). It is highly likely that the system is at equilibrium if isolation by distance is detected by the means used here (Ouborg *et al.* 1999), increasing the confidence one can have in the gene flow estimate produced by the indirect methodology used in Chapter 2. Isolation by distance has been detected in a wide variety of organisms, from forest herbs (Williams and Guries 1994), to coral (Hellberg 1994) and butterflies (Peterson 1996). Williams and Guries (1994) investigated genetic differentiation between population subdivisions in three ecologically similar forest herb species. They measured these differences at discrete scales; between population subdivisions of a few metres apart, between populations at tens of kilometres, and between drainages at hundreds of kilometres. As predicted, for all three species there was increasing genetic differentiation as geographic distance increased. There was also a difference between the species in terms of their hypothesised seed dispersal abilities, based on seed morphology. At the smallest scale, gene flow estimates were significantly greater for species with seeds better adapted for attachment to fur.

Hellberg (1994) used both *F*-statistics and regression to describe gene flow in a coral species. The regression of log gene flow against log distance showed the expected increased isolation over greater distances. The estimates of gene flow calculated from θ were higher for patches separated by up to 30 m within populations than between populations, however the between population scale included distances of up to 3000 km.

In a species of sedentary butterfly it was found that, although gene flow estimates over distances of 100 km between populations often exceeded $N_M = 10$, a regression methodology estimated neighbourhood size to be only approximately 39 individuals (Peterson 1996). The regression also demonstrated that gene flow decreased with distance over the scale of several hundred kilometres.

In these studies, isolation by distance was detected only if populations were sampled over a geographically large scale. Gene flow at smaller scales was usually sufficient to homogenise genetic variation between sampling units. In contrast, the indications from the present study are that isolation by distance in *H. carinata* is operating at the smallest sampling scale, between sampled sites within populations.

The hierarchical *F*-statistics presented here suggest that gene flow is greater over long distances than short distances. Genetic differences were greatest at the smallest scale, between populations and the extent of this differentiation was substantial, with the θ_P value giving a gene flow estimate of $N_M = 0.31$. The genetic differences were less at the highest scale between groups where the gene flow estimate exceeded unity ($N_M = 1.42$). There are two main explanations for this observed pattern. Firstly the high degree of self fertilisation demonstrated in this species creates patterns of differentiation between neighbouring populations, but the pattern weakens and is lost as the scale increases. The low rate of outcrossing, in some populations estimated as zero (Chapter 2), has led to fixed genetic differences within populations. As greater distance cannot increase this differentiation between sampling units, comparisons of genetic differences over larger scales is meaningless. Secondly, the narrowness of the genetic base, as demonstrated by the low allelic diversity ($A = 1.53$, Chapter 2), means

there are only a limited number of multilocus genotypes detected by the electrophoresis. Hence, at larger scales the subdivisions are between groups containing repeated small scale patterns.

The notion of increasing genetic differentiation with greater distances between pairs of populations is supported by the regression model. In particular, the estimate of neighbourhood size that results from this model suggests that dispersal is extremely limited within populations as five individuals is very much less than the size of most of the sampled populations. Genes appear to be exchanged only between plants in small clumps of a few individuals. The comparison between all pairwise combinations of populations, used in the regression analysis, showed that in 80% of estimates gene flow was much lower than one migrant per generation.

I have defined populations as relatively continuous distributions of individuals, clearly separated from the nearest occurrence of the species. In *H. carinata* populations, plants are not distributed regularly within stands. Populations occur as repeated clusters of often ten or more plants with occasional isolated plants scattered between clusters. I have defined any separation between stands of greater than 500 m as being the division between populations. This arbitrary classification of populations has been used because *H. carinata* is not the dominant species in its habitat, but is distributed in gaps wherever it apparently has had the opportunity to establish. The clustering seems to be the result of several seeds establishing in the same gap simultaneously, or of dispersed seeds having greater success in establishing close to the parent plant.

The combination of pollen movement mainly within plants and successful seed establishment predominantly near the parent best explains the low neighbourhood size of approximately five plants. In previous studies genetic structure has been attributed to restricted dispersal in either pollen or seed. Loveless and Hamrick (1984) concluded in their review that the breeding system, and hence pollen dispersal, is the principal factor determining genetic structure in plant populations. In particular, selfing species are more likely than outcrossing species to demonstrate considerable levels of local differentiation (Heywood 1991). In contrast, Williams and Guries (1994) concluded that in *Sanicula odorata*, *Osmorhiza claytonii*, and *Cryptotaenia*

canadensis it is seed dispersal that predominantly affects the level of gene flow within each species. These species have different seed morphologies that directly influence their dispersal capabilities and thus the scale of gene flow. In either instance the assumption is that the alternative mode of dispersal is more restricted and doesn't contribute significantly to gene flow. The indications in *H. carinata* are that both pollen and seed dispersal are highly restricted, without suggesting which is the predominant influence on genetic structure.

Rare long distance dispersal has been invoked to explain weak relationships between genetic differentiation and geographic distance (Ritland 1989, Hellberg 1994, Williams and Guries 1994). This explanation is not supported by the findings for *H. carinata*. For long distance gene flow to produce the patterns of genetic differentiation indicated for *H. carinata* it would have to be more frequent than local gene flow between populations separated by a few kilometres, or even between plants within populations. It is more likely that the small neighbourhood sizes that are apparent in these populations cause the equilibrium between gene flow and drift to be heavily biased toward drift as the determining force in genetic differentiation. Drift has been postulated as being a major cause of noise in the relationship between genetic differentiation and geographic distance (Peterson 1996). This would account for the lack of strength in the regressions of gene flow and genetic difference in *H. carinata* despite the obviously very low levels of gene flow between populations.

The importance of sampling at various spatial scales to distinguish between processes occurring at regional, local and sub-population level has been emphasised (Loveless and Hamrick 1984). Regression models enable estimates to be made of the scale at which isolation by distance occurs in species. The indications from this study are that substantial genetic structuring occurs within populations of *H. carinata*, and additional work to investigate this phenomenon at the smallest possible scale is presented in the following chapter.

CHAPTER 4. SPATIAL STRUCTURE WITHIN POPULATION

4.1 Introduction

Population genetic differentiation is shaped by restricted gene flow, selection and genetic drift (Levin and Kerster 1974). Gene flow is an important constraining force in evolution as it homogenises genetic variation between populations and prevents speciation. In contrast, restricted gene flow can lead to adaptive evolution by allowing local gene pools to evolve independently (Wright 1977). Studies of genetic differentiation between and within populations are therefore of substantial evolutionary interest.

Most plant populations are genetically structured. This can result from a variety of factors including the mating system and dispersal capabilities. Many life history traits of plant species have been correlated with population genetic differentiation (Hamrick *et al.* 1979, Loveless and Hamrick 1984, Hamrick and Godt 1989) and with spatial sub-structuring within populations (Epperson 1995, Heywood 1991). The mating system is a significant determinant of structuring, with selfing species exhibiting far more genetic differentiation than outcrossing species. Spatial structure of population genetic variation is readily described but the description is incomplete without inferences about the evolutionary forces that have caused the observed pattern.

Substantial theoretical work exists on the spatial patterns created by evolutionary forces in populations, in particular examining various combinations of life history traits. These studies have used mainly computer simulations, with their advantages of being able to follow changes in populations over many generations and incorporate various combinations of evolutionary processes (Slatkin and Arter 1991, Sokal and Jacquez 1991). Little has been done to apply these models to empirical data. Analyses to date of spatial genetic structure in plant populations have found little significant structuring except at very short distances, usually only a few metres eg. Berg and Hamrick (1995), Campbell and Dooley (1992), Geburek and Tripp-

Knowles (1994), Montalvo *et al.* (1997), Perry and Knowles (1991), Schnabel *et al.* (1991). An opportunity exists to apply these models to *H. carinata*, which has been shown to have a very small neighbourhood sizes and therefore may display substantial structuring (Chapter 3). This chapter will examine the extent to which the models can suggest the evolutionary forces creating spatial structure within populations of *H. carinata*.

Selection, and the spatial genetic pattern created by it, has been difficult to detect in continuously distributed populations. These populations often occur in habitats that are substantially homogeneous, which suggests that selection may also be homogeneous there. In contrast, patchiness or neighbourhoods will develop within continuous populations in species where dispersal is limited because of isolation by distance (IBD), rather than selection. Wright's (1946) definition of the neighbourhood, as the group with which an individual mates at random, is based on IBD as the cause of population sub-structuring. Wright (1946) concluded that if neighbourhood size is less than 20 there can develop substantial differences between neighbourhoods in a continuous population. IBD has been used to explain spatial genetic structure within plant populations in homogeneous environments (Geburek and Tripp-Knowles 1994, Schaal 1975, Schoen and Latta 1989, Shapcott 1995). Selection has rarely been proposed as a significant cause of structure in these homogeneous environments. This hypothesis has yet to be tested as a statistical test to differentiate between patterns caused by the different evolutionary forces has been lacking.

Sokal and Jacquez (1991) proposed a solution to this problem. They used a simulation study to test whether statistical analyses of spatial genetic structure can be used to differentiate between spatial patterns caused by either selection, long distance migration or IBD, or various combinations of these. This methodology, although not widely adopted, is potentially useful because of the way it compares competing hypotheses.

Hakea carinata is a suitable subject species on which to test these hypotheses. It is highly selfing, a trait which itself may create substantial population sub-structuring.

Population differentiation between 30 populations from the entire range of the species suggested that populations are substantially isolated (F_{ST} = 0.481) (Chapter 2), and that neighbourhood size is approximately 5 individuals (Chapter 3). The hierarchical analysis of population differentiation using a nested ANOVA of *F*-statistics in *H. carinata* (Chapter 3) enabled the genetic structuring to be studied at discrete scales. The degree of differentiation between populations, however, is such that they are substantially isolated, even over short distances. The use of spatial autocorrelation analysis within a continuously distributed population can provide a more complete description of spatial genetic structure (Heywood 1991) because of the ability to use distance as a continuous rather than a discrete variable. This study applies spatial autocorrelation analysis, using both interval and nominal data, to a genetic data set from one large population of *H. carinata*, and uses the methods of Sokal and Jacquez (1991) to infer microevolutionary processes.

4.2 Materials and Methods

4.2.1 Study Organism and Site

The study organism has been described previously (Chapter 2). The data used for analyses in previous chapters was taken from 30 populations covering the range of the species. Sampled plants did not have their precise geographic location within the population recorded. A single geographic reference point was taken instead to represent the entire population. The present analysis required a large sample to be taken from a single continuous population. The geographic reference for each plant was recorded to enable accurate distances to be calculated between all pairwise combinations of plants. The sample was taken from a large population, Humbug Scrub (HS), that was previously included in earlier analyses, however an entirely new sample was taken and none of the data collected in the original sample were used in the present analysis. This population was chosen for a number of reasons; the previous sample from this population displayed genetic polymorphism at most of the loci assayed, it was one of the largest extant populations observed, it was surrounded by large areas of relatively undisturbed native vegetation thereby minimising edge effects, and it was close enough to Adelaide to enable frequent visits.

The study site covered approximately 100 ha and was part of the Para Wirra Recreation Park, approximately 40 km north east of Adelaide. The sampled population of *H. carinata* occurred in the southern part of the park, which carried the local name of 'Humbug Scrub'. The vegetation was dry sclerophyll woodland dominated by *Eucalyptus goniocalyx* and *E. fasciculosa*.

The population of *H. carinata* comprised approximately 2000 plants distributed in a manner typical for this species. There were many dense clumps of twenty or more plants and these clumps were irregularly spaced throughout the population, between which there are numerous solitary individuals. The species did not dominate the understorey except for where it had formed these dense clumps and was able to exclude other species. The remainder of the understorey was characterised by *Xanthorrhoea semiplana*. The next nearest population of *H. carinata* occurred at a distance of 500 m. *Hakea carinata* was absent from steep gullies and creekbeds, but appeared to have no preference for any of the remaining habitat, which was fairly homogeneous. The study population was bounded in an area approximately 600 m by 600 m. The sample was taken from the entire area of the population by randomly sampling several plants from every clump and sampling every solitary individual. In total 400 plants were sampled. All plants were accurately located to ±5 m on the UTM (Universal Transverse Mercator) coordinate scale using a differential GPS (Global Positioning System) unit.

Maternal genotypes were determined from progeny arrays as mature leaf tissue proved to be unsuccessful in producing isoenzyme activity under electrophoresis. Eight seeds were taken from each parent plant and germinated in petri dishes. It was not necessary to assay all of these seeds for every plant as the maternal genotype could be inferred from fewer seeds, particularly in those circumstances where a species is highly selfing and has high homozygosity, as in *H. carinata*. The efficiency of progeny sampling was maximised by using design IV of Brown (1975) where initially two seedlings per family were assayed. If both seeds were homozygous for the same allele then this was accepted as the maternal genotype. If either of the seeds were heterozygous then the sample was increased to the full eight seeds. This method was developed for diallelic loci (Brown 1975). All loci sampled in this population were diallelic. The maternal

genotype was inferred from the arrays of eight seeds using the computer program MLTR (Ritland 1990).

Cellulose acetate gel electrophoresis was performed on cotyledons of sprouted seeds. Five putative loci from four enzyme systems showed polymorphism in this population; peptidase-D (PEP-D, EC 3.4.13.9 using substrate Phe-Pro), shikimate 5-dehydrogenase (SKDH, EC 1.1.1.25) (2 loci), diaphorase or dihydrolipoamide dehydrogenase (DIA, EC 1.8.1.4), and phosphoglycerate kinase (PGK, EC 2.7.2.3).

4.2.2 Data Analysis

Loci were analysed for linkage disequilibrium using *G*-tests with Williams' correction (Sokal and Rohlf 1981). Locus Pep-D showed linkage with both Skdh-2 and Pgk and was therefore omitted from further analysis. No other significant disequilibria were found for any combinations of the remaining four loci.

Spatial genetic structure was analysed using spatial autocorrelation of allele frequencies in 50 m by 50 m quadrats, and gene frequency surfaces (spatial maps of frequencies in quadrats). This analysis follows the procedures of Sokal and Jacquez (1991). The theoretical expectations are that comparisons across loci and between correlograms and surfaces should differ under different microevolutionary regimes (Table 4.1).

The processes of interest are selection, migration, and IBD. Each operating alone will create non-random spatial distributions of alleles that are, in theory, measurably different. Selection will affect allele frequencies at one or at most only a few loci at a time. Different selection pressures should be uncorrelated and produce spatial patterns of different size and location. Under selection it is expected that the correlograms from the spatial autocorrelation analysis will be dissimilar and that the gene frequency surfaces will also be dissimilar (Sokal and Jacquez 1991) (Table 4.1).

Migration, or long distance gene flow, will cause similar spatial patterns at all loci at which the immigrant gene frequencies differ substantially from the population. At loci where these frequency differences are small it may be difficult to detect migration.

These similar gene frequency surfaces should produce correspondingly similar correlograms (Table 4.1). Strongly similar surfaces and dissimilar correlograms is a combination that is mathematically impossible (Sokal and Jacquez 1991).

IBD operates independently at every locus and therefore the gene frequency surfaces should be uncorrelated and dissimilar. However as the spatial process, ie. restricted dispersal distance, is the same at every locus their correlograms should be similar (Table 4.1).

	Similar correlograms	Dissimilar correlograms
Similar gene surfaces	Migration	Not possible
Dissimilar gene surfaces	Isolation-by-distance	Selection

Table 4.1 Combinations of correlograms and gene frequency surfaces at multiple loci, and expected causes of significant spatial genetic sub-structuring

Correlograms were generated by spatial autocorrelation analysis of allele frequencies in quadrats for one allele at each of the four loci. Each locus was diallelic therefore the correlogram for each alternate allele was exactly the same (Slatkin and Arter 1991). The population was divided into 50 m quadrats using UTM coordinates derived from the GPS. Allele frequencies were calculated from maternal genotypes within each quadrat, which contained from 1 to 32 plants. Distances between quadrats were measured as Euclidean distance between coordinates taken as the central point in each quadrat. Autocorrelation coefficients between all pairwise comparisons of quadrats were calculated as Moran's I, using the computer program SAAP v4.3 (Spatial Autocorrelation Analysis Program) (Wartenberg 1989). This program also provides significance testing of individual autocorrelation coefficients and the significance of entire correlograms using Bonferroni approximation. The autocorrelation coefficients were calculated at 13 equal size distance classes up to the highest distance of 651.9 m, giving intervals of approximately 50 m.

Gene frequency surfaces were tested for spatial heterogeneity using a *G*-test (Sokal and Rohlf 1981). All pairwise comparisons of surfaces were tested for correlation to determine if there were any significant similarities between them. Average Manhattan distances (Sneath and Sokal 1973) were calculated between all pairs of correlograms. There are, however, at present no suitable tests for significant differences between correlograms (Sokal and Oden 1991) and this procedure has only been used for simulation data where a large number of correlograms can be grouped into classes of similarity. There are only six possible pairwise comparisons of correlograms in this study and therefore all combinations are used regardless of similarity.

The analysis of gene frequencies in quadrats does not give information on processes occurring at distances smaller than quadrat size ie. 50 m in this example. The above-mentioned analysis required that the same quadrats be used for both autocorrelation and gene surfaces. Additional information on spatial structure is available if diploid genotypes of individual adult plants are used rather than converting these into gene frequencies in quadrats and calculating Moran's I statistics (Epperson 1995). Join count autocorrelation examines every pairwise combination (join) between adult plants by comparing whether their diploid genotypes at each locus are identical (like joins) or different (unlike joins). Join count autocorrelation provides information on the concentration of homozygotes in patches as well as the scale of intermixing of heterozygotes and homozygotes (Epperson 1995). Differences in cluster sizes between genotypes may be due to spatial variation in the mating system, and this information is not provided by the spatial distribution of allele frequencies (Heywood 1991). Locus Dia was excluded from the analysis as 99.5% of the genotypes in this population were of the common homozygote and no alternate homozygotes were detected.

Genotypes were designated according to whether alleles were common (A, $p>0.5$) or uncommon (a, $p<0.5$). Heterozygotes were rare in this species at all loci examined. Pairwise comparisons of each plant to every other plant provided counts of the number of joins in each of the six possible combinations of genotypes at these diallelic loci. These combinations were AA:AA, aa:aa, AA:aa, AA:Aa, aa:Aa, and Aa:Aa. These counts were divided into distance classes at 10 m intervals and compared to the

number of joins expected if genotypes were randomly distributed. In each distance class the SND (standard normal deviate) (Sokal and Oden 1978) was calculated and plotted on a correlogram. This was done here for distance classes up to 200 m. Distance matrices and SNDs for each distance class were calculated using the R Package (Legendre and Vaudor 1991).

Values of these spatial autocorrelation statistics vary with levels of dispersal, which affects the scale of spatial distribution, and with sample size (Epperson and Li 1997). The statistics estimated for the shortest distance class in each of the comparisons between like homozygotes, unlike homozygotes, and total unlike joins were compared to predicted values provided by Epperson and Li (1997). These predicted values are necessary as the expected probabilities of identity by descent in isolation by distance models cannot be observed directly in actual populations. The variance of these statistics are unknown from empirical data as only one outcome from the range of probable outcomes of the evolutionary process exists.

As such it is necessary to use computer simulations to determine the distribution of these statistics. The distributions of these statistics are influenced by the simulated dispersal model, that is an expression of dispersal distances as the number of surrounding individuals from which the parents of an offspring are chosen. A greater number of potential parents equals a greater dispersal distance. The distributions of these statistics are also affected by the sampling, in terms of what proportion of individuals from a given area are included in the sample. With these two conditions in mind, Epperson and Li (1997) generated computer simulations of evolutionary scenarios of isolation by distance, in the absence of selection, that yielded distributions of the spatial autocorrelation statistics. From these distributions, expected values for pure isolation by distance models were produced in the tables they published (Epperson and Li 1997).

The expected spatial autocorrelation statistics produced by Epperson and Li (1997) suit sampling schemes that exactly match their simulations. To make practical use of these tables where actual sampling differed from that used in the simulations, it is necessary to extrapolate values from those in the tables that are nearest to the actual.

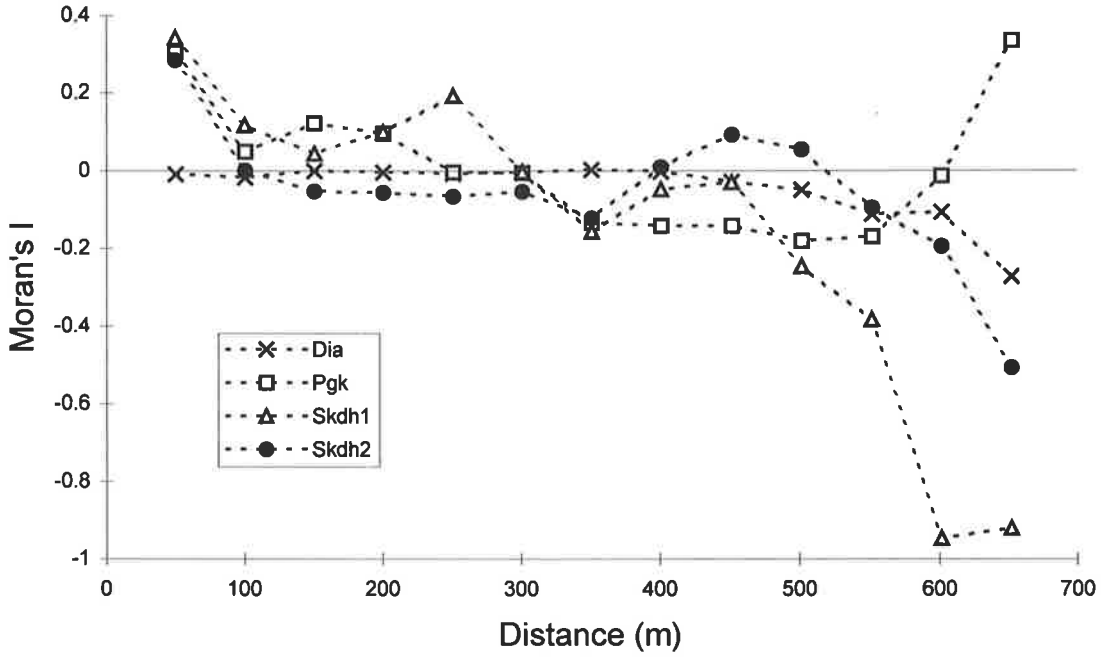
The procedure for making these adjustments was kindly provided by Bryan Epperson (pers comm.). Corrections to predicted SND values were made for variations in sample size by multiplying predicted SNDs by the square root of the ratio between the actual sample size at each locus and the next nearest size in the tables (n = 625). The porosity of sampling, or the proportion of the population included in the sample, was taken as 1/4 in the tables of Epperson and Li (1997), which was closest to the estimated value of 400/2000 = 1/5. The mean observed SNDs were compared to corrected predicted values for the three simulation dispersal models closest to observed patterns. These were models with neighbourhood sizes of 4.2, 8.4, and 12.6 (Epperson and Li 1997), and compare to previous estimations of neighbourhood size in *H. carinata* of 5 (Chapter 3).

4.3 Results

The correlograms for gene frequencies in quadrats revealed significant clustering of alleles at all four loci (Figure 4.1a). All correlograms were significant at either $\alpha = 0.05$ (Skdh-1, $p < 0.001$; Skdh-2, $p = 0.050$; Dia, $p = 0.048$) or $\alpha = 0.1$ (Pgk, $p = 0.055$). Correlograms for loci Skdh-2, Skdh-1 and Pgk indicated positive correlations at shorter distances and negative correlations at longer distances, with the highest correlation coefficients being approximately 0.3 within the shortest distance (50 m) class. All significantly positive coefficients were in distance classes less than 400 m and significantly negative coefficients at distances greater than 400 m (Figure 4.1b). The correlograms did however vary, with averaged Manhattan distances of standardised Moran's I ranging from 0.83 to 1.51.

Gene frequency surfaces were each highly heterogeneous, with the exception of locus Dia in which all but one quadrat were totally homozygous for the common allele (Figure 4.2). This locus was omitted from the remaining spatial autocorrelation analysis of join counts. *G*-tests for heterogeneity were significant at $p < 0.001$ for the other three loci. Gene surfaces were not correlated between loci, with correlation coefficients for pairwise comparisons ranging from $r = 0.017$ for Skdh-1/Pgk to $r = 0.127$ for Dia/Pgk.

a)



b)

	Distance class (m)												
Locus	50	100	150	200	250	300	350	400	450	500	550	600	650
Skdh-2	+ +												–
Skdh-1	+ +				+ +					– –	– –	– –	– –
Dia							+				–		– –
Pgk	+ +		+							–			

Figure 4.1

a) Correlograms of spatial autocorrelation of alleles in 50 m × 50 m quadrats at Humbug Scrub population. Moran's I calculated for one allele at each of four diallelic loci.

b) significance of association in each distance class for each locus: positive, + P<0.05, + + P<0.01; negative, – P<0.05, – – P<0.01

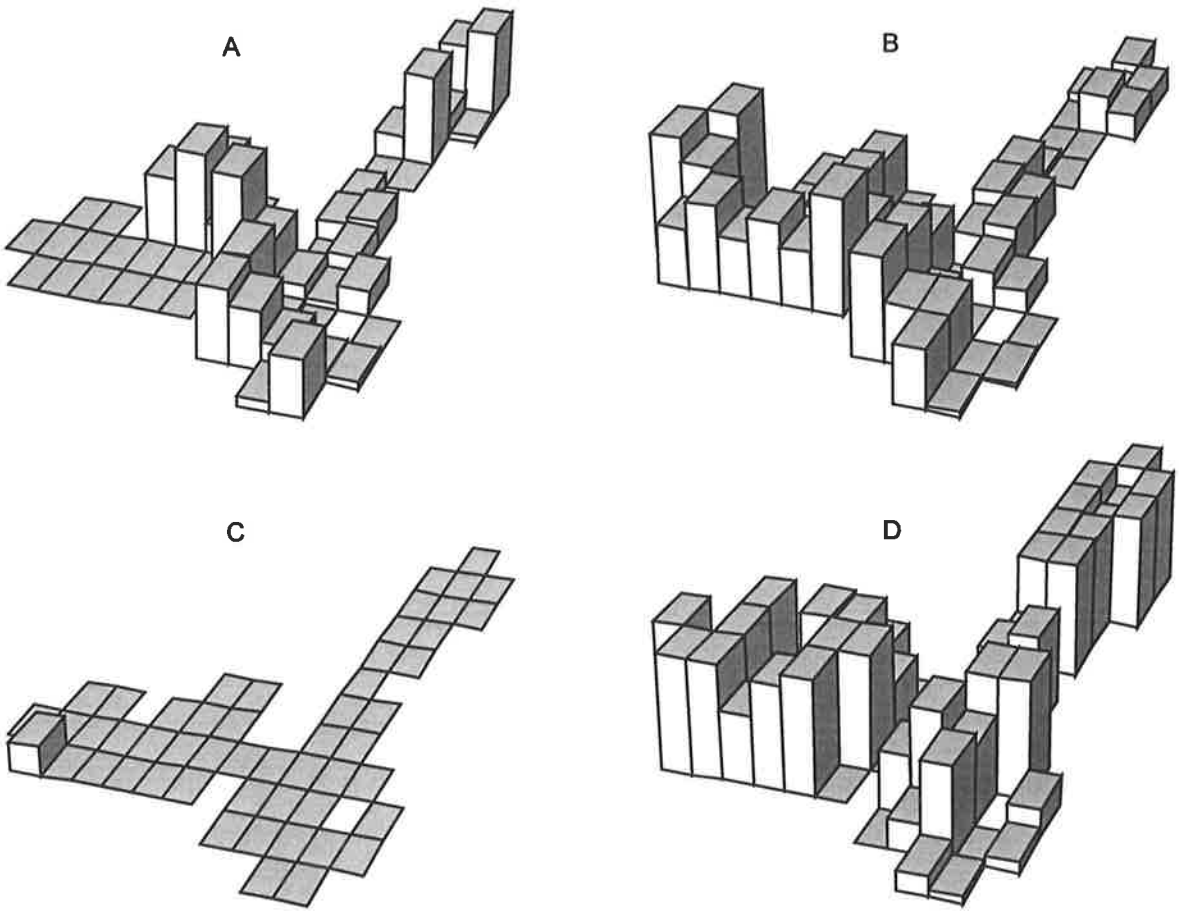


Figure 4.2 Gene frequency surfaces for single alleles at diallelic loci in *H. carinata* at Humbug Scrub population. Frequencies are means for sampled individuals in 50 m × 50 m quadrats. Columns are all to the same scale, with highest columns representing frequency of 1.0 Quadrats are shown in geographic relation in population; absence of bar indicates plant species not present. Loci represented; Skdh-2, A; Skdh-1, B; Dia, C; Pgk, D

		Genotype frequency	
Locus	Sample size	AA	aa
Skdh-2	396	0.667	0.212
Skdh-1	389	0.622	0.201
Pgk	333	0.655	0.276

Table 4.2 Summary of sample sizes and genotype frequencies for spatial autocorrelation analysis of individual plants at Humbug Scrub population

Spatial autocorrelation of join counts between individual plant genotypes generally agreed with the patterns expected from computer simulations (Epperson 1995, Epperson and Li 1997). The genotype frequencies and sample sizes for each locus are given in Table 4.2. Joins between like homozygotes were in excess, and joins between unlike homozygotes and between all unlike genotypes were in deficit at shorter distances (Figure 4.3). Joins between like homozygotes significantly exceeded expectations at distances up to around 100 m in most instances. The intercepts of the correlogram with the abscissa for joins between unlike homozygotes indicate that homozygote clusters were separated by distances scarcely larger than the size of the clusters themselves. Heterozygotes were rare and the inclusion of them in the analysis of all unlike joins only marginally alters the pattern from that of unlike joins between alternate homozygotes (Figure 4.3). An example of the pattern of clustering of like genotypes, and the paucity of heterozygotes, is given for locus Skdh-2 in Figure 4.4. The proportion of positive joins between like genotypes that were statistically significant ranged from 21 to 92 percent, and negative joins between unlike genotypes from 60 to 91 percent (Table 4.3).

Observed spatial autocorrelation statistics in the shortest distance class were sometimes within the range of values predicted for neighbourhood sizes between 4.2 and 12.6 (Table 4.4). The differences observed were most probably due to sampling error, as the number of loci used was small and a much greater number of loci would have reduced the effect of high variability between loci.

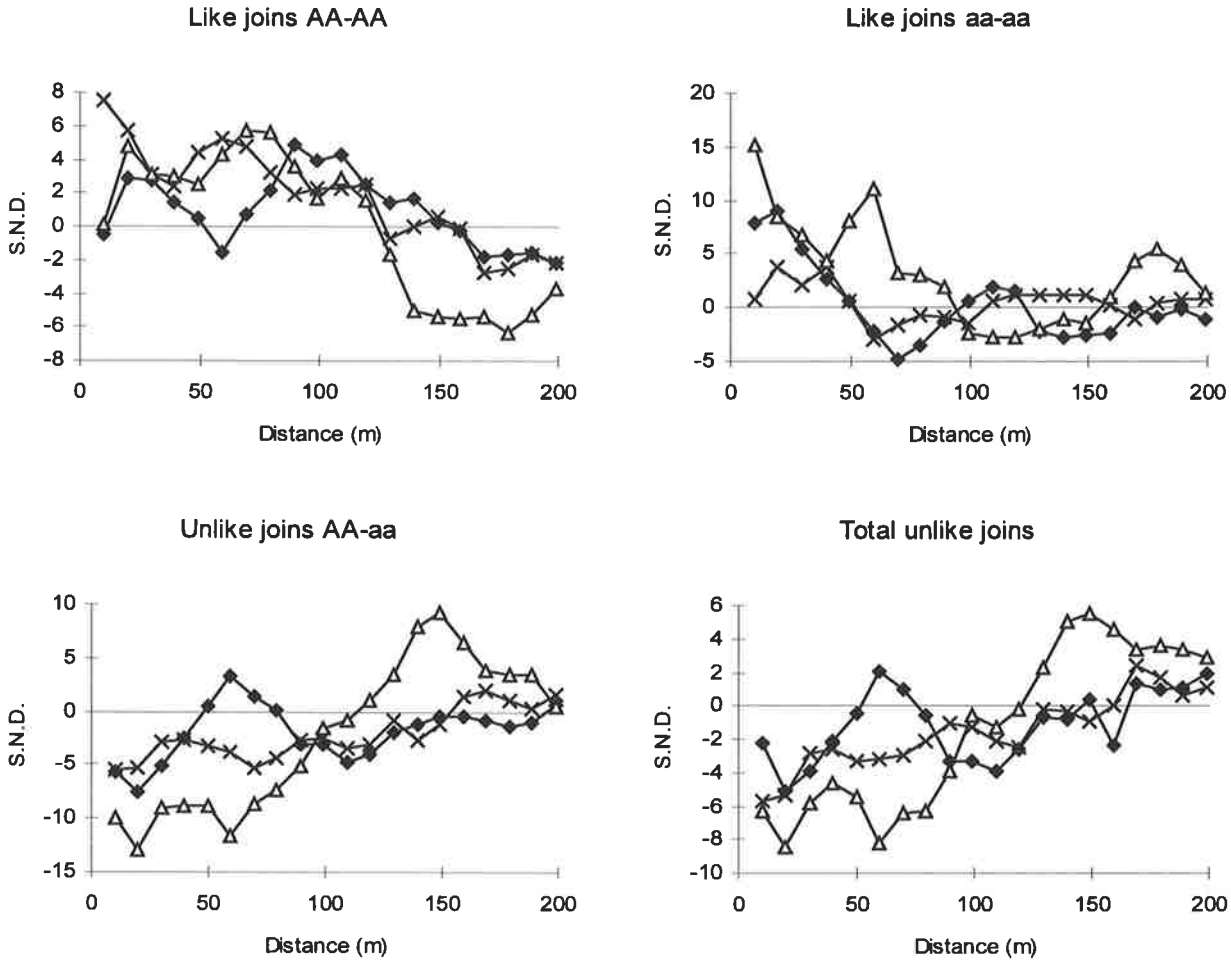


Figure 4.3 Correlograms showing the relatedness of genotypes with increasing distance. Standard normal deviates (SND) were calculated from join counts of like-homozygote, unlike-homozygote and total unlike genotype joins for each distance class. Loci are plotted separately; Skdh-2, ♦; Skdh-1, ×; Pgk, ▽. Significances of SNDs are given in Table 4.3

Type of join	Locus	% of +ve SNDs	% of –ve SNDs
Like (AA-AA)	Skdh-2	62	43
	Skdh-1	92	57
	Pgk	75	100
Like (aa-aa)	Skdh-2	63	58
	Skdh-1	21	33
	Pgk	86	67
Unlike (AA-aa)	Skdh-2	20	60
	Skdh-1	20	87
	Pgk	78	91
Total unlike	Skdh-2	25	67
	Skdh-1	40	67
	Pgk	100	75

Table 4.3 Percentage of positive and negative SNDs (standard normal deviates) that are statistically significant at $\alpha = 0.05$. SNDs are of join counts between like-homozygotes, unlike-homozygotes and total unlike joins as shown in correlograms in Figure 4.3

Locus	N	Observed SND	Predicted SND		
			$N_e = 4.2$	$N_e = 8.4$	$N_e = 12.6$
Like homozygotes AA					
Skdh-2	396	-0.5	9.1	7.8	5.4
Skdh-1	389	7.5	9.0	7.8	5.4
Pgk	333	0.1	8.3	7.2	5.0
Mean		**2.4**	**8.8**	**7.6**	**5.3**
Like homozygotes aa					
Skdh-2	396	7.9	9.1	7.8	5.4
Skdh-1	389	0.7	9.0	7.8	5.4
Pgk	333	15.1	8.3	7.2	5.0
Mean		**7.9**	**8.8**	**7.6**	**5.3**
Unlike homozygotes					
Skdh-2	396	-5.6	-11.3	-9.7	-6.9
Skdh-1	389	-5.5	-11.2	-9.6	-6.8
Pgk	333	-9.9	-10.3	-8.9	-6.3
Mean		**-7.0**	**-10.9**	**-9.4**	**-6.7**
Total unlike joins					
Skdh-2	396	-2.2	-10.3	-8.8	-5.5
Skdh-1	389	-5.7	-10.2	-8.7	-5.4
Pgk	333	-6.3	-9.4	-8.1	-5.0
Mean		**-4.7**	**-10.0**	**-8.5**	**-5.3**

Table 4.4 Comparison of predicted SND values for selected neighbourhood sizes and observed SND values from Humbug Scrub population of *Hakea carinata*. Predicted values are taken from Epperson and Li (1997) with corrections for actual sample size

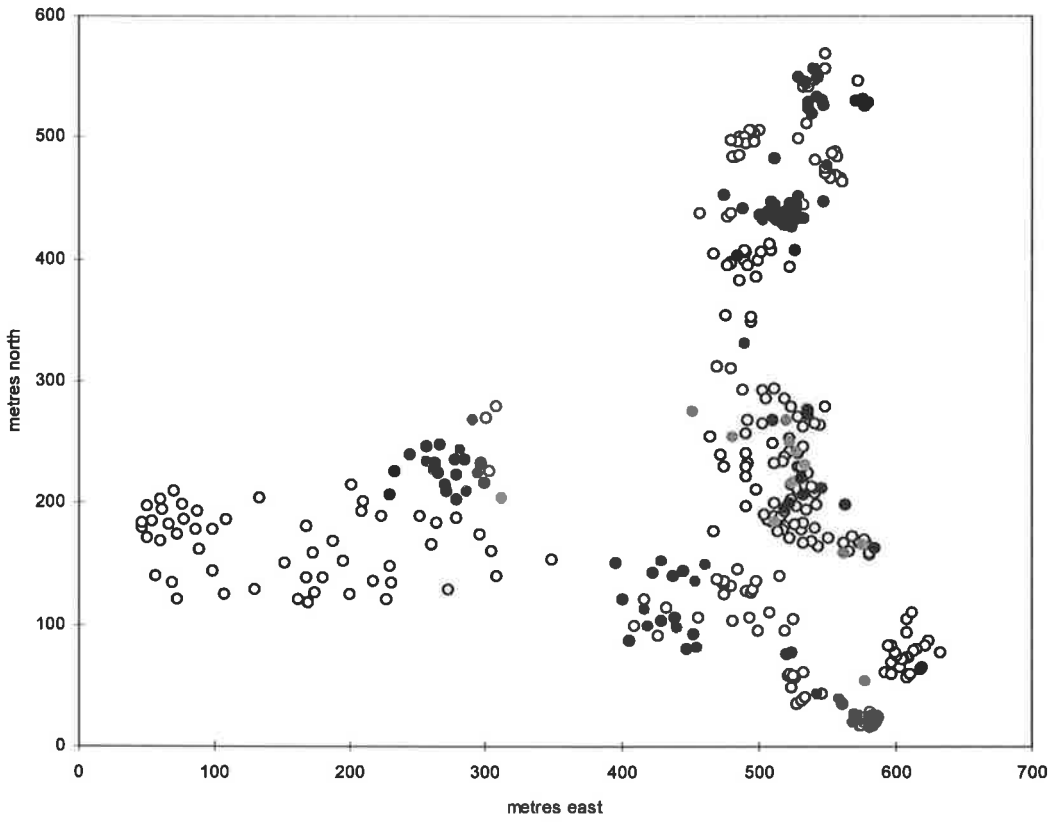


Figure 4.4 Map of geographic relations of Skdh-2 genotypes for 396 individuals of *Hakea carinata* in Humbug Scrub population. Genotypes; AA homozygotes, •; Aa heterozygotes, ◦; aa homozygotes, o

4.4 Discussion

This chapter has demonstrated that substantial spatial structuring of genetic variation exists in the Humbug Scrub population of *H. carinata*. A previous chapter indicated that neighbourhood size is approximately five individuals (Chapter 3). The results of the present chapter generally support this estimate of localised dispersal. This finding is unusual for most Proteaceous species, although note some *Grevillea* species (*Grevillea barklyana*, Hogbin *et al.*, 1998; *G. mucronulata* and *G. sphacelata*, Richardson *et al.*, 2000). It even displays a greater degree of isolation than has been shown in most plant species.

The two main components of the analysis proposed by Sokal and Jacquez (1991), gene frequency surfaces and spatial autocorrelation analysis, both show a distinct departure from a random spatial distribution of genetic variation in this population of *H. carinata*. Gene frequency surfaces were each significantly heterogeneous and the pattern between loci was uncorrelated indicating their independence. The variation in allele frequencies between quadrats was pronounced, with all but one locus having frequencies covering the maximum range from zero to one.

The correlograms of gene frequencies in quadrats showed significant patterns of positive correlations at distances less than 400 m, and negative correlations at distances greater than that (Figure 4.1a). The indication is that for the three most variable loci the diameter of clusters of related individuals is between 50 and 100 m. Variation between loci in the patterns of correlation are probably due to two factors; firstly, the very few loci sampled gives little power to the comparisons of correlograms, and secondly, the small geographic area of the population leads to variability in the numbers of clusters at each locus and hence to variation in correlations at higher distances.

The combination of uncorrelated gene surfaces and similar correlograms observed in this study is best explained by a simple IBD model. Each locus appears to behave independently as shown by the substantial differences in the geographic location of related clusters between surfaces, unlike the expected pattern of correlated clustering

if migration were a substantial influence on gene frequencies. The microevolutionary processes appear similar at each locus however, as indicated by the correlograms. This would tend to eliminate selection as a major force in this population as it would not be expected to influence all loci equally.

Previous work on *H. carinata* suggested that dispersal is very low (Chapter 3), mainly due to a high degree of inbreeding (Chapter 2). Particular care was taken with sampling in this study to include the scale at which isolation processes are occurring. The spatial analysis above suggests that there are processes occurring at distances less than the size of the sampling quadrats, ie less than 50 m. An advantage of further investigating structure at the scale of metres between individuals is that IBD is expected to create structure between pairs of genotypes involving diploid individuals that is not detectable using Moran's I as an index (Epperson 1995). The information about genotypes containing the same allele is treated as a weighted average by Moran's I, whereas with join counts the patterns for each genotype can be examined separately.

The correlograms of join counts of genotypes in *H. carinata* agree in general with theoretical expectations as detailed in Epperson (1995). Joins between like homozygotes are expected to be in excess at short distances and joins between unlike genotypes in deficit at the same distances. At the scale of 0 to 200 m in *H. carinata*, joins between like homozygotes are in excess at short distances while there are deficiencies of joins between alternate homozygotes and all unlike genotypes. These deviations exist for several distance classes and their extent indicates the scale of patch structure of homozygotes in the population. Heterozygotes are rare in this species, and in any case it is difficult to characterise their spatial structure (Epperson 1995). Rather than forming clusters, heterozygotes occur where distinct patches of alternate homozygotes overlap, eg. locus Skdh-2 (Figure 4.4), and their extent is influenced by the variability of rare outcrossing events (Epperson 1995).

Joins between like homozygotes, for both the common (AA) and uncommon (aa) alleles, show different patterns suggesting that cluster size is not the same for both alleles at a locus. Despite some non-significant values at 10 m, positive values at shorter distance classes indicate clusters of homozygotes at up to approximately 120 m diameter (Figure 4.3). Large clusters of this magnitude occur for both alleles at locus Pgk, while the common allele displays larger clusters than the uncommon allele at locus Skdh-1. Clustering at locus Skdh-2 appears more complex with small (~50 m) clusters very close to each other producing two positive peaks in the correlograms.

The correlogram of unlike homozygotes can be interpreted as indicating the distance between clusters of alternate homozygotes by the first intercept of the correlogram with the abscissa, as well as being affected by dispersal. Higher dispersal causes clusters of homozygote to be further apart even though the size of the cluster may not change (Epperson 1995). The pattern shown here is similar at loci Pgk and Skdh-1 with clusters more than 100 m apart, and less at locus Skdh-2. Finally, the total unlike joins displays a similar pattern, as the addition of heterozygotes has little influence on the detected pattern. The pattern of different cluster size did not show up in the analysis of quadrat data because the resolution of that analysis was not sufficient to detect it. The differences between loci are minor however as variation would be expected in a discrete population of such small size. The distinct spatial structure detected supports the suggestion that dispersal is extremely limited within this species.

These results summarise the spatial structure in one population of *H. carinata* but are consistent with expectations in all populations, based on the previous estimates of neighbourhood size. As evident in the examples below, the degree of spatial structuring in *H. carinata* appears to exceed that found in other plant species.

Several studies have found significant autocorrelation at the smallest distance sampled. Berg and Hamrick (1995) found significant clustering at < 10 m in a forest tree, *Quercus laevis*, using both interval and nominal data. This was thought to be due to limited seed dispersal rather than inbreeding in this wind-pollinated species. At any larger scale there was no evidence of any well developed genetic structure. Wind

pollination in forest trees is capable of significant amounts of long distance dispersal. The rather large acorns of *Quercus laevis* are suggested to establish immediately beneath the parent tree and therefore be responsible for the small scale genetic structure in that species.

Another forest tree that is capable of wind pollination, although partially entomophilous, has genetic structuring that has been described as 'moderate' (Perry and Knowles 1991). Seed dispersal in *Acer saccharum* can reach hundred of metres due to the winged samara and the heights from which the seeds may be released. Nevertheless, small scale genetic structure with patch widths of approximately 20-30 m was found in this species. Rather than being explained as isolation by distance it was thought that demographic factors such as cohort recruitment were responsible for the observed patterns.

Another species with similar spatial coefficients to *Acer saccharum* was bur oak, *Quercus macrocarpa* (Geburek and Tripp-Knowles 1994). As with the other species of Quercus, the small genetic patch size was attributed to low seed dispersal and was used to explain the smaller patches from similar spatial coefficient values. A third study of a Quercus species found very similar results (Montalvo *et al.* 1997).

Perhaps the species that has shown the greatest autocorrelation structure is *Gleditsia triacanthos* (Schnabel *et al.* 1991). There was shown to be significant positive autocorrelation between near neighbours, mainly within 10 m. This species forms dense clumps and seed dispersal is extremely limited, however genetic diversity is high and genetic structure is not apparent over greater distances.

The main pattern emerging from these studies is that where pollen dispersal occurs over reasonable distances there is no observation of isolation by distance at medium to large scales. Small scale genetic structure can develop if seed dispersal is limited and many seeds establish very close to the parent plant. As such the area of patches of genetically autocorrelated individuals can be measured as only a few metres.

Patterns in selfing species have only found spatial autocorrelation at distances less than 5 m eg. Schoen and Latta (1989), Maki and Yahara (1997). In both these studies it was suggested that the highly restricted pollen dispersal occasioned by the mating system was more strongly responsible for the appearance of small scale structure. A combination, therefore, of restrictions in both seed and pollen dispersal is perhaps necessary before spatial autocorrelation over longer distances in populations can be detected. The findings in the present study of substantial structure over longer distances in *H. carinata* suggest that both pollen and seed dispersal are severely limited.

In summary, *H. carinata* shows evidence of spatial genetic structure concomitant with an IBD model. This evidence is apparent at all loci examined. Pollen dispersal is mainly within plants rather than between plants. Homozygotes of alternate alleles are found in close proximity to each other but heterozygotes are rare. This may be associated with a loss of pollinators due to human disturbance and introduction of competitors, but further ecological work is required in this area. Seed dispersal also appears highly limited due to the clumped spatial distribution of plants.

The application of spatial autocorrelation analysis for the detection of spatial genetic structure in small populations is not without its problems. Population sizes in *H. carinata* are small, ranging from a few individuals to several thousand (Chapter 2), and the effect of population edges may cause deviations from the theoretical patterns expected in continuous populations. Such population edges have been shown to behave differently to centrally located patches in simulation studies. Simulation studies that characterise spatial statistics are able to create large populations of regularly spaced individuals and eliminate edge effects by sampling from the centre of the population (Epperson 1995, Slatkin and Arter 1991, Sokal and Jacquez 1991). I have used sampled individuals from the entire population including the edges, and the irregular spacing of plants has meant that individuals cannot be sampled on a regular lattice. The time and cost constraints of genotyping individuals from progeny arrays has also placed limits on the possible sample size, which is below that used for computer simulations. Despite these hindrances to applying spatial analyses to empirical data, the methods have been successfully used in this study to characterise

spatial structure and elucidate microevolutionary forces within populations, and it is hoped that these methods will be applied to other species in the future.

CHAPTER 5. FLOWERING OF *HAKEA CARINATA*

5.1 Introduction

The behaviour and movement of animals visiting flowers has a profound influence on the mating systems of plants (Barrett and Harder 1996). This occurs because the foraging behaviour of animals influences pollen dispersal distances. Movement between flowers over short distances, particularly within a plant, will increase levels of inbreeding, whereas movement predominantly between distant plants enhances outcrossing. "Who mates with whom and how often" determines the patterns of inheritance of all genes in populations (Barrett *et al.* 1996).

Animals can be wholly or partially responsible for pollination in a plant species. They may pick up pollen and remove it from a flower whilst in the process of visiting. Dependent on their subsequent behaviour, the pollen may be deposited on another flower on the same plant, another plant of the same species, or be lost to the system entirely. The animal may also merely move the pollen around within the same flower in which it was collected. Successful transfer of pollen by an animal is when fertilisation occurs. A plant species that relies substantially on animal pollination is subject to the behavioural characteristics of the animals. Where these characteristics are highly variable or unreliable, sexual reproduction in the plant may suffer.

Animals may visit flowers but not be a major participant in the process of pollination. Some animals may avoid pollination mechanisms and take the rewards offered by the flower without aiding plant reproduction. Others will perhaps contact only the male or female sexual parts of the flower. In these instances pollen may or may not be removed but there is no significant deposition of pollen on flowers that will result in fertilisation. Where pollen is removed under these circumstances, the plant may suffer detrimental effects of animal visitation by a loss of reproductive effort. There is an inherent dichotomy in the purposes for which animals visit flowers and the services required by the plant for successful pollination (Waser and Price 1983). Animals seek

to maximise their metabolic gain with the least effort, and plants require abundant pollen of the best quality. Even if animals are successful pollinators of flowers, there is evidence that these competing goals result in pollinators transferring pollen over shorter distances than what appears to be optimal for the plant (Waser and Price 1983). Evidently, the result of attracting animals to a plant through floral displays and the provision of rewards is not always efficient fertilisation. Studies of the behaviours of animals visiting flowers are therefore central to understanding pollination and plant mating systems.

Hakea carinata flowers are visited by a wide range of insects. These tend to be generalists and visit many other different flower species. The generalist nature of these insects suggests the lack of a close evolutionary relationship between them and the plant. Furthermore, evidence from the genetic analyses in the previous chapters suggests that if these insects are pollinating the flowers then their behaviour involves movement predominantly between flowers on the same or closely related neighbouring plants, resulting in high levels of inbreeding. The genetic analyses used indirect methods to identify the mating patterns of *H. carinata* and to infer the relationships between the plant and the insect species that visit the flowers. The present chapter focuses on the use of direct methods for investigating the ecological relations between the plant and insects, using observations of the flowers and their insect visitors.

Direct observations of pollinator behaviour are important in helping to establish patterns of movement of pollen between flowers. The mating system of *H. carinata* has been shown to be predominantly selfing/inbreeding (Chapter 2). The low level of genetic diversity within populations has meant however that it is not possible to distinguish between modes of inbreeding ie. biparental inbreeding, geitonogamy, or autogamy, by identifying paternal input. The clustering of related individuals (Chapter 4) has resulted in many genotypes in a small area that are genetically identical at the level of resolution achieved by the genetic assays used in this study. It has been shown that autogamy may be possible in *H. carinata* (Loram 1994), suggesting that insects may not be effecting pollination but that fertilisation occurs automatically within the flower.

These points pose the following questions; are the insect visitors to flowers of *H. carinata* involved in pollinating flowers and if so where are they transporting and depositing pollen; and if they are not involved in pollination, what effect does insect visitation have on the reproductive output of the plant?

5.2 Materials and Methods

5.2.1 Study Species

Hakea carinata flowers during September and early October, in the early southern hemisphere Spring. At this time there is abundant soil moisture and sunshine, although daytime maximum temperatures can still be quite low (< 10° C) at some sites. The flowering period for each adult plant is brief, usually occurring over a period of two to three weeks. Within this time, flowers open fairly synchronously within each plant giving a massed display, and then rapidly decline as fruit matures. Plants within populations vary in time of flowering with some plants beginning flowering just as other plants have passed their peak and are in decline. However plants within populations overlap in flowering to a great extent. Greater differences in the timing of flowering are noted between populations. There appears to be no geographical pattern to this, but several populations in the Mt Lofty Ranges were noted to commence flowering after nearby populations had finished.

Like most plants from the family Proteaceae, flowers of *H. carinata* are protandrous, with male and female phases separated in time and the male phase occurring first. Protandry is a mechanism that enhances outcrossing by minimising pollen transfer within the flower. Pollen is available for distribution from a pollen presenter in the flower before the stigma is mature and can receive pollen (Ladd 1994). The usual process in species with protandrous flower morphology is that during the male phase most or all of the pollen is removed by visitors and in the female phase there is only pollen brought from other flowers that is available for fertilisation.

In *H. carinata*, anthesis occurs prior to the opening of the tepals and during this stage pollen is deposited on the end of the style. The stigma is immature at this stage, and

the enlarged end of the style acts as a pollen presenter. The prominence of the style makes the pollen load readily available to floral visitors. The stigma matures and becomes receptive to pollen a minimum of 24 hours after anthesis (Loram 1994). The stigma remains receptive for up to four days after anthesis, and once flowers display signs of senescence eg. the tepals are pale and positioned away from the style, then the stigma is no longer receptive to pollen (Loram 1994).

5.2.2 Flowering Phenology

The temporal sequence of flowering from bud to fruit formation was observed during the flowering season of 1996. This was to discover the rate of pollen removal in relation to flower maturity, and also to observe the lifespan of flowers. Protandry generally indicates that a flower must be visited at least twice for fertilisation to occur, once for pollen to be removed from the presenter while the flower is in the male phase, and once for pollen from another flower to be deposited on the mature stigma. Pollen that is not removed from the presenter before the maturation of the stigma may possibly self-fertilise the flower, therefore it is important to establish whether this occurs. The rate at which pollen was removed from flowers was here established by direct observation of pollen remaining on the flowers, and later in the methods observations are made of the rate of insect visitation to flowers. Flowers of *H. carinata* that receive pollen on a mature receptive stigma demonstrate withering of the style tissue within one day of fertilisation (Loram 1994). Therefore flowers that show no signs of senescence several days after all pollen has been removed and the stigma has become receptive have most probably not received pollen through insect visitation. These patterns of pollen removal and the longevity of flowers after pollen removal were used to infer the pattern of pollination of *H. carinata* flowers by insect visitors.

The observations of flowering phenology were made in a roadside population on Frank Barker Road at Humbug Scrub near Adelaide (site reference latitude 34° 43′ S, longitude 138° 49′ E). This population is near to the one used in Chapter 4 for the study of spatial genetic structure. The road is used by only a few cars per day and is

surrounded by extensive natural bushland. It provides access to only the southern part of Para Wirra Recreation Park and is not a through road.

Ten plants from the population were selected at random from a 150 m section of track that passes through the middle of the population. Five branches from each plant were randomly selected, and on each of these branches a randomly chosen inflorescence was tagged with a piece of coloured cotton tied around the base of the inflorescence. Inflorescences were sampled while the flowers were still enclosed by the bracts and consequently it was not possible to know *a priori* the number of flowers per inflorescence. The average inflorescence contains approximately 12 individual flowers. Once the bracts surrounding the inflorescence had fallen and exposed the flower buds, the number of flowers in each inflorescence was reduced to ten by removing outside flowers by cutting the pedicel with small scissors. This gave a sample size of 50 flowers per plant and 500 flowers in total.

Observations of the flowering stage of each flower were made on a daily basis. The maturation of each flower was classified in stages from closed bud through to the senescence of the flower as indicated by withering of the style. Flowers were classified as being still in bud if the tepals were joined at the distal end and the style tip remained enclosed by the tepals. Once the style was exposed by the opening tepals, flowers were classified according to the amount of pollen on the pollen presenter. Full pollen loads, indicating no visits to the flower, were clearly visible as yellow masses that completely covered the pollen presenter. Pollen presenters having less than a full pollen load were examined using a 10x lens for evidence of remaining pollen. These were classified as having either partial pollen load or no pollen remaining. Flowers showed the first signs of senescence when the region of the style immediately below the stigma changed from white to pink and the style began to shrivel. Loram (1994) showed that flowers at this stage, which were described as also displaying pale tepals and a mottled pedicel, do not have a receptive stigma.

Flowering stage was recorded at dusk on sequential days from the first emergence of inflorescences from their bracts until all marked flowers were open and all pollen had been removed. Thereafter flower senescence and fruit development was recorded later

in spring and during the subsequent autumn. Flower life was assessed per inflorescence as number of days from first open flower to first signs of senescence.

5.2.3 Pollinator Visitation and Fruit Set

The rate at which insects visit a flower can have a significant bearing on the probability of that flower being fertilised. As mentioned before, in protandrous flowers such as *H. carinata* the flower requires a visit in the male phase to remove pollen and another visit after the stigma has become receptive to receive pollen. If these flowers are mainly visited while they offer a pollen resource, and rarely visited when the pollen has been taken and the stigma is receptive, then few flowers should set fruit and the insect visitors are not effective pollinators.

Observations were made of the behaviour of visiting insects to determine the principal types of insect visitors to inflorescences, their rates of visitation, and any possible relationships between visitation and fruit set rates. Observations of floral visitation and fruit set were made at ten populations in the northern Mt Lofty Ranges (Table 5.1). Three plants were randomly selected for observation in each population. A single flowering branch approximately 1.5 m above the ground on each plant was used for observations, and these branches were tagged with flagging tape. *Hakea carinata* flowers appear on the terminal part of a branch on the new growth of the previous season, and therefore the last 30 cm of the tagged branches was used for observations. The number of inflorescences per branch was not standardised across plants or populations, and the number of flowers per branch could not be known until the bracts covering the inflorescence had dropped, exposing the flowers. The flowers were counted on each tagged branch as inflorescences opened. The total number of flowers observed in each of the ten populations is shown in Table 5.1.

Population	Lat. S	Long. E	# flowers
Bundy	34° 46.0′	138° 56.7′	2614
Brewer's Corner	34° 46.3′	138° 49.2′	2612
Crick's Mill	34° 44.5′	138° 58.7′	1842
Jenkins Scrub	34° 42.7′	138° 57.6′	2438
Humbug Scrub FB	34° 43.1′	138° 48.6′	1636
Humbug Scrub RT	34° 43.0′	138° 48.7′	1716
Birdwood/Gumeracha Rds	34° 44.3′	138° 57.5	2433
Cromer	34° 46.8′	138° 58.6′	1941
Kersbrook Rd	34° 45.1′	138° 48.2′	1639
Yettie Rd	34° 39.8′	138° 48.6′	2033

Table 5.1 Location of ten populations used for observations of insect visitors to flowers of *H. carinata*, with number of flowers observed in each population

Observations were conducted for one hour on each of three separate days in each population and were spaced in time so that they began once each at 10 AM, 12 PM and 2 PM. The hour of observation was divided into 20 minutes each for the three sampled branches per population. Sites were chosen randomly each day from those populations that were currently in flower. Flowering did not occur synchronously in all populations. For example, flowering had begun by 6 September 1995 at Brewer's Corner Road and was well into decline when observations ceased there on 18 September 1995. At this time flowering had just begun at Crick's Mill Road and observations continued there from 19 to 26 September 1995. At the start of each observation session an estimate was made of the proportion of flowers that were open on each marked branch. This estimate was to the nearest 10%. This proportion was used to estimate the total number of flowers on the branch that were available to insect visitors at the time of observation. Insects visits were recorded as the number of inflorescences an insect touched during a foraging bout. It was not feasible to observe contact with individual flowers as being so close would have disturbed the insect.

Insects were classified into broad groups eg. flies, native bees, honeybees. Capture of insects was not possible without disturbing the foraging behaviour of the insect or causing mechanical damage to the flowers and therefore identification of insects could only be made from a distance. The low number of insects that visited the marked flowers during the 30 hours of observations meant that there was little statistical power in classifying insects to family or genus. The broad classifications used were based on body size and colour where a more accurate identification was not possible.

All of the observed flowers were followed through to the end of the flowering season and the number of fruit set on all of the tagged branches was recorded at two months after the end of flowering. At this stage the fruit produced during the flowering season were at full size and beginning to turn brown, indicating their maturity.

The activity and behaviour of insects visiting flowers can be affected by the ambient temperature and the number of flowers displayed. Temperatures in early Spring can be low enough to inhibit the activity of flying insects. Ambient temperature was recorded at the start of each observation session, and relations between temperature and visitation rates of insects investigated using Spearman rank correlation analysis. A similar procedure was followed for the number of open flowers and visitation rates. Correlations between visitation rates and fruit set were calculated to determine if higher rates of visitation by insects resulted in greater fruit set.

5.2.4 Autogamy Test

Previous work indicated that *H. carinata* may be capable of being fertilised by autogamy (Loram 1994). Autogamy ensures that at least some fruit is set in the absence of suitable pollination by insects. This is achieved by using pollen that remains on the maturing stigma to effect fertilisation. The low rate of visitation observed suggests that there may be insufficient delivery of pollen to receptive stigmas, and that some fertilisation may occur through autogamy. The high level of inbreeding demonstrated by the genetic data in Chapter 2 supports the suggestion of autogamy as a possible mechanism of fertilisation.

The possibility of fruit set via autogamy in *H. carinata* was tested by excluding insect visitors to flowers using mesh bags to enclose the flowers for the duration of the flowering season. The experiment was replicated in two populations in the northern Mt Lofty Ranges that had been sampled as part of the genetic survey in Chapter 2. These populations were Humbug Scrub (RT) (34°43′ S 138°49′ E) and Jenkins Scrub (JS) (34°43′ S 138°58′ E). The terminal 15 cm of a flowering branch on each of ten replicate plants in each population was covered with an insect proof bag. The bags were installed in mid August just as the flower buds were bursting from the bracts but before any flowers had opened. They remained on the branches until late November when all flowering had ceased and any fruit produced had matured. Each bag enclosed approximately ten inflorescences.

The bags were fabricated from a 30 cm by 30 cm sheet of mosquito netting folded in two and sealed along the seam with silicone sealant. Each bag was secured to the stem of the plant with a length of string. Once positioned on the branches the bags were held clear of the flowers by the stiff leaves of the plant (the inflorescences appear in the axis between the leaf and stem). Sunlight, rain and wind were all able to pass through the material of the bags. It was possible to see through the material to inspect the flowers at stages through the flowering season.

The installation of the insect exclusion bags prior to the opening of the majority of the inflorescence buds meant that the number of flowers within the bags could not be controlled or counted. The number of fruits formed within the bags was therefore not quantified in terms of a flower-to-fruit ratio but treated as an observation as to whether fertilisation by autogamy in the absence of pollinators was possible.

5.3 Results

5.3.1 Flowering Phenology

Inflorescences of *H. carinata* emerge from their surrounding bracts as partially developed buds. Flowers undergo an increase in size of perhaps double before the tepals open. Concomitant with this maturation is a separation of the previously compact flowers to form a roughly hemispherical inflorescence approximately 1 cm in

diameter. Individual flowers within an inflorescence mature and open sequentially over several days to a week.

The observed inflorescences opened a mean of 2.2 flowers per day from the day the first flower opened (S.D. = 1.9, n = 49, range 0-9 flowers per day). There was a mean of 4.5 days (range 2-7 days) over which all flowers in an inflorescence opened. It should be noted that this applies only to the inflorescences that were artificially trimmed to ten flowers for the purposes of standardisation and may not apply to undisturbed inflorescences.

Pollen presented on newly open flowers of *H. carinata* appeared to be rapidly removed. Flowers that had been noted to have opened the previous day rarely had even slight traces of pollen remaining on the presenter. The mean number of days from flower opening to complete pollen removal was 1.16 days (S.D. = 0.4, n = 452). The majority of the lifespan of these flowers was not as a pollen donor but as a pollen recipient. Observed mean flower life was 7.47 days (S.D. = 1.17, n = 19).

Stigma receptivity of *H. carinata* has been shown to occur within the range of 24 to 54 hours from anthesis (Loram 1994). This would tend to indicate that pollen is mostly removed from flowers before the stigma has become receptive. Flowers that receive pollen on a receptive stigma show signs of senescence within one day of fertilisation (Loram 1994). The persistence of flowers beyond the 54 hours from anthesis when stigma receptivity has substantially declined or stopped, and for several more days, indicates that very few flowers are fertilised. The 500 flowers observed from bud to fruit formation set a total of five fruit (ie. 1%).

5.3.2 Pollinator Visitation and Fruit Set

Insect visitors to flowers of *H. carinata* were generalist pollinators. Many other plant species flower at the same time as *H. carinata*, and these insect species were observed to visit the flowers of these plants as well. Observed visitors included European honeybees *Apis mellifera*, native bees, flies and beetles. Despite the large floral displays observed on bushes of *H. carinata* during the experimental period, visitation to flowers by insects was low. A total of 30 hours of observation were made of insect

visitors to randomly selected branches. In the observation period only 36 individual insects were observed to visit flowers.

Inflorescences were small in relation to the size of the insect visitors. The diameter of an inflorescence was approximately 1 cm. The mean number of flowers in an inflorescence was 11.8 (n = 1784) and ranged up to 20 flowers. Insect behaviour involved landing or climbing on top of the inflorescence and walking over the tops of the flowers. Insects were too large to enter a flower so the parts of the insects that came into contact with the styles were legs, heads, and ventral surfaces. Some insects used their mouthparts to probe between the tepals at the base of the style. Other insects appeared to be concerned mainly with the ends of the styles where pollen may have been available. The abundance of nectar, obvious from the strong scent of the flowers, suggests that nectar was the main target of the insect visitors. The rapid rate of pollen removal may have been either a target of other insects or just inadvertent removal by insects seeking nectar.

It was not possible to discern the number of flowers visited or manipulated for each visit of an insect to an inflorescence. This was due to the rapid movement of the insects, the large number of flowers in close proximity, and the inability to observe insect movements at close enough range to identify which flower they were manipulating. Pollen may be collected from or deposited on several flowers by insects in the action of walking over the inflorescence. Observations of insect visitors to flowers therefore involved counts of the number of inflorescences visited rather than of individual flowers.

The total number of flowers per marked branch ranged from 295 to 1385, and a total of 20,904 flowers were observed from bud to fruit set. The mean number of flowers open on a marked branch at any one time was 510 (S.E. = 29, n = 30). The number of flowers observed on a plant represented much less than 10% of the total flowers on the plant.

Insect visitors were classified according to general descriptions. These categories, and the number of each type observed, are shown in Table 5.2. The most common visitors

were flies. Identification of these was difficult in the short time they were visible and they have been roughly classified according to their appearance. Flies with a metallic gold sheen on their abdomen were classified as "flies (gold)" and all other flies were plain black unless they were clearly identified as blowflies.

Insect type	Number observed
fly (gold)	7
blowfly	7
native bee	6
fly (black)	5
beetle	4
unidentified	3
honeybee	3
butterfly	1
Total	36

Table 5.2 Type and number of insects observed to visit flowers of *H. carinata*

Honeybees were the most abundant flying insects in the vegetation at that time of the year and yet were among the least frequent visitors observed on flowers of *H. carinata*. They visited more inflorescences on average in a foraging bout however than other observed insects. The mean number of inflorescences visited in a foraging bout on a marked branch, by the categories of insects described above, are shown in Figure 5.1. The numbers of inflorescences visited were highly skewed with a median value of 2 inflorescences, and a range of 1 to 56 inflorescences.

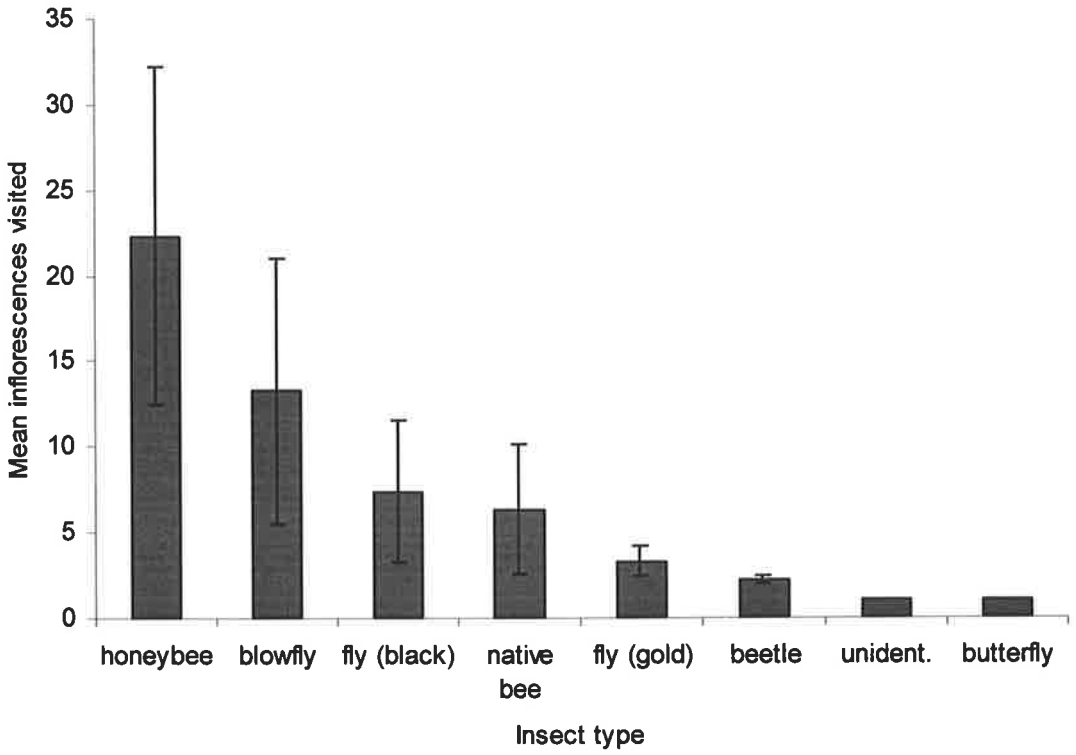


Figure 5.1 Mean number of *H. carinata* inflorescences visited in a foraging bout, by insect type. Bars are standard errors

The movements of insects visiting flowers varied considerably. Blowflies and honeybees concentrated on visiting many inflorescences in a small area, often revisiting inflorescences in the process. Other insects usually visited only one or a few inflorescences and then left the plant. Pollen movement could potentially vary either within plants or between plants but there was no evidence from the observations as to whether insects were removing or depositing pollen. Most of the insects did not display fidelity to this plant species but were observed to visit a variety of species. Honeybees were most likely to remain foraging on *H. carinata* as evidenced by the number of inflorescences visited (Figure 5.1).

The number of open flowers on *H. carinata* was significantly positively correlated with the number of insect visitors (rank correlation, $R_S = 0.339$, $p < 0.001$) and with the number of inflorescences visited in a foraging bout on the marked branches (rank correlation, $R_S = 0.266$, $p < 0.001$) (Figures 5.2 and 5.3).

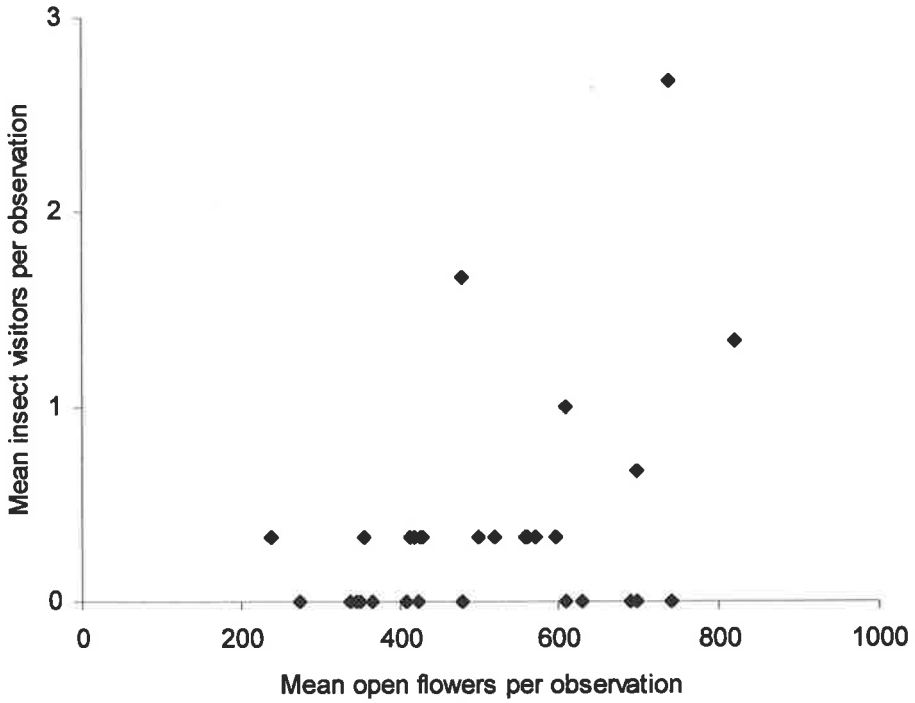


Figure 5.2 Visitors to flowers by number of open flowers. Rank correlation $R_S = 0.339$, $p < 0.001$

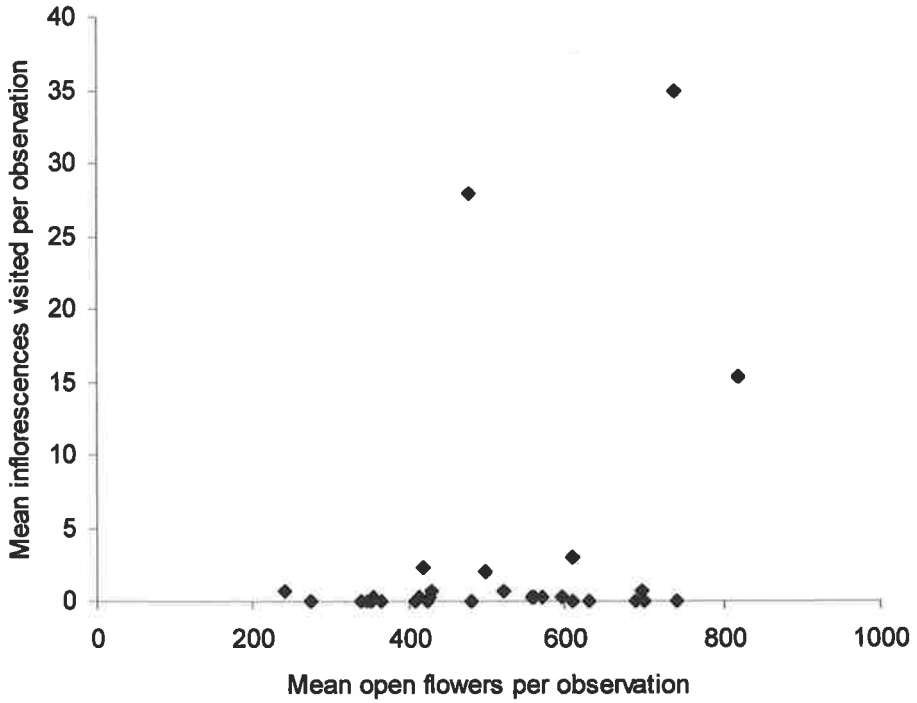


Figure 5.3 Number of inflorescences visited by number of open flowers. Rank correlation $R_S = 0.266$, $p < 0.001$

The temperature during the period of observations was fairly evenly distributed between cold (< 10° C max) and warm (> 20° C max) spring days. The visitation rate of insects to inflorescences, expressed as the number of insect visitors, was significantly positively correlated with ambient temperature (rank correlation, $R_S = 0.410$, $p < 0.001$) (Figure 5.4). Temperature was also significantly positively correlated with the mean number of inflorescences visited per branch per observation (rank correlation, $R_S = 0.369$, $p < 0.001$) (Figure 5.5). Interaction between temperature and the number of open flowers was tested by linear regression. There was no significant relationship between temperature and the number of open flowers (adjusted $R^2 = -0.03$, ANOVA $p = 0.99$).

Fruit set for the observed flowers was low with a mean number of 7.9 fruit set per 1000 flowers (S.E. = 1.6, n = 26). This flower to fruit ratio of less than 1% is even lower than the already low ratios found in many Proteaceous species. Ayre and Whelan (1989) reported fruit set of usually less than 5% in the Proteaceae. Fruit set was not correlated with either visitation rate or the number of flowers per plant (Table 5.3). Both measures of visitation rates had negative correlation coefficients with fruit set suggesting that increased visitation reduced fruit set. The number of fruit set was not associated with the number of observed flowers per plant (Table 5.3).

Visitation rate and flower density	Fruit set
Insect visitors /hr /1000 open flowers	-0.339 P = 0.091
Inflorescences visited /hr /1000 open flowers	-0.320 P = 0.111
Number of flowers /plant	0.006 P = 0.977

Table 5.3 Spearman rank correlations (R_S) of visitation rates and total number of flowers per plant with fruit set

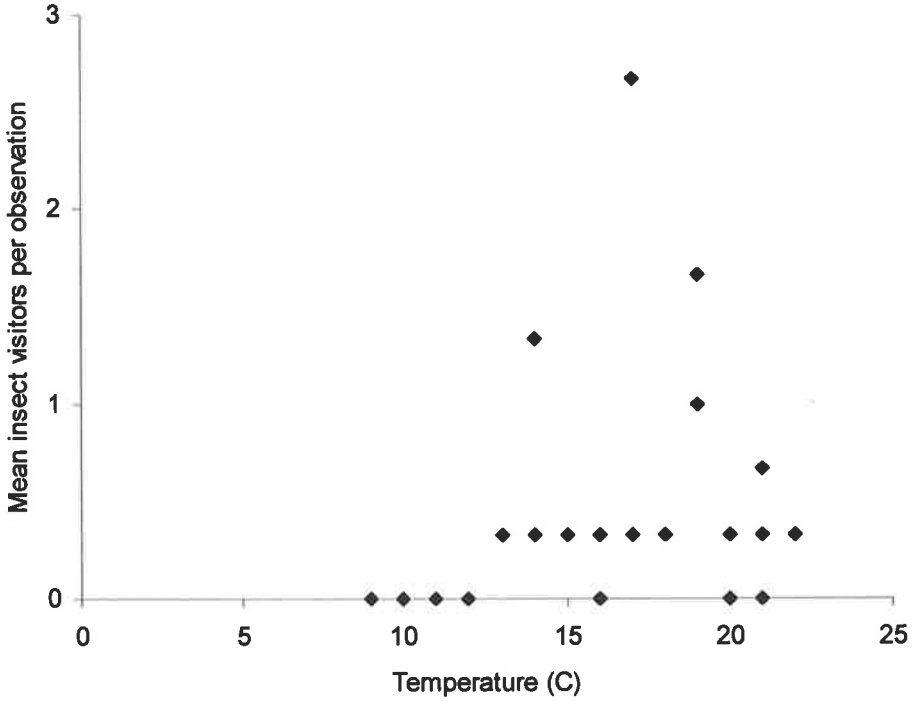


Figure 5.4 Visitors to flowers by temperature. Rank correlation $R_S = 0.410$, $p < 0.001$

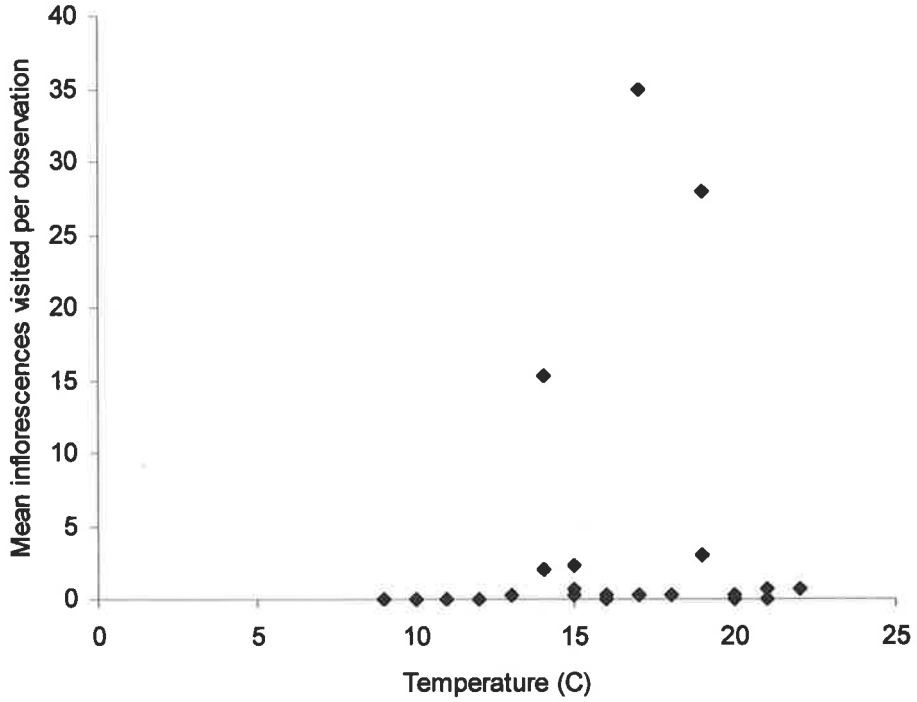


Figure 5.5 Number of inflorescences visited by temperature. Rank correlation $R_S = 0.369$, $p < 0.001$

5.3.3 Autogamy Test

Inspection of the autogamy test bags during flowering revealed that pollen could be plainly seen on the presenter at the end of the style of all flowers in the bags. This pollen remained on the presenter right through until the end of flowering. This indicates that pollen adheres to the presenter and is not readily dislodged by wind or rain, and confirms that insects were not able to enter the bags and remove the pollen. Flowers from which insects were excluded set fruit on eight of ten treated plants in Jenkins Scrub population and six of ten in Humbug Scrub (RT) population. Of these the number of fruit set per treated branch ranged from one to eight with a mean of 3.6 fruit (S.D. = 2.5), although the number of flowers per branch was not standardised as mentioned before. Fruit set on the remainder of the plant outside the insect exclusion bags ranged from one to several hundred. Two plants in the Humbug Scrub (RT) population each set five fruit within the insect exclusion bag and only one fruit on the remainder of the plant.

5.4 Discussion

Hakea carinata plants present large masses of flowers in a short burst of flowering that lasts about two weeks in early spring. At the beginning of the flowering season, flowers of *H. carinata* present abundant pollen that is quickly removed by insect visitors. Little pollen remains on the flowers the day after they have opened. The flowers develop mature stigmas at this time and are ready to receive pollen for fertilisation (Loram 1994). Those flowers that are fertilised quickly become senescent and are discoloured and wither within a day (Loram 1994). Fertilisation does not occur however for the great majority of flowers and they retain the appearance of newly opened flowers for an average of seven days.

These open flowers provide a display that is dense and almost entirely covers the plant. Despite the obviously showy nature of the floral presentation and the abundance of flying insects present during the flowering period, visitation to flowers of this species is very low. The daytime temperatures experienced during flowering were positively correlated with visitation rates in the flying insect species that visit flowers

of *H. carinata*. Some days in September in the Mt Lofty Ranges can exceed 20° C but the majority of days are in the teens, and often do not exceed 9° C at sites such as Mt Crawford Forest. Warmer days resulted in greater insect activity, yet there were never more than eight insects seen to visit marked flowers in any one hour observation period. The massed displays of flowers also appeared to attract more insects. There was a positive correlation between the number of flowers open at the time of any observation period and the number of insects visitors observed. In contrast, the rate of insect visitation to flowers was associated negatively with fruit set, although not statistically significantly so. This would tend to suggest that fruit set is inhibited by insect visitation. This is a surprising result and experiments should now be conducted to determine if this is due to insects mainly removing pollen when flowers are in the male phase and not effectively delivering pollen to female phase flowers.

The small size of the flowers made it difficult to observe contact between the insects and the reproductive parts of the flowers. Thus, no conclusions could be made from the observations on the relative effectiveness of the insect species in transferring pollen between flowers. Foraging behaviour did differ between the insect categories. Honeybees moved short distances between flowers and visited many flowers in a foraging bout, while some other insects visited only one or a few flowers before leaving the plant altogether. However, the small sample sizes in each category make it difficult however to come to firm conclusions about differences between insect species in foraging behaviour.

Inadequate pollen transfer by pollen vectors can result in failure to produce seed or evolutionary pressure for selfing (Schoen *et al.* 1996). The reproductive assurance hypothesis states that there is a selective advantage in self-pollination if pollinators are lost or absent and pollen remaining on a flower can fertilise ovules (Baker 1955). *Hakea carinata* can produce seed by autogamy, and seed set may actually be increased in the absence of pollinators. Whenever pollen removal is incomplete, the pollen that remains on the style is capable of fertilising the flower, as demonstrated by the autogamy test. It is not possible to say whether the majority of seed set in *H. carinata* was the result of autogamous fertilisation. Substantial numbers of fruit were produced when insects were excluded. Insect visitors appear to be removing pollen that could

result in fertilisation if left on the style. Insect visitors are however not reciprocating by depositing on a receptive stigma the pollen they have collected. This is contrary to expectations, as most other plant species in the Proteaceae are predominantly outcrossing and rely on animal vectors to transport pollen between flowers and plants (Goldingay and Carthew 1998).

Hakea carinata has, however, been shown to be an atypical Proteaceous species in terms of its mating system and population genetic structure (Chapter 2). The development of selfing in previously outcrossing species demonstrates that function is an essential part of adaptation and is not secondary to phenotype (Barrett *et al.* 1996). The degree of inbreeding in *H. carinata* is much higher than most other Proteaceous species studied to date, although there is a bias toward studies of other highly outcrossed genera such as *Banksia* and *Grevillea*.

The small neighbourhood size and clustering of related individuals in *H. carinata* confirm that pollen and seed dispersal are very limited. The high degree of population genetic differentiation suggests that long distance pollen dispersal does not occur. Dispersal of pollen over long distances need only be a rare occurrence for it to unite populations and neighbourhoods. The apparent absence of it in *H. carinata* points to insect visitors to flowers not being effective at transferring pollen to receptive flowers, otherwise it is to be expected that the occasional long distance episode would occur.

The conclusions reached from this chapter are that insect visitors to flowers of *H. carinata* are rarely involved in effective pollination of flowers. In the absence of effective pollination the species has adapted to use the pollen that remains on the style after insect visits to achieve self-fertilisation through autogamy. The result of such a reproductive strategy in terms of population genetic structure agrees closely with the patterns found by the genetic analyses detailed in the previous three chapters. These are; a high level of inbreeding and low heterozygosity, very low gene flow between populations and substantial population genetic differentiation, small neighbourhood sizes, and the primary evolutionary force shaping population genetic structure being isolation-by-distance.

CHAPTER 6. DISCUSSION

This study has provided a detailed analysis of the population genetic structure and levels of genetic diversity in the Australian sclerophyllous plant species *Hakea carinata*. As a genus, *Hakea* is less well studied than other genera in the family Proteaceae. There are numerous species of *Hakea*, particularly in Australia, that warrant attention to match the amount of study done on genera such as *Banksia* and *Grevillea*. Despite this contribution to more understanding of the genus *Hakea*, the subject species has been shown to be quite unlike other species studied so far in the Proteaceae.

Hakea carinata has a widespread geographic range over which it is patchily distributed. Where it occurs it has a low population density and never dominates the vegetation community. Populations often number only a few hundred individuals, usually scattered within *Eucalyptus* woodland. Populations are geographically separated by considerable distances, usually at least one kilometre but often much more. These populations are genetically quite distinct and appear to be evolving independently. The genetic diversity held within each population is low, with some populations, particularly at the extremities of the range eg. Mount Remarkable, exhibiting no detectable genetic variation at all.

A variety of direct and indirect methodologies were used to study gene flow within and between populations of *H. carinata*. The combination of genetic and ecological investigation has provided new insights on the evolutionary history and possible future of the species. The use of genetics is a valuable tool in uncovering the evolutionary processes responsible for population genetic patterns (Holsinger 1996). More studies of gene flow are needed in a variety of widespread species to provide a better understanding of the scale of genetic differentiation and the probable consequences of fragmentation. Although it is species with continuous distributions over a broad geographic range that are more likely to be affected by fragmentation (Holsinger 1993), the contributions made by the study of naturally fragmented species such as *H. carinata* are valuable in understanding population dynamics. In particular, tests of

isolation by distance models of genetic differentiation in continuously distributed populations are a valuable source of knowledge.

This study of *H. carinata* has described genetic diversity from a sample of 30 populations across the range of the species. This has enabled a highly detailed picture of the genetic relations between populations covering a range of some 500 km in a north-south direction. The range covers a variety of habitats from steep, rocky, low rainfall to flat, sandy, higher rainfall. To date, this is the most comprehensive survey of genetic variation in a *Hakea* species.

The geographic range of the species has been highly modified by human activity in the years since European settlement of the state in 1836. The extent of the species prior to settlement is unknown, with present populations being restricted to those sites and aspects unsuitable for vegetation clearance for agriculture. The evidence from populations in large expanses of relatively undisturbed vegetation is, however, that *H. carinata* occurs most often in the least hospitable habitats. It is present on exposed sites with poor soils more often than on better sites nearby, suggesting that it may have always been restricted to the remnants in which it now exists.

The pattern of genetic differentiation between populations of *H. carinata* exhibits a degree of genetic isolation to be expected if populations rarely, if ever, experience migration. Allele frequencies differ markedly over distances even as small as 1 km, and fixation of alleles occurs frequently. Fixation of alternate alleles occurs several times over the range of the species. This pattern of genetic differentiation between populations suggests either a very low level of migration for a long period of time ie. longer than can be accounted for by human activity fragmenting the landscape, or very strong genetic drift resulting from small population sizes.

There is no doubt that present population sizes are small enough for drift to be a considerable force. The evidence for this is the high degree of fixation within populations (Chapter 2). It is not necessary for these conditions to have existed for a long period of time for the present pattern to occur, however rapid drift from a situation of high diversity would cause deleterious effects from the exposure of the

genetic load. It may well be in *H. carinata* that the genetic load has been purged in all populations, both large and small.

The evidence against the argument that drift has become stronger since human activity has caused habitat fragmentation is in the estimation of neighbourhood size. The neighbourhood size estimated by regression analysis across 27 populations was approximately five individuals ie. the size of the pool in which individuals are exchanging genes through mating is just five plants. This indication of mating pools within populations means that any population size reduction due to habitat fragmentation has not been sufficient to perturb or alter the genetic drift already caused by small neighbourhood sizes. The spatial evidence from the autocorrelation analysis confirms the presence of clusters of related individuals, with substantial isolation between individuals over distances greater than 150 m. Small neighbourhood sizes with concomitant high genetic drift is therefore not a function of small population sizes, however it is they might be caused.

To understand the pattern of genetic variation in *H. carinata*, it is necessary to consider the pattern of mating as shown by observations of pollination. How is it that neighbourhood sizes should be so small in a plant species whose flowers are showy, offer abundant rewards and attract flying insects?

The observations of insect behaviour in visiting flowers of *H. carinata*, and the response of the plant in terms of seed set, indicate that there is very little movement of genes through pollen between plants over any distance. The level of self fertilisation is high eg. Chapter 2, mean estimated outcrossing rate over 30 populations, $t = 0.111$. It is most probable that this is achieved through autogamous pollination rather than insect mediated pollination. Insect visitors to flowers evidently rob the flowers of pollen by taking it from flowers while in the male phase but not delivering it to flowers that are receptive to pollen in the female phase. Flowers from which insect visitors are excluded are able to enhance the level of seed set.

Without a means of exchanging genes between plants over the distances between populations, let alone within populations, populations of *H. carinata* must diverge

genetically. It appears as though this situation has existed for some time. One possible explanation for the present pattern is that *H. carinata* was previously continuously distributed and predominantly outcrossing. The evolution of a predominantly outcrossing mating system, as shared by nearly all studied species in the Proteaceae (Goldingay and Carthew 1998), into a selfing system in the absence of effective pollination by animals must occur over a considerable period of time. This may have occurred as an example of the 'reproductive assurance hypothesis' (Schoen *et al.* 1996), where there is a selective advantage in self-pollination in the absence of sufficient pollinators to achieve full seed set. If so, the species appears to have adapted well to the apparent absence of outcrossing using an animal vector. An alternative is that *H. carinata* has always been selfing, and the patchy distribution is due to rare long distance seed dispersal. More work needs to be done on *Hakea* species to see whether the biology of *H. carinata* is as unusual as it appears, or whether selfing is typical of many species and the present day population distributions owe much to this life history trait.

The pattern of genetic variation in *H. carinata* shown by this study provides a number of important points. The study has been valuable in advancing knowledge in the area of isolation by distance. This has rarely been demonstrated in continuous distributions before, however such isolation is a major effect of fragmentation of natural distributions. The study of isolation by distance gives a better understanding of genetic structure in widespread continuously distributed species. Such species are expected to be more affected by habitat fragmentation than patchy species, however *H. carinata* has shown the possible evolutionary consequences of the disturbance to processes that can be caused by fragmentation.

The genetic analysis presented here has been possible using the fairly low resolution of isozymes. This is appropriate for an broad scale species and population approach to describing genetic variation. The interesting result of very small neighbourhood size and clustering of related individuals has run into the problem of a lack of resolution to differentiate individual genotypes. Modern molecular techniques can now be brought into play to observe the processes between individuals, including assigning paternity to seeds as a way of describing pollen flow between plants. These techniques are

costly and time consuming when used at the survey scale presented here, but any further investigation into the population genetic structure of *H. carinata* would be well advised to use them. Foremost of the modern molecular techniques are AFLPs (Amplified Fragment Length Polymorphisms) and microsatellites. These enable the unique genotype of each individual to be described. An extension of this study into describing movements of genes between individual plants would add substantially to our understanding. This must be left however to another study.

The patterns of genetic differentiation within and between populations shown in *H. carinata* also raise questions of the conservation and management of rare and fragmented species. Species can be considered rare if they have a low population density where they occur, even though they have a wide geographic distribution (Kruckeberg and Rabinowitz 1985). Loss of genetic diversity has severe consequences for the future of species as it will retard the evolutionary response to environmental change. This study of *H. carinata* has shown that diversity can be maintained in naturally fragmented species. Local diversity within populations is maintained as neighbourhood sizes are smaller than the population size. The species would face increased risk of extinction if remaining populations were too small to maintain several genetically independent patches (Holsinger 1993). Despite substantial separation between populations and low gene flow between them, diversity among populations is also maintained. Overall species genetic variation is maintained at a level comparable to other plant species with similar life history traits. Further study is required of the responses of other plant species to fragmentation. This study has provided valuable data for comparison with those species, and demonstrated the evolutionary consequences of isolation by distance.

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APPENDIX 1. ALLELE FREQUENCIES

The following tables present mean allele frequencies for six allozyme loci in 30 sampled populations of *H. carinata*. Values are averaged over seeds per plant and plants per population. Population definitions are given in Table 2.1. These tables have previously been published as the Appendix to Starr and Carthew (1998).

	BC	BU	CL	CM	CR	CS	GO	HO	HS	JS	LW	M1	M2	MB	MC
PepD															
(N)	10	10	8	10	10	10	9	10	10	12	10	10	10	12	10
A	0.000	0.775	0.000	0.730	0.480	0.027	0.097	0.000	0.160	1.000	0.000	0.000	0.000	0.217	0.000
B	0.000	0.000	0.000	0.000	0.000	0.206	0.000	0.000	0.000	0.000	0.000	0.050	0.000	0.783	0.375
C	1.000	0.225	1.000	0.270	0.520	0.768	0.903	1.000	0.840	0.000	1.000	0.950	1.000	0.000	0.625
Skdh-2															
(N)	10	10	8	9	10	10	9	10	10	12	10	10	10	12	10
B	1.000	0.788	0.063	0.796	0.213	0.385	0.889	0.200	0.970	0.283	0.603	0.780	0.540	0.000	0.148
C	0.000	0.213	0.938	0.204	0.788	0.615	0.111	0.800	0.030	0.717	0.397	0.220	0.460	1.000	0.853
Skdh-1															
(N)	10	10	8	10	10	10	5	9	10	10	10	10	10	10	10
A	0.700	0.000	0.000	0.000	0.000	0.000	0.200	0.000	0.335	0.000	0.000	0.000	0.000	0.190	0.053
B	0.300	1.000	1.000	1.000	1.000	1.000	0.800	1.000	0.665	1.000	1.000	1.000	1.000	0.810	0.948
Dia															
(N)	10	10	8	10	10	10	5	10	10	10	10	10	10	10	10
A	0.000	0.000	0.188	0.000	0.000	0.544	0.000	0.100	0.070	0.000	0.316	0.850	0.410	0.400	0.000
B	1.000	1.000	0.813	1.000	1.000	0.456	1.000	0.900	0.930	1.000	0.684	0.150	0.590	0.600	1.000
Pgk															
(N)	7	10	6	10	9	10	3	7	10	10	10	10	10	10	7
A	0.726	0.650	1.000	0.435	0.978	0.883	1.000	1.000	0.713	0.990	1.000	1.000	1.000	0.000	0.714
B	0.274	0.350	0.000	0.565	0.022	0.117	0.000	0.000	0.288	0.010	0.000	0.000	0.000	1.000	0.286
Gpi															
(N)	10	10	7	10	10	10	5	10	10	10	10	10	10	10	10
A	0.000	0.000	0.071	0.017	0.000	0.010	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
B	1.000	1.000	0.714	0.983	1.000	0.750	1.000	1.000	1.000	1.000	0.800	1.000	0.980	0.933	0.840
C	0.000	0.000	0.214	0.000	0.000	0.240	0.000	0.000	0.000	0.000	0.200	0.000	0.020	0.000	0.160
D	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
E	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.067	0.000

	MM	MO	MR	MY	NS	OD	PA	RT	SH	SM	VR	WA	WC	WS	YU
PepD															
(N)	10	10	16	10	10	10	16	10	5	10	39	5	2	10	8
A	0.040	0.000	0.000	0.000	0.000	0.000	0.016	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
B	0.050	0.000	0.000	0.000	0.000	0.000	0.984	0.000	0.000	0.000	0.000	0.000	0.000	0.100	0.224
C	0.910	1.000	1.000	1.000	1.000	1.000	0.000	1.000	1.000	1.000	1.000	1.000	1.000	0.900	0.776
Skdh-2															
(N)	10	10	16	10	10	10	16	10	5	10	35	5	2	10	8
B	0.617	0.450	1.000	0.150	0.545	0.060	0.000	1.000	0.200	0.129	0.486	0.000	0.000	0.429	0.266
C	0.383	0.550	0.000	0.850	0.455	0.940	1.000	0.000	0.800	0.871	0.514	1.000	1.000	0.571	0.734
Skdh-1															
(N)	10	10	14	10	10	10	10	10	5	10	35	5	2	10	7
A	0.000	0.000	0.000	0.025	0.218	0.000	0.000	0.075	0.000	0.033	0.000	0.000	0.000	0.000	0.000
B	1.000	1.000	1.000	0.975	0.782	1.000	1.000	0.925	1.000	0.967	1.000	1.000	1.000	1.000	1.000
Dia															
(N)	10	10	14	10	10	10	10	10	5	10	39	5	2	10	8
A	0.700	0.000	1.000	0.933	0.800	0.300	0.000	0.000	0.000	0.110	0.333	0.000	1.000	0.483	0.213
B	0.300	1.000	0.000	0.067	0.200	0.700	1.000	1.000	1.000	0.890	0.667	1.000	0.000	0.517	0.788
Pgk															
(N)	9	9	6	8	10	9	9	10	3	10	27	5	2	7	7
A	0.806	1.000	1.000	0.750	0.708	1.000	0.000	0.578	1.000	0.380	1.000	1.000	1.000	0.857	0.619
B	0.194	0.000	0.000	0.250	0.292	0.000	1.000	0.422	0.000	0.620	0.000	0.000	0.000	0.143	0.381
Gpi															
(N)	9	10	10	10	10	10	9	10	5	10	30	5	2	10	8
A	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
B	0.644	1.000	1.000	0.850	1.000	1.000	0.833	1.000	0.800	0.983	1.000	1.000	0.875	0.633	0.666
C	0.356	0.000	0.000	0.150	0.000	0.000	0.167	0.000	0.040	0.017	0.000	0.000	0.125	0.367	0.334
D	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.160	0.000	0.000	0.000	0.000	0.000	0.000
E	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000

APPENDIX 2. HERBARIUM VOUCHER SPECIMENS

Voucher specimens of *Hakea carinata* lodged with State Herbarium of South Australia

Date collected	Plant ID	Location description	Lat (UTM)	Long (UTM)
11/4/95	G1-01	Northern Lofty	34°39′S	138°50′E
11/4/95	G1-03	Northern Lofty	34°39′S	138°50′E
11/4/95	AR-03	Northern Lofty	34°39′S	138°49′E
11/4/95	GG-01	Northern Lofty	34°39′S	138°50′E
11/4/95	GG-05	Northern Lofty	34°39′S	138°50′E
11/4/95	QT-01	Northern Lofty	34°40′S	138°50′E
11/4/95	QT-04	Northern Lofty	34°40′S	138°50′E
29/4/95	MR-01	Mount Remarkable	32°43.30′S	138°04.12′E
29/4/95	MR-02	Mount Remarkable	32°43.45′S	138°03.95′E
29/4/95	MR-07	Mount Remarkable	32°43.98′S	138°03.03′E
9/8/95	HS-10	Humbug Scrub	34°43.19′S	138°48.91′E
9/8/95	RT-10	Humbug Scrub	34°43.02′S	138°48.67′E
11/8/95	JS-09	Jenkins Scrub	34°42′41″S	138°57′40″E
11/8/95	JS-10	Jenkins Scrub	32°42′41″S	138°57′40″E
6/11/95	MC-07	Mount Compass	35°19′42″S	138°35′01″E
6/11/95	NS-10	Nixon-Skinner	35°24′46″S	138°26′01″E
6/11/95	YU-10	Yulte	35°25′04″S	138°28′36″E
6/11/95	MY-10	Myponga	35°27′17″S	138°26′15″E
21/1/96	MM-10	Mount Magnificent	35°18′36″S	138°40′24″E
21/1/96	WC-02	Mount Compass	35°23′17″S	138°35′57″E
21/1/96	WS-04	Mount Compass	35°24′54″S	138°35′45″E
21/1/96	WS-10	Mount Compass	35°25′07″S	138°35′40″E