Landscape structure and population size effects on genetic pattern and process in *Banksia ilicifolia* R.Br.: consequences for conservation and ecological restoration

Michalie Foley
BSc (Hons)

This thesis is presented for the degree of Doctor of Philosophy
University of Western Australia
2013
I, Michalie Foley, declare that this thesis is submitted in its entirety for the fulfillment of the degree Doctor of Philosophy, School of Plant Biology, University of Western Australia. I declare that it is my sole work, unless otherwise referenced and acknowledged and has not been submitted for publication.

..................................................  
Michalie Foley
Abstract

Habitat fragmentation is an issue of conservation concern around the world. An understanding of how fragmentation affects populations on a landscape and local scale will underpin better conservation and ecological restoration outcomes. Habitat fragmentation reduces population size, can reduce connectivity among remnants within a matrix that is altered from the original, and potentially impacts the demographics and genetics of the species affected. A landscape genetic approach can reveal historical and contemporary genetic processes to inform better management choices for conservation and restoration. In this thesis, I take this approach to understand landscape scale genetic diversity and to assess how urbanization affects the important ecosystem function of pollen dispersal in *Banksia ilicifolia* R.Br. (Proteaceae). I also quantify population size effects on fitness parameters for *B. ilicifolia* seedlings, and how they respond to environmental stress.

The current 700 km range-wide spatial genetic structure of *B. ilicifolia* was assessed and the impact of historical climatic changes on these genetic patterns inferred. This information provides an insight into how the species may respond to future climate change. Microsatellite markers were developed for *B. ilicifolia*, and the levels and structuring of genetic variation within and among populations assessed by Mantel tests, principal components analysis and Bayesian clustering. Two broad regional scale genetic clusters were identified. Further analysis of the spatial structure of the allele frequencies strongly suggested a secondary contact zone between these the two regions, following greater separation during the last glacial maximum. This is the first time a secondary contact zone has been demonstrated for a southwest Australian plant species, and shows an impact of past climate change on species distributions in the region. Current climate change may be impacting the distribution of *B. ilicifolia* in a similar way, and this needs to be considered for conservation management.
Understanding how changes in population size and habitat fragmentation affect gene flow at a local scale is important for conservation, as a decrease in pollen input from outside the population as well as disruption to pollen dispersal within the population can lead to elevated inbreeding, a reduction in genetic diversity, and ultimately impact population viability. Pollen flow and genetic diversity were assessed in small (n=37), intermediate (n=97) and large (n>500) populations of *B. ilicifolia* by using microsatellite markers and conducting paternity assignments of seeds collected from within each population. The small population had greatly elevated nearest neighbour mating at approximately 40% compared with less than 5% for the larger populations. Genetic diversity decreased more between generations in the small population compared to the larger populations, despite comparable estimates of gene flow through pollen, with approximately 16% of siring from pollen originating from outside the local population. This study has shown through genetic analysis of pollen flow that in small fragmented populations of fewer than 40 plants, pollinator behaviour and/or composition has changed, leading to a negative outcome for the next generation. From these conclusions it is recommended for the management and future development planning that bush-land remnants be large enough to ensure that pollinator services are not compromised.

The third objective of this study was to assess the effect of population size on the fitness and capacity to respond to environmental change of progeny from small and large populations. This was done by evaluating the reproductive success of four small and four large populations of *B. ilicifolia*, and then subjecting seedlings from these populations to drought stress in a glasshouse trial. The small populations had a lower reproductive success due to a higher amount of aborted seeds. Germination and biomass of the seeds of small populations, however, were equivalent to those of the larger populations. When water was withheld, the seedlings of the larger populations survived longer. This may be due to the greater mass of roots in the top 20 cm of the soil profile compared with smaller populations, possibly leading to tighter stomatal control. From this study it is recommended that for restoration, seeds be sourced from larger populations to enable greater resilience of the population to a changing climate.
This thesis provides novel data underpinning the conservation and restoration of bird-pollinated species, especially in urban environments. A negative effect of fragmentation on pollination in small populations that has resulted in decreases in genetic diversity on a short time scale has been demonstrated. A negative effect of small population size on the fitness of progeny has also been demonstrated with small population seedlings having lower survival in drought conditions. Genetic structure has given indications of the possible response of *B. ilicifolia* to climate change and should be taken into account when thinking of restoration in the longer term. Together, this information provides managers and planners essential information to help restore and conserve natural populations for long-term viability.
Table of contents

Declaration i
Abstract ii
Table of Contents v
Acknowledgements vii

Chapter 1 General Introduction 1
1.1 Habitat fragmentation effects on natural populations 1
  1.1.1 Habitat fragmentation 1
  1.1.2 Pollination a key ecosystem service affected by fragmentation 1
  1.1.3 Genetic threats to fragmented populations 3
1.2 The Southwest Australian Floristic Region - a unique biodiversity hotspot 5
1.3 Approaches to understanding habitat fragmentation 6
1.4 This study 8
  1.4.1 Biology of Banksia ilicifolia R.Br. 8
  1.4.2 Outline and questions addressed in thesis 12

Chapter 2 Characterisation and cross amplification of novel microsatellite markers for Banksia ilicifolia 14
2.1 Introduction 14
2.2 Methods and Results 15
2.3 Conclusion 19

Chapter 3 Present genetic structure reflects past demographic situations and informs likely impacts of future climate change 20
3.1 Introduction 20
3.2 Methods 23
  3.2.1 Study species 23
  3.2.2 Sampling design 23
  3.2.3 DNA extraction and microsatellite analysis 25
  3.2.4 Statistical analysis 25
3.3 Results 29
  3.3.1 Genetic diversity 29
  3.3.2 Principal Components Analysis 29
  3.3.3 Population Genetic Structure 33
  3.3.4 Mantel test 38
  3.3.5 Spatial Analysis of Allele Frequencies 40
3.4 Discussion 44

Chapter 4 Urban fragmentation alters pollen-dispersal patterns in Banksia ilicifolia populations 50
4.1 Introduction 51
4.2 Methods 54
Acknowledgements

I would like to thank my principal supervisor Dr Siegy Krauss for his invaluable input with the design and direction of the project and his patience to deal with draft after draft. I would also like to thank my other supervisors Professor Erik Veneklaas and Winthrop Professor Hans Lambers for their skills and instrumental motivation and support.

I wish to thank the University of Western Australia and the Botanic Garden and Parks Authority for hosting me during my PhD. I also would like to thank Dr Janet Anthony for her help and support and Dr Carole Elliot for reading through my drafts. Thanks go to my fellow PhD students for providing friendship and support through this long process with a special thank you to Donna Bradbury for her help in the field and providing an ear when I needed it.

Last, I would like to give a big thank you to my parents, Robyn and Brian Foley, for all your help in the field and glasshouse, encouragement, support and believing that I would get to this point eventually. The very last thank you goes to my partner, Sacha Ruoss, for his help with fieldwork and editing, and his constant love and support.
Chapter 1 General Introduction

1.1 The impact of habitat fragmentation on natural populations

1.1.1 Habitat fragmentation

Habitat fragmentation is one of the most significant concerns for the conservation of biodiversity (Young and Clarke, 2000). Habitat fragmentation involves the reduction of continuous natural habitat through anthropogenic means into smaller remnants that are often separated by a matrix that can be very different from the original habitat (Wilcove et al., 1986, Saunders et al., 1991, Young et al., 1996). Globally, forested areas are being reduced at a rate of 13 million hectares per year, though this is reduced from the 1990’s (FAO, 2010). While the largest areas of deforestation are currently in South America and Brazil, Australia has cleared vast areas of native vegetation for agriculture over the past 100 years (Saunders et al., 1991, FAO, 2010). From 2007 to 2010, land clearing of forested areas in Australia was offset by forest expansion. However, the historical clearing has left a legacy of extensive fragmentation (State of the Environment 2011 Committee, 2011).

Due to the concerns about the negative effect of fragmentation on ecosystem processes and species extinction, research into the effects of fragmentation has been a major focus for conservation biology (Lande, 1988, Hobbs and Yates, 2003). Fragmentation is known to have a negative effect on biodiversity directly via habitat loss, reduction in population size, species richness and genetic diversity, as well as indirectly by increasing the effect of environmental variables such as weather, herbivory, pollinators and edge effects (Fahrig, 2003, Oostermeijer et al., 2003). One of the key gaps in our knowledge, that is crucial to the understanding of ecosystem function and sustainability, is pollination and and how pollinator functions and genetic consequences are affected by habitat fragmentation (Hadley and Betts, 2012).

1.1.2 Pollination a key ecosystem service affected by fragmentation

Pollen dispersal is a significant ecosystem function that is affected by habitat fragmentation and there is a need to understand how fragmentation affects the species that contribute to pollination (Rathcke, 1993) Despite examples showing the negative impact of fragmentation on pollination (Aguilar et al., 2006, Isagi et al., 2007, González-Varo et al., 2009) there is still a paucity of
studies on how habitat loss and fragmentation affects pollination (Hadley and Betts, 2012). It is essential to get an understanding on how fragmentation and pollination are linked as pollination and reproductive success can be affected by both a reduced number of pollinators or a reduced number of available mates which occurs in disturbed landscapes (Duncan et al., 2004). It is also difficult to gauge the extent of these effects when many ways of investigating pollination are used in the wider literature. These include indirect measurements such as examining pollen loads on plants (Duncan et al., 2004), reproductive success and output (Brys et al., 2004) as well as direct estimation of gene flow and paternity analysis (Llorens et al., 2012). If these approaches were integrated it would provide a greater understanding of the ecological implications of fragmentation on pollination.

Pollinators in habitats that are fragmented are particularly important as they provide a link between populations and isolation can limit movement of pollinators between the remnants (Steffan-Dewenter and Tscharntke, 1999). The distance that pollinators will travel is largely dependent on the pollinator species themselves and their foraging area (Kwak et al., 2009). Generally smaller bodied pollinators such as insects will not travel as far as larger bodied pollinators such as birds (Steffan-Dewenter and Tscharntke, 1999).

The functional diversity of a pollination guild is highly important as many plant species have morphology evolved for specific pollinators. For example, plants with long tube flowers rely on pollinators with long beaks (Pauw and Louw, 2012). If landscape change and fragmentation reduces the functional diversity of the pollinator guild then the reproductive success of plant species will be compromised with reduction of seed production and increased inbreeding (Şekercioğlu et al., 2004). In areas in need of restoration these processes are crucial. Restoration efforts need to include attempts to restore pollinator services for the population to be successful however there is little information available in this area (Menz et al., 2011).

Much of the research conducted on landscape change and pollination has occurred in the tropics (Dick et al., 2003, Ward et al., 2005). There are few
studies on the effect fragmentation has on pollination in temperate regions (Steffan-Dewenter and Westphal, 2008) and of these many are based in the northern hemisphere (Kunin, 1997, Cheptou and Avendaño V, 2006, Peterson et al., 2008, Van Rossum, 2010) with few in the southern hemisphere (Vaughton, 1995, Wooler and Wooler, 2003).

While there is an abundance of literature on insect and wind pollination (Steffan-Dewenter et al., 2002, Ghazoul, 2005, Peterson et al., 2008), the function and ecological significance of bird pollination is a neglected area that requires more attention (Phillips et al., 2010). Studies from around Australia have concentrated on insect pollination syndromes (Cunningham, 2000, Harris and Johnson, 2004, Ottewell et al., 2009) and while this is important to understand bird pollination is an essential ecosystem service for many species of plants and has a few key differences to insect pollination. Birds have a high energy requirement and may forage more widely, intensively and faster than insects which may increase the chance of outcrossing (Ford et al., 1979). The larger size of birds may in fact allow for travelling greater distances (Stiles, 1978). Insect pollination often results with a high majority of pollen deposited on plants next to the pollen donor (Kwak et al., 2009). In contrast, bird pollination can result in a departure from nearest neighbour with plants further away being prevalent pollen donors (Krauss et al., 2009).

If bird numbers decline then there is a threat of pollen limitation for the dependent bird-pollinated species. Bird pollinated plants have flowers evolved to maximise pollination by birds and studies have shown that in some cases insects (eg. honeybees) will reduce seed set due to inefficient pollen deposition and outcrossing rates because of the smaller distance traveled between inflorescences (Paton, 1993, England et al., 2000, Celebrezze and Paton, 2004). Reduction of efficient pollinators can lead to reduced seed set and a decline to the plant species (Robertson et al., 2001, Şekercioğlu et al., 2004). As habitats are fragmented and the size and shape of natural remnants change there may be loss of bird species that are of the required morphology for pollination (Elliott et al., 2012, Pauw and Louw, 2012). How these losses of species impacts on plant reproductive function is an important area of study and one that is critical to the conservation of natural remnants. It is also crucial to
understand how pollination limitation affects the genetic diversity of small populations.

1.1.2 Genetic consequences of habitat fragmentation

Habitat fragmentation often leads to negative genetic consequences and is a concern in conservation biology because of the possible detrimental affect on a population’s viability (Young et al., 1996). Frankham (1996) demonstrated that genetic variation was positively related to population size, therefore a reduction in population size leads to a loss of genetic diversity. This occurs because reduced population size increases the effect of genetic drift and inbreeding, and habitat fragmentation leads to reduced connectivity of remnants (Dudash and Fenster, 2000, Charlesworth, 2003). Genetic drift involves random change of allele frequencies due to chance and is experienced by all populations. However, these changes are relatively minor in large populations and become more pronounced as population size decreases (Ellstrand and Elam, 1993). Genetic drift may lead to a reduction in heterozygosity and fixation of alleles and increase the differentiation among populations (Ellstrand and Elam, 1993). Genetic drift may actually cause the fixation of deleterious alleles. With the fixation of alleles and reduction in genetic diversity, extinction may occur when low genetic variation does not allow for a species to adapt to changing environments (Wright, 1931, Ellstrand, 1992).

Historically small, fragmented populations may not have genetic concerns. While populations may lose heterozygosity and increase the fixation of deleterious alleles (genetic load), some populations may be able to purge the genetic load through selection after a bottleneck (Frankham et al., 2002). Purging of the genetic load is dependent on the severity of the lethal allele and the degree of inbreeding. There is debate about the evidence of this phenomenon and its extent in natural populations (Crnokrak and Barrett, 2002). With the possibility of purging the genetic load are populations that have low heterozygosity but not reduced fitness as can be seen in the Wollemi pine (Peakall et al., 2003a).

The other concern of reduced population size is elevated inbreeding due to increases in selfing and biparental inbreeding because of the likelihood that the individuals in the remaining population are related (Young et al., 1996).
Inbreeding may occur because the genetic linkers (such as pollinators) are no longer present or functioning in a way to increase mating between closely related individuals (eg. nearest neighbour mating). Increased inbreeding serves to reduce genetic diversity by increasing homozygosity and reducing the effective frequency of recombination (Charlesworth, 2003, Ouborg et al., 2006). Inbreeding is a concern because of inbreeding depression associated with the increased homozygosity and affects various fitness components of the population (Dudash and Fenster, 2000, Reed and Frankham, 2003). Inbreeding depression can be detrimental to the viability of populations and can lead the populations towards extinction (Frankham et al., 2002).

Gene flow can counteract the negative consequences of genetic drift and inbreeding by introducing new variation or reintroducing lost alleles. Gene flow in plants occurs via seed or pollen and has been shown to be affected by patch size (Ellstrand and Elam, 1993) The nature of habitat fragmentation means that remnants often become spatially isolated which can the lower the ability for gene flow to occur (Fahrig, 2003). Without the exchange of alleles the populations will become increasingly differentiated (Wright, 1969a, Wright, 1969b). However, this may not always be the case and fragmentation may increase gene flow into small populations and the negative associations of small population size may not apply to all species (Kramer et al., 2008).

1.2 The Southwest Australian Floristic Region – a unique biodiversity hotspot

The Southwest Australian Floristic Region (SWAFR) is a species rich area dominated by old landscapes that have been unglaciated since the Permian (Hopper and Gioia, 2004). The SWAFR is flat and stable with nutrient impoverished soils and this is believed to drive the high species diversity as well as high diversity in form and function of the flora (Lambers et al., 2010). There is a high turnover of species along habitat gradients and landscapes (Cowling et al., 1996). The SWAFR is characterized by a high amount of endemism, with 49% of species endemic to small areas of the region (Hopper and Gioia, 2004) with the highest percent of vertebrate pollinated plants globally at around 15% (Hopper, 2009).
There are many threats to the biodiversity in the SWAFR, with habitat fragmentation a major one. Large areas of native vegetation have been cleared for agriculture and urban development with some areas left with just 2-3% of the original vegetation (Hobbs, 2002). This has resulted in 2240 taxa in the SWAFR being placed in a conservation category (Western Australian Herbarium, 1998) with unknown numbers of un-named species with conservation needs still to be identified (Hopper and Gioia, 2004). Furthermore, there are threats of introduced herbivores which can decimate the native vegetation and invasive exotic species that compete for resources in degraded habitats (Hobbs, 2002). Consequently, because of the threats to this highly biodiverse region, the SWAFR is an international biodiversity hotspot for conservation, the only one in Australia (Myers et al., 2000).

Within the threatened flora of the SWAFR, 40% is bird pollinated by nectarivorous bird species (Hopper, 2009). With this very large proportion with this pollination syndrome there is very little known on the impacts of fragmentation on gene flow in these species. While there are some studies beginning to fill this gap (Byrne et al., 2007, Llorens et al., 2012) there is an urgency to better understand the impacts that fragmentation has on the pollination web of nectarivorous birds and the species they pollinate.

Within the SWAFR there has been extensive clearing not only for agriculture but also for urbanisation. Urbanisation is a conservation concern as it is a more permanent type of habitat destruction and brings to it a whole suite of concerns such as habitat loss, isolation, pollution and invasive species (McKinney, 2002). The Perth metropolitan region is a rapidly expanding city that is placing natural bushland into isolated pockets throughout the city. It is important to understand how these remnants function for future viability.

1.3 Approaches to understanding habitat fragmentation on genetic processes
There are many ways to approach the challenge of habitat fragmentation and its effect on populations depending on the questions being asked and the perceived importance of threats. Here, I am concentrating on the genetic
consequences of habitat fragmentation. It is important to start at a landscape scale and understand the drivers of genetic diversity of the species of interest to be able to put in place strategies to see these drivers persist in the future. There are now many tools to examine genetic structure and the processes creating the patterns at a landscape scale. It is also important to understand ecosystem processes that affect genetic diversity at a population level to ensure individual populations can be viable for the long-term.

Landscape genetics is a recently emerged field of study that incorporates molecular population genetic studies with landscape scale ecological studies (Manel et al., 2003). It has become more popular as molecular tools have improved in ease of use and cost as well as advances in geographic information systems and spatial statistics (Sork et al., 1999). This combination of approaches allows for a deeper understanding of how landscape and ecological processes shape the genetic structure that are present and allow researchers to move past tests of isolation by distance into more complex spatial testing (Storfer et al., 2006).

The number of studies that incorporate landscape genetics has increased ten fold in the first decade of the 21st century (Storfer et al., 2010). These studies have included a wide variety of taxa and used many analysis techniques to explore a number of different questions (Storfer et al., 2010). Questions include understanding how environmental variables influence genetic structure, identifying barriers to gene flow and whether perceived barriers (eg. roads and waterways) have an effect on genetic structure, understanding dispersal and exploring spatial and temporal scales (Manel et al., 2003).

The main advantage of landscape genetics is the ability to use a suite of analytical techniques from a variety of disciplines. The use of traditional analysis of isolation by distance and matrix correlations (Mantel test) are still widely used to compare genetic distance and geographic distance (Storfer et al., 2010) but are complemented with more complex analysis. Analyses include spatial autocorrelation, ordination, interpolation, Monmonier algorithm, partial Mantel tests and Bayesian clustering (Manel, 2003).
Understanding population level drivers of genetic diversity and the response to habitat fragmentation is also an increasing area of interest. Many techniques are available to understand fine-scale genetic structure as well as gene flow through dispersal patterns. With the advent of powerful hypervariable markers such as microsatellite markers, gene flow can be directly estimated with the use of parental reconstruction and paternity analysis (Gerber et al., 2000, Jones et al., 2010). There are many types of ways to conduct parentage analysis, and Jones et al. (2010) provide a good review of each method. There are six types of parentage analysis and each has positives and negatives. The type and polymorphism of the chosen molecular marker as well as sampling strategy will determine the approach for paternity analysis. Ideally offspring would be collected from known mothers and all possible fathers in the population sampled and genotyped with highly polymorphic molecular markers (Jones et al., 2010). Once paternity is assigned the sire can be traced to location and patterns of pollen dispersal will be revealed (He et al., 2004, Llorens et al., 2012).

1.4 This study

In this study, I use the tools of landscape genetics and paternity analysis to explore historical and current spatial genetic structure at a landscape scale, as well as the effect of habitat fragmentation at a population level. I use Banksia ilicifolia R.Br. (Proteaceae) as a model species to understand the drivers of, and habitat fragmentation impacts on, spatial genetic structure and pollen dispersal. I aim through this study to provide a greater understanding of how habitat fragmentation has affected the viability of small populations.

1.5 Biology of Banksia ilicifolia

Banksia ilicifolia is a member of the Isostylis subgenus of Banksia and is characterized by the holly shape of its leaves and short inflorescences that contain less than 100 flowers (Broadhurst and Coates, 2004). The other members of Isostylis are B. cuneata and B. oligantha. Banksia ilicifolia is wide spread with a 700 km range that is from east of Albany on the south cost of Western Australia to Cervantes in the north, while B. cuneata and B. oligantha are restricted to pockets inland in the drier agricultural region. The distribution of B. ilicifolia is limited to low-lying areas in the landscape (Zencich et al., 2002)
and because of this its distribution can be naturally fragmented. *Banksia ilicifolia* habitat occurs in areas that are being cleared for urbanisation on a large scale. This has resulted in the already patchy populations of becoming smaller and more isolated.

*Banksia ilicifolia* has been shown (Zencich et al. 2002) to use groundwater as its main water source except following major rainfall events. Studies conducted at the main aquifer supplying Perth City water have shown *B. ilicifolia* is restricted to areas of shallow ground water (within 10 m). With abstraction of water from the aquifer and the drawdown of groundwater there has been substantial death of *B. ilicifolia* trees indicating its low tolerance for drought conditions (Groom et al., 2000). This with a combination of habitat degradation and loss makes for significant conservation issues that may be linked.

*Banksia ilicifolia* flowers with a peak occurring August through October. Inflorescences change colour from yellow to red as they age and are primarily pollinated by birds (Lamont and Collins, 1988). However, unlike many bird pollinated plants, in which red flower colour attracts bird pollinators, it has been demonstrated that the birds will visit the *B. ilicifolia* flowers while they are yellow and not red and hence is considered a signal of nectar depletion (Lamont and Collins, 1988). The study by Lamont and Collins (1988) also demonstrated that insects also visit the *B. ilicifolia* inflorescences but birds carry far more pollen than native insects. Previous studies have shown that *B. ilicifolia* is preferentially outcrossing and has a low conversion of flowers to fruit (Heliyanto et al., 2005). *Banksia ilicifolia* is not a serotinous species; seeds are released from the follicles in early autumn. The seeds do not have any dormancy and natural recruitment is very low.

This species provides an interesting model to study landscape genetics because of the widespread distribution and the specific ecological conditions needed for survival. Previous studies on mating systems of this species also provide vital and useful information such as the tendency for preferential outcrossing(Heliyanto et al., 2005). This species occurs in areas that are being extensively cleared for housing and development. An understanding of its genetic diversity and pollination biology is essential to be able to conserve this
species in the small populations left in urban remnants and inform future development strategies. This combined with the previous knowledge of the species make it an ideal and relevant candidate for pollination biological and ecophysiological studies.
Figure 1. *Banksia ilicifolia* from the Perth region. A) mature tree with new and old inflorescences B) new unopened inflorescence (yellow) C) old opened inflorescence (red) D) *B. ilicifolia* tree in *Banksia* woodland community on deep sands
Figure 2. A) Banksia ilicifolia community from the southern extent of range. B) old inflorescence from southern region has a different colour (yellow) than the northern region (red)
1.4.1 Outline and aims addressed in thesis

The structure of the thesis is the chapters are a series of papers that are in the format for publication. Therefore each chapter contains its own literature review in the introduction and there may be some overlap of information encompassed in the methods. The thesis aims to address the following questions in the relevant chapters as outlined:

Q1 Can microsatellite markers be developed for B. ilicifolia to use in landscape and pollen dispersal studies?

In Chapter 2, my aim is to describe the development of microsatellite markers to enable genetic analysis of B. ilicifolia. I present the primer note that explains the methods used to test cross-transfer of microsatellite markers from other Banksia species to B. ilicifolia. Development of B. ilicifolia specific microsatellite markers will also be outlined and proven to be useful for further study with relevant genetic diversity measures presented.

Q2 What are the possible drivers of current spatial genetic structure in B. ilicifolia?

In Chapter 3, I aim to characterize landscape scale spatial genetic structure in B. ilicifolia. I assess the genetic structure of B. ilicifolia using the microsatellite markers developed in Chapter 2. I use the statistical tools developed for landscape genetics to understand genetic spatial structure across the range of the species and present theories of how this structure came to be.

Q3 How is pollen dispersal affected by habitat fragmentation and small population size?

In Chapter 4, I aim to investigate whether population size has a negative affect on pollen dispersal. I use paternity analysis to construct pollen dispersal curves for small, intermediate and large populations of B. ilicifolia to understand the effect of habitat fragmentation on pollen flow. Within this chapter I also assess the effect of small population size on genetic diversity between generations.
Q4 Does habitat fragmentation and plant fitness affect reproductive success, germination and plant growth?

And

Q5 Is there an effect of population size on affect under environmental stress?
In Chapter 5, I aim to test whether plant reproductive fitness and growth are compromised by small population size. I also aim to demonstrate that plants from smaller populations have a lower ability to cope with environmental stress. I compare the reproductive success of small and large populations of *B. ilicifolia*. I also assess the effect of environmental stress on the seedlings from small and large populations to establish if seed used from small populations for restoration will be detrimental to the success of population.

Q6 What are the practical conservation implications of this study?
In Chapter 6, I will give an overview of all results as a whole. I will draw conclusions on the impact of population size on pollen flow and seedling fitness and how this will affect population viability and restoration attempts. I will also discuss long-term conservation under the current models of climate change.
Chapter 2

Characterization and cross-species amplification of novel microsatellite markers for *Banksia ilicifolia* R.Br. (Proteaceae)

Abstract

I developed 8 novel polymorphic microsatellite markers for the Australian tree *Banksia ilicifolia* R.Br. and cross-species amplified a further 6 polymorphic loci from 56 developed for other *Banksia* species to study landscape genetics and pollen dispersal. In a sample of 30 individuals for the *B. ilicifolia* primers, the number of alleles over the 8 loci ranged from 4 to 12, observed- and expected-heterozygosities ranged from 0.32-0.84 and 0.55-0.88, respectively. One locus showed a deviation from Hardy-Weinberg equilibrium expectations, and was the only one to show evidence for null alleles. For the remaining 7 primers the number of alleles ranged from 4-10, observed and expected heterozygosity ranged from 0.258-0.93 and 0.286-0.737 respectively and no locus showed a departure from Hardy-Weinberg equilibrium.

2.1 Introduction

Molecular markers have long been used to study genetic processes such as gene flow and genetic spatial structure (Selkoe and Toonen, 2006) Microsatellite markers are highly variable codominant markers that are ideal for these kinds of studies (Ouborg et al., 1999). However they are species specific with limited cross-transferability and can be costly to develop (Sunnucks, 2000). Microsatellite markers can be developed by a number of methods including searching sequence databases for existing microsatellite sequences, transferring existing microsatellites from related species, cloning new microsatellites (Baker, 2009). Next generation sequencing (NGS) is now becoming the preferred method of microsatellite development (Gardner et al., 2011). NGS provides a much larger and greater numbers of fragments during a single read that will contain by chance a great many microsatellite repeat motifs thus providing a cheaper and more efficient method of development (Abdelkrim et al., 2009, Davey et al., 2011)
Banksia ilicifolia R.Br. is an over-story species endemic to the sandy coastal plain of southwest Australia. Much of its northern distribution has been impacted by urbanization of the Perth metropolitan area, where it largely remains in fragmented urban bushland remnants. To better understand the consequences of these impacts on key population processes and their impacts on pollinators (Ramsey, 1989, Bradshaw et al., 2007) research is required into metapopulation dynamics, pollination biology and population genetics of B. ilicifolia. Therefore, microsatellite markers previously developed for Banksia attenuata R.Br., Banksia hookeriana Meisn., and Banksia sphaerocarpa (He et al., 2007, He et al., 2008, Nistelberger et al., 2009) were tested for cross-transferability to B. ilicifolia, and subsequently microsatellite markers were developed specifically for B. ilicifolia.

2.2 Methods and Results

Leaves of B. ilicifolia were collected and stored at -80 °C until DNA extraction. Total genomic DNA was extracted following the protocol of Jobes et al. (1995). Genomic libraries were constructed from 100 µg of DNA by Genetic Identification Services (GIS, www.genetic-id-services.com) following the methods of Jones et al. (2002). Briefly, genomic DNA was partially digested with a mixture of seven blunt-end restriction enzymes (RsaI, Hae III, Bsr B1, Pvu II, Stu I, Sca I, Eco RV). Fragments 300 to 750 bp were adapted and captured with magnetic bead capture using biotinylated capture molecules. Captured fragments were then digested with Hind III to remove adapter sequences. The resulting fragments were then ligated onto the Hind III site of pUC19 and cloned into the Escherichia coli strain DH5a. Inserts from 100 recombinant clones were sequenced on an ABI PRISM 377 DNA autosequencer (Applied Biosystems, Carlsbad, California, USA) using Amersham’s DYEnamic Terminator Cycle Sequencing Kit (Amersham Bioscience P/N US81050). There were 114 inserts found to contain microsatellite sequences. PCR primers were designed for 75 of the microsatellites using DesignerPCR version 1.03 (Research Genetics Inc.)

From these, 24 loci were initially assessed with unlabeled primers for seven samples of B. ilicifolia. DNA PCR reactions were carried out in 10 µL total
volume containing 3-5 ng of template DNA, 2 µL of 5x buffer (Fisher Biotec: final concentration of 67 mM TRIS (pH 8.8) 16.6 mM (NH₄)₂SO₄, 0.45% Triton X-100, 0.2 mg/mL gelatin, 0.2 mM of each dNTP), 0.025 U/µL Taq DNA polymerase (Fisher Biotec, Perth, Australia), 1.5-3 mM MgCl₂ and 0.75 µM of forward and reverse primer. PCR reactions for each primer pair were carried out separately with initial activation of 94 °C for 3 minutes followed by 35 cycles of denaturation at 94 °C for 40 seconds, annealing at 51-60 °C for 40 seconds and extension at 72 °C for 30 seconds with a final extension at 72 °C for 15 minutes. PCR products were visualised on a 2% agarose gel and primers that amplified clear bands were selected for further genotyping and 5’ end labeled with Wellred D2, D3 or D4 fluorescent dyes (Sigma Aldrich Corp, Missouri, USA.). Testing of labeled primers was carried out on 30 samples collected from one population with the same PCR conditions as for unlabeled primers. Amplified products were visualised using a CEQ 8800 Genetic Analysis System and CEQ fragment analysis software (Beckman Coulter, California, USA). This procedure was also carried out on the labeled primers developed from the other species of Banksia to test for amplification, polymorphy and reproducibility.

Expected heterozygosity, observed heterozygosity, allelic diversity, and departures from Hardy-Weinberg equilibrium expectations were calculated with GenALEX v6.41 (Peakall and Smouse, 2006). Microchecker (Van Oosterhout et al., 2004) was used to test for the presence of null alleles. To test for Mendelian inheritance, 20 open pollinated offspring from one maternal plant were genotyped.

Of the 24 B. attenuata primers, 8 amplified, of which 3 were consistently scoreable and polymorphic. Within the 24 B. hookeriana primers, 9 amplified, of which 2 were consistently scoreable and polymorphic. Of the 8 B. sphaerocarpa primers, 6 amplified, 4 were polymorphic but only one was consistently scoreable. Number of alleles per locus ranged from 4 to 10 and expected heterozygosity ranged from 0.29 to 0.69 (Table 2.1). Only one locus was not in Hardy-Weinberg equilibrium. All loci showed Mendelian inheritance.
Of the 24 *B. ilicifolia* microsatellite loci for which primers were designed, 4 failed to amplify, 7 were monomorphic and a further 4 were discarded due to severe stuttering. Eight loci were consistently scoreable and polymorphic. Number of alleles per locus ranged from 4 to 12 and expected heterozygosity ranged from 0.55 to 0.88 (Table 2.2). One locus was not in Hardy-Weinberg equilibrium, most likely due to null alleles as identified by Micro-Checker (Van Oosterhout et al., 2004).

### Table 2.1. Six microsatellite markers cross-transferred to *Banksia ilicifolia*. Sample size (N), annealing temperature (Ta), observed heterozygosity (Ho), expected heterozygosity (He), number of alleles (Na), Hardy-Weinberg equilibrium (HWE) where ns is non-significant and * is significant

<table>
<thead>
<tr>
<th>Locus</th>
<th>Species</th>
<th>Reference</th>
<th>N</th>
<th>Ta (°C)</th>
<th>Na</th>
<th>Ho</th>
<th>He</th>
<th>HWE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bs108</td>
<td><em>Banksia sphaerocarpa</em></td>
<td>Nistelberger et al. (2009)</td>
<td>31</td>
<td>50</td>
<td>5</td>
<td>0.742</td>
<td>0.691</td>
<td>ns</td>
</tr>
<tr>
<td>BaA3</td>
<td><em>Banksia attenuata</em></td>
<td>He et al. (2007)</td>
<td>31</td>
<td>52</td>
<td>10</td>
<td>0.677</td>
<td>0.643</td>
<td>ns</td>
</tr>
<tr>
<td>BaD115</td>
<td><em>Banksia attenuata</em></td>
<td>He et al. (2007)</td>
<td>31</td>
<td>53</td>
<td>4</td>
<td>0.258</td>
<td>0.286</td>
<td>ns</td>
</tr>
<tr>
<td>BaC8</td>
<td><em>Banksia attenuata</em></td>
<td>He et al. (2007)</td>
<td>31</td>
<td>54</td>
<td>6</td>
<td>0.581</td>
<td>0.584</td>
<td>ns</td>
</tr>
<tr>
<td>BhB5</td>
<td><em>Banksia hookeriana</em></td>
<td>He et al. (2008)</td>
<td>31</td>
<td>52</td>
<td>5</td>
<td>0.839</td>
<td>0.649</td>
<td>ns</td>
</tr>
<tr>
<td>BhA3</td>
<td><em>Banksia hookeriana</em></td>
<td>He et al. (2008)</td>
<td>31</td>
<td>52</td>
<td>6</td>
<td>0.935</td>
<td>0.737</td>
<td>*</td>
</tr>
<tr>
<td>Locus</td>
<td>Sequence</td>
<td>Ta (°C)</td>
<td>Size Range (bp)</td>
<td>Repeat</td>
<td>Wellred Dye</td>
<td>N</td>
<td>Na</td>
<td>Ho</td>
</tr>
<tr>
<td>-------</td>
<td>----------</td>
<td>---------</td>
<td>----------------</td>
<td>---------</td>
<td>-------------</td>
<td>---</td>
<td>----</td>
<td>----</td>
</tr>
<tr>
<td>B6</td>
<td>F: TTTCCCTTTACACATCATCATGAG</td>
<td>56</td>
<td>245-268</td>
<td>(CT)(<em>{13})(CA)(</em>{6})</td>
<td>D3</td>
<td>31</td>
<td>7</td>
<td>0.40</td>
</tr>
<tr>
<td></td>
<td>R: GCATTATTTTACTACCTCCCMTGC</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D1</td>
<td>F: GAGTGGTATGTTGCACTATGAGAAGG</td>
<td>56</td>
<td>176-202</td>
<td>(TCC)(_6)</td>
<td>D2</td>
<td>31</td>
<td>6</td>
<td>0.62</td>
</tr>
<tr>
<td></td>
<td>R: GATAACGACTTGAAGCAACGAAAGAG</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B104</td>
<td>F: CACACTTTCACTGCACAC</td>
<td>53</td>
<td>215-249</td>
<td>(AG)(_{14})</td>
<td>D3</td>
<td>31</td>
<td>12</td>
<td>0.80</td>
</tr>
<tr>
<td></td>
<td>R: CGTAAACCGAAATGTGTAC</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A3</td>
<td>F: AGGCCAACAGAGATATGC</td>
<td>56</td>
<td>196-200</td>
<td>(CA)(_{13})</td>
<td>D2</td>
<td>31</td>
<td>8</td>
<td>0.81</td>
</tr>
<tr>
<td></td>
<td>R: ATACGAAAGCAGCACATACAA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D3</td>
<td>F: TGGGCTTCATTCGACTACATCGCCG</td>
<td>54</td>
<td>102-129</td>
<td>(TGA)(_{13})</td>
<td>D4</td>
<td>31</td>
<td>8</td>
<td>0.84</td>
</tr>
<tr>
<td></td>
<td>R: TTCTGCTCACCACATAAACCTT</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C103</td>
<td>F: GATTTGTGCAAGTCGCTAGCTAC</td>
<td>56</td>
<td>257-279</td>
<td>(CAA)(_{13})</td>
<td>D4</td>
<td>31</td>
<td>3</td>
<td>0.68</td>
</tr>
<tr>
<td></td>
<td>R: TGCTTTTTGGATCATCATATG</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B105</td>
<td>F: CCTGCTCAGATGTTGAGACTGCTAC</td>
<td>56</td>
<td>162-200</td>
<td>(CT)(_{20})</td>
<td>D3</td>
<td>31</td>
<td>10</td>
<td>0.72</td>
</tr>
<tr>
<td></td>
<td>R: TGAGCAAGAGAGTAGGAGAGAC</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A110</td>
<td>F: ATCCCGATTTACTCTAAACAAGCA</td>
<td>55</td>
<td>155-189</td>
<td>(CT)(<em>{14})(CA)(</em>{13})</td>
<td>D2</td>
<td>31</td>
<td>10</td>
<td>0.75</td>
</tr>
<tr>
<td></td>
<td>R: GTGAGCAGGCTGCCATAT</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
2.3 Conclusion

Cross-species amplification of microsatellites from *B. attenuata*, *B. hookeriana* and *B. sphaerocarpa* to *B. ilicifolia* was successful. However, of the 23 loci that amplified only 6 were consistent, reproducible and polymorphic. This is likely to be a consequence of the phylogenetic divergence between these three species and *B. ilicifolia*, which is placed in the tribe Isostylis and believed to have diverged more than 20 Mya (He et al., 2011). The eight microsatellite markers developed here for *B. ilicifolia* and the six cross-transferred from other *Banksia* species, provide highly polymorphic molecular markers for further studies to assess landscape scale genetic structure, mating system, and pollen dispersal through paternity analysis in this species.
Chapter 3

Present genetic structure reflects past demographic situations and informs potential impacts of future climate change

Abstract
An understanding of current genetic structure can give an insight into how historic landscapes shaped population structure. The biodiversity hotspot of southwest Australia exists on an ancient stable landscape and the tools of landscape genetics can be used to understand how subtle landscape and climate changes can influence spatial genetic structure. Using microsatellite molecular markers I explore the genetic structure of Banksia ilicifolia R. Br. Through the use of Mantel tests, principal components analysis and Bayesian clustering I show that there is a significant correlation of genetic and geographic distance with two distinct genetic regions likely to have segregated at the Last Glacial Maximum. Inspection of assignment probabilities and the spatial variation of allele frequencies reveal a secondary contact zone as the two regions came into proximity again. The broad-scale spatial genetic structure is best explained by historical events that have resulted in two clear regions. This is the first time a secondary contact zone has been identified in southwest Australia. This historic pattern may be repeated if the models of climate change prove to be true and the area becomes drier. Banksia ilicifolia may once again be forced into range contraction and segregating the species once more.

3.1 Introduction
There are many factors that influence spatial genetic structure within plant species, including gene flow via pollen and seed dispersal, mating systems, genetic drift, adaptation and selection. However, demographic history of populations, including range contractions and expansions, is also potentially a significant contributor to current patterns of landscape-scale spatial genetic structure (Holzhauer et al., 2006). For example, spatial genetic structure is impacted by historical climate changes, through range expansion and contraction, local extinctions and founder effects (Turgeon and Bernatchez, 2001, Hewitt, 2004).
The consequences of climatic impacts on spatial genetic structure have been documented for many northern hemisphere species (Petit et al., 2003, Knowles and Richards, 2005, Schönswetter et al., 2005). Research into effects of glacial periods in this region, including ice core data and the fossil record, is expansive, and as a consequence, studies of spatial genetic structure and phylogeography can be placed into a reasonably accurate context of historical climate changes. The last glacial maximum (LGM) resulted in the southward expansion of the northern ice sheet and permafrost driving species into southern refugia (Hewitt, 2004). Interglacial periods then allowed recolonisation and possible secondary contact between diverged lineages (Turgeon and Bernatchez, 2001, François et al., 2008).

During the Pleistocene in Australia, however, there were no ice sheets expanding and contracting as at northern latitudes, and the cooler and drier LGM made increasing aridity the main driving factor impacting species distributions (Byrne, 2008). Climatic conditions of the Pleistocene have been shown to influence genetic diversity through the creation of bottlenecks in Eastern Australia in the conifer species Atherosperma moschatum (Worth et al., 2011). Another Eastern Australian study has shown different climatic drivers in the northern and southern range of a single species can lead to different distributional shifts and genetic signals (Mellick et al., 2012). Both of these studies utilise available data on past climatic conditions and historical distributions shown by fossil records and demonstrate the importance of this information for the interpretation of molecular data.

The southwest of Australia has not been impacted by volcanic activity or glaciation for 250My leading to one of the most ancient stable landscapes in the world (Hopper and Gioia, 2004). Detailed studies into the past climate and fossil record of the southwest Australia are limited. Little is known about the effect of past climates and population history on the genetic structure of species in this region. Combined with a relative paucity of studies on spatial genetic structure in southwest Australia, the result is a currently limited knowledge base of historical demography as a driver for genetic structure compared to studies in the northern hemisphere.
While there are few current studies in southwest Australia, interest into understanding the genetic structure of species is increasing. Phylogeographic research has focused on species in the more arid inland regions of the southwest, with 300 to 600mm annual rainfall, and shown a genetic disjunction separating the species into northern and southern regions (Byrne et al., 2003, Byrne and Hines, 2004). Of three phylogeographic studies in the more mesic regions of southwest Australia with 600 to 1200mm annual rainfall, one on *Eucalyptus marginata* has attributed genetic structuring to formation of landscapes rather than climate changes during the Pleistocene (Wheeler and Byrne, 2006). Studies on native frogs *Metacrinia nichollii* and *Crinia georgiana* attribute a north-south division to possible climate change during the Pleistocene (Edwards et al., 2007, Edwards et al., 2008).

Southwest Australia is a global biodiversity hotspot (Hopper and Gioia, 2004), which makes it more pertinent to understand the drivers of spatial genetic structure in this region. This information can be used to inform conservation efforts and to gain an understanding of evolutionary history. The bird pollinated species *Banksia ilicifolia* will be used to study these concepts in southwest Australia. It is a common and widespread species that inhabits the more mesic sand plains of southwest Australia. It has a range that extends over 700 km from north to south with a patchy distribution that is associated with dependency on groundwater (Groom et al., 2000). It is hypothesized that because of the nature of the distribution patterns of *B. ilicifolia* there will be significant spatial structure. The drivers of this possible structure are unknown and one of the major aims of this study will be to explore possible drivers of spatial structure using the tools of spatial and population genetics including Mantel tests for isolation by distance and Bayesian analysis to assess genetic grouping along a geographical range.
3.2 Methods

3.2.1 Study species

*Banksia ilicifolia* is an over-story species that occurs on the coastal plain of southwest Australia with a range of over 700 kilometers. The location of *B. ilicifolia* depends on access to groundwater and hence needs to grow in an area where there is close proximity to the water table (Zencich et al., 2002). As a consequence, it is found in low-lying areas near swamps and lower dune slopes (Dodd and Bell, 1989). This dependency on groundwater has led to a patchy distribution of the species with population sizes ranging from 300 to greater than 1000 individuals growing close to the higher rainfall coastal areas (Broadhurst and Coates, 2004). Peak flowering occurs from August through September and the flowers are yellow when first opened, changing to red or orange as they age (Lamont and Collins, 1988). It is a preferentially outcrossing species (Heliyanto et al., 2005) and is primarily pollinated by birds (Lamont and Collins, 1988). *Banksia ilicifolia* is not a serotinous species; seeds are released from the follicles in early autumn. The seeds do not have any dormancy but recruitment is low (Personal observation).

3.2.2 Sampling design

Samples were taken from populations across the range of the species (Table 3.1, Fig. 3.1.), which was determined by herbarium records. Sites were chosen at roughly equal distances along the range of the species in areas that are easily accessible and not disturbed by agriculture or urbanisation. All sample sites contained greater than 100 individuals. Leaf samples were collected from 30 arbitrarily chosen trees from each population with a distance of ten or more metres between individuals to avoid the sampling of closely related individuals. In the field leaf samples were immediately stored at 4 °C. In the laboratory samples were fresh frozen at -80 °C until DNA extraction.
Figure 3.1. The locations of sampled populations of *Banksia ilicifolia* (Red dots) within Western Australia. The grey dots indicate species range (DEC, 2007)


3.2.3 DNA extraction and microsatellite profiling

Frozen leaf material was ground in liquid nitrogen and DNA was extracted using the protocol of Doyle and Doyle (1990) with the additional purification steps after the chloroform extraction of adding the same volume of cold 5 M potassium acetate followed by an isopropanol precipitation and then washing the dissolved pellet in 2/3 volume of 5 M sodium chloride. DNA was then suspended in Tris-EDTA buffer pH 8. For genotyping, eight microsatellite primers developed for B. ilicifolia were used (Chapter 2). Polymerase chain reactions (PCR) were carried out using Qiagen Multiplex PCR kits with the following reaction formula: 2x Qiagen Multiplex master mix, 2 µM of each primer, 5x Q-solution, dH2O, 2 ng DNA. The reaction conditions were as recommended by the manufacturer with an initial activation of 95 °C for 15 minutes followed by 35 cycles of denaturation at 94 °C for 30 seconds, annealing at 56 °C for 90 seconds and extension at 72 °C for 90 seconds with a final extension at 72 °C for 15 minutes. All reactions were carried out on a Veriti thermocycler. The multiplex PCR products were diluted with a 1:3 dilution and run on a Beckman Coulter 8800 Capillary sequencer with a 400 DNA size standard (Beckman Coulter Brea, California, USA). Amplified products were visualised using a CEQ 8800 Genetic Analysis System and CEQ fragment analysis software (Beckman Coulter, California, USA). Allele sizes were scored for each locus and individual.

3.2.4 Statistical analysis

Genetic diversity

Each microsatellite locus was assessed for polymorphism by calculating the number of alleles across all populations. Each population was assessed for allelic richness (Rs) using FSTAT version 2.9.3.2 (Goudet, 1995), which standardises for sample size. Number of alleles (Na), observed heterozygosity (Ho), expected heterozygosity (He) and fixation index (F) were calculated using Genalex V6.4 (Peakall and Smouse, 2006) for each population across all loci. Genetic differentiation among the populations was assessed by pairwise and overall Fst and Analysis of Molecular Variance (AMOVA) using Genalex (Peakall and Smouse, 2006)
Population genetic structure

As *B. ilicifolia* distribution is confined to the coastal region of southwest Australia, analysis were conducted to assess whether lack of habitat between the northern and southern populations acts as a barrier to gene flow. A bivariate correlation between pairwise Fst and coastal and Euclidean distance was conducted as the Euclidean distance cuts across areas of uninhabited land. These two correlations were then compared with a Stieger Z-test in FZT computator (C.Garbin). A Mantel test was conducted to assess the relationship between genetic distance and geographic distance in Genalex (Peakall and Smouse, 2006). Pairwise Fst was then plotted against geographic distance. A principal components analysis for pairwise Fst values between sampled populations was conducted in Genalex. Eigenvalues from the analysis were then plotted against distance from northern most population.

To assess the genetic structure in terms of the number of genetically distinct clusters the data were subjected to Bayesian clustering analysis using STRUCTURE (Pritchard et al., 2000), which has a non-spatial prior distribution. The number of clusters (K) considered ranged from one to fourteen, which was the total number of locations sampled plus three. Ten runs per K value were conducted. Each run had a burn-in of 50000 with 100000 MCMC iterations. The parameters used for each run assumed no prior population knowledge, admixture and uncorrelated alleles. The optimal number of clusters was determined by assessing the LnPd against K curve as well as ΔK against K which has been suggested as being a more accurate measure of clusters (Evanno et al, 2005). ΔK was calculated by the method outlined by Evanno et al. (2005) via the online program Structure Harvester (Earl and vonHoldt, 2011).

TESS (Chen et al., 2007) is also a Bayesian clustering analysis but unlike the non-spatial prior distribution of STRUCTURE, the prior distribution is based on a Hidden Markov Random Field (HMRF), which also takes into account spatial interactions. TESS was run for K max two to eleven with 50000 sweeps and a burnin of 10000 with 100 runs per K. The model used was with admixture under the Besag, York and Mollié (BYM) model. TESS gives a value for each run known as a deviance information criterion (DIC). The average DIC for each K was plotted and the K at which the curve plateaus is recognised as the
minimal number of population clusters that explains the data. TESS outputs were run through the program CLUMPP (Jakobsson and Rosenberg, 2007) to obtain an average over all runs and visualised with the program DISTRUCT (Rosenberg, 2004). To assess hybrid zones TESS was run with admixture under the BYM model and a trend degree of one for K=2.

Spatial variation of allele frequencies
Spatial variation of allele frequencies was examined by calculating allele frequencies across all loci and all populations with Genalex (Peakall and Smouse, 2006). The allele frequencies of all loci and alleles were plotted against distance from northern most population. The frequencies of alleles from loci that were correlated with geography were averaged and plotted over the distance from northern most population.
3.3 Results

3.3.1 Genetic diversity

Eight microsatellite loci scored were polymorphic with a range of 6 to 24 alleles per locus with an average of 15 alleles per locus. Average allelic richness per locus was 6.74 with a minimum of 5.68 at the most southeastern population of Albany to 7.65 in Bunbury (Table 3.1). Expected heterozygosity ranged from 0.59 in Albany to 0.75 in Busselton. The average He and Ho across all populations and loci were 0.70 and 0.67, respectively.

Overall genetic differentiation among populations was moderate, with Fst=0.163 (p<0.01). Average pairwise Fst=0.083, and all pairwise Fst comparisons were significantly greater than 0 (P<0.01). Populations that were the most geographically distant showed greater FST values. AMOVA partitioned 79% of the total genetic variation within populations and 21% among.

3.3.2 Principal Components Analysis (PCA)

The PCA plot clustered populations in a north-south direction along coordinates 1 and 2 (Fig. 3.3). There was a strong division between the southern group of Lakes Cave to Albany and the Northern Group of Busselton to Perth along coordinate 1. Along coordinate 2 there was an east to west trend for the southern group, with Albany being quite isolated, and with strong grouping of the Boat Harbour, Broke inlet and Windy Harbour populations. For the northern group there was a south to north trend along coordinate 2 with the exception of the Busselton population, which was drawn to the eastern side of the plot placing it intermediately between the two clusters. Eigen values for PCA 1 plotted against geographic distance revealed a sigmoidal curve with a sharp transition through the Bunbury and Busselton populations at 320 and 390 km, respectively (Fig. 3.3).
Table 3.1. Genetic diversity parameters for *Banksia ilicifolia*. Number of Alleles per locus (Na), Allelic Richness (Rs), Observed Heterozygosity (Ho), Expected Heterozygosity (He) and Fixation Index (F). Standard error is in italics.

<table>
<thead>
<tr>
<th>Population</th>
<th>Latitude</th>
<th>Longitude</th>
<th>Sample Size</th>
<th>Na</th>
<th>Rs</th>
<th>Ho</th>
<th>He</th>
<th>F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Albany</td>
<td>35° 5'36.46&quot;S</td>
<td>117°54'42.52&quot;E</td>
<td>22</td>
<td>6.50</td>
<td>5.69</td>
<td>0.54</td>
<td>0.59</td>
<td>0.07</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.02</td>
<td>0.75</td>
<td>0.05</td>
<td>0.05</td>
<td>0.04</td>
</tr>
<tr>
<td>Boat Harbour</td>
<td>35° 0'32.76&quot;S</td>
<td>117°54'42.97&quot;E</td>
<td>23</td>
<td>7.00</td>
<td>7.32</td>
<td>0.68</td>
<td>0.71</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.03</td>
<td>0.96</td>
<td>0.04</td>
<td>0.04</td>
<td>0.07</td>
</tr>
<tr>
<td>Broke Inlet</td>
<td>34°54'42.37&quot;S</td>
<td>116°28'3.50&quot;E</td>
<td>25</td>
<td>5.87</td>
<td>6.06</td>
<td>0.67</td>
<td>0.69</td>
<td>0.00</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.97</td>
<td>0.90</td>
<td>0.06</td>
<td>0.05</td>
<td>0.09</td>
</tr>
<tr>
<td>Windy Harbour</td>
<td>34°50'1.18&quot;S</td>
<td>116°21'6.22&quot;E</td>
<td>20</td>
<td>6.00</td>
<td>6.52</td>
<td>0.61</td>
<td>0.70</td>
<td>0.14</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.90</td>
<td>0.88</td>
<td>0.07</td>
<td>0.04</td>
<td>0.08</td>
</tr>
<tr>
<td>Scott River</td>
<td>34°17'2.90&quot;S</td>
<td>115°16'12.58&quot;E</td>
<td>26</td>
<td>6.37</td>
<td>5.98</td>
<td>0.65</td>
<td>0.69</td>
<td>0.05</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.96</td>
<td>0.84</td>
<td>0.06</td>
<td>0.06</td>
<td>0.05</td>
</tr>
<tr>
<td>Lake Cave</td>
<td>34° 2'45.28&quot;S</td>
<td>115°1'25.14&quot;E</td>
<td>29</td>
<td>7.75</td>
<td>7.01</td>
<td>0.69</td>
<td>0.72</td>
<td>0.04</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.90</td>
<td>0.67</td>
<td>0.06</td>
<td>0.04</td>
<td>0.06</td>
</tr>
<tr>
<td>Busselton</td>
<td>33°38'37.79&quot;S</td>
<td>115°30'22.14&quot;E</td>
<td>21</td>
<td>7.62</td>
<td>7.43</td>
<td>0.75</td>
<td>0.75</td>
<td>0.00</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.10</td>
<td>0.99</td>
<td>0.04</td>
<td>0.05</td>
<td>0.03</td>
</tr>
<tr>
<td>Bunbury</td>
<td>33°14'39.62&quot;S</td>
<td>115°43'34.7&quot;E</td>
<td>20</td>
<td>8.12</td>
<td>7.51</td>
<td>0.64</td>
<td>0.75</td>
<td>0.14</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.89</td>
<td>0.77</td>
<td>0.06</td>
<td>0.04</td>
<td>0.07</td>
</tr>
<tr>
<td>Perth</td>
<td>31°42'53.06&quot;S</td>
<td>115°48'14.08&quot;E</td>
<td>24</td>
<td>6.25</td>
<td>6.06</td>
<td>0.71</td>
<td>0.69</td>
<td>-0.06</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.78</td>
<td>0.65</td>
<td>0.03</td>
<td>0.03</td>
<td>0.06</td>
</tr>
<tr>
<td>Lancelin</td>
<td>31° 1'32.27&quot;S</td>
<td>115°32'50.17&quot;E</td>
<td>25</td>
<td>6.62</td>
<td>7.58</td>
<td>0.68</td>
<td>0.69</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.96</td>
<td>0.95</td>
<td>0.06</td>
<td>0.04</td>
<td>0.07</td>
</tr>
<tr>
<td>Cervantes</td>
<td>30°18'53.46&quot;S</td>
<td>115°12'9.14&quot;E</td>
<td>21</td>
<td>7.00</td>
<td>6.79</td>
<td>0.69</td>
<td>0.68</td>
<td>-0.03</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.80</td>
<td>0.71</td>
<td>0.03</td>
<td>0.04</td>
<td>0.05</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td>256</td>
<td>6.86</td>
<td>6.73</td>
<td>0.67</td>
<td>0.69</td>
<td>0.04</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.28</td>
<td>0.37</td>
<td>0.02</td>
<td>0.01</td>
<td>0.02</td>
</tr>
</tbody>
</table>
Figure 3.2. Principal Coordinates Analysis for pairwise $F_{st}$ between populations along the range of *Banksia ilicifolia*. Southern populations fall in the right hand quadrants and northern populations fall in the left hand quadrants.
Figure 3.3. Principal Component Analysis Eigen values for coordinate 1 against geographic distance for 11 populations of *Banksia ilicifolia*. Grey box indicates putative admixture zone.
3.3.4 Population genetic structure
The program STRUCTURE yielded a LnP(d) against K curve that plateued at seven clusters (Fig 3.4): Albany, Boat Harbour, Broke inlet and Windy Harbour, Scott River and Lakes Cave, Busselton, Bunbury, Perth, Lancelin and Cervantes (Fig 3.5). The membership proportions for populations into clusters (Q) were only above 0.85 in four of the populations. The lowest Q being that of Busselton with 0.587 for its own cluster as well as Q values for the Bunbury cluster and Perth-Lancelin clusters at 0.22 and 0.173 respectively.

Plotting ΔK against K indicated the most likely number of clusters was 2 as this was the maximum of the curve (Fig 3.4). The cluster plot for K=2 shows clear northern and southern clusters (Fig. 3.6). The northern group consists of Cervantes, Lancelin, Perth, Bunbury and Busselton, and the southern group consisted of Lakes Cave, Scott River, Windy Harbour, Broke Inlet, Boat Harbour and Albany. The Q values were above 0.9 for all populations in their respective clusters. Fst between the two clusters was 0.09.

The TESS analysis indicates DIC plateaued at K(max)=7 clusters (Fig. 3.8). However, the seventh cluster had less than five individuals assigned an admixture proportion of less than 0.008 and hence was excluded. The next smallest DIC value equated with K=6. All clusters were assigned individuals with admixture proportions above 0.85. The clusters were as follows: the first was Albany, the second was Boat Harbour, Broke Inlet and Windy Harbour, the third was Scott River and Lakes Cave, the fourth was Busselton, Bunbury and Lancelin, the fifth was Perth, the sixth was Cervantes. A three-way AMOVA conducted for when k=2 and k=7 produce the same partitioning of variation with 78% within individuals, 8% among regions, 6% among populations and 8% among individuals indicating neither scenario has greater validity.
Figure 3.4 A) Ln of the probability of data for different K against K for each population of *Banksia ilicifolia*, the highest point is the probable K; B) ΔK against K as analysed by Structure Harvester where highest point before the drop to the lowest point is the probable K (Earl and vonHoldt, 2011)
Figure 3.5. Clusterplot of admixture proportions for each individual from STRUCTURE when K=7. Columns represent assignment proportion to individual clusters. AL=Albany, BH=Boat Harbour, BI= Broke Inlet, WH= Windy Harbour, SR= Scott River, LC=Lake Cave, BS= Busselton, BN= Bunbury, PER= Perth, LN- Lancelin, CV=Cervantes
Figure 3.6. Clusterplot of admixture proportions for each individual in the population from STRUCTURE when K=2. Columns represent assignment proportion to individual clusters. AL=Albany, BH=Boat Harbour, BI=Broke Inlet, WH=Windy Harbour, SR=Scott River, LC=Lake Cave, BS=Busselton, BN=Bunbury, PER=Perth, LN=Lancelin, CV=Cervantes
Figure 3.7. Deviance Information Criterion (DIC) for *Banksia ilicifolia* against number of possible clusters [K(max)]. The first dip in the curve indicates optimal K from TESS.
When K was set at 2 the assignment probabilities for 45% of the individuals in the Busselton and Bunbury populations were of mixed ancestry, being less than 0.85 for either region (Fig. 3.7). Of all individuals, 23% had assignment probabilities at roughly 0.5 for each cluster (Fig 3.7). The other populations sampled had assignment probabilities over 0.85 for most individuals for one of the two clusters.

3.3.3 Mantel test

There were significant correlations of pairwise Fst with both coastal distance ($r=0.89\ p<0.001$) and Euclidian distance ($r=0.77\ p<0.001$). These correlations were significantly different from each other ($Z=3.8\ p<0.001$) indicating there is a barrier to gene flow along the less correlated Euclidean distance. The Mantel test showed a significant correlation of genetic distance with geographic distance ($P<0.01$). The regression coefficient was 0.401. Plotting geographic distance against pairwise Fst revealed a pattern with pairs assigned to the same cluster having small Fst values, and the population pairs assigned to different clusters having larger Fst values as expected (Fig. 3.9.). The interesting result is that the populations of Bunbury and Busselton fell into the middle of the correlation between the two different population pairs as well as the greater spread of Fst for these two populations. There were also some population pairs in this group with larger distances that were showing smaller FST values. This can be seen with the Busselton and Lancelin populations with a geographic distance of 290 km having a Fst of 0.08 whereas the populations of Albany and Lakes Cave had a similar distance at 298 km and a Fst that was more than double at 0.17.
Figure 3.8. Clusterplot of admixture proportions from TESS when $K=2$ for *Banksia ilicifolia*. Each column represents one individual. The two colours represent the two different clusters.
Figure 3.9. Genetic distance (pairwise F\textit{st}) and geographic distance for the range of \textit{Banksia ilicifolia}. The blue diamonds are for populations assigned to the same cluster as designated by STRUCTURE and TESS. The red squares are for populations assigned to different clusters as designated by STRUCTURE and TESS and the green triangles are for the two populations that are in the possible transition zone.
3.3.5 Spatial analysis of allele frequencies

The geographic structuring of allele frequencies across the species range was examined for loci displaying marked differences between northern and southern populations. Of all alleles 12.4% were unique to southern populations (Lake Cave to Albany), and 5.8% of all alleles were unique to northern populations (Perth to Lancelin). Within the geographically intermediate (Busselton and Bunbury) populations 10.5% of all alleles were not found in the populations to the north and 11.5% of all alleles in the geographically intermediate populations were not found in the populations to the south of this area. Within-population allele frequency displayed a loose sigmoidal distribution against geographic distance for 12 alleles from 5 loci (9.9% of all alleles with frequency 0.05<x<0.95). For these alleles, frequency was relatively high or low through the northern or southern population clusters, with a sharp transition through the geographically intermediate populations Busselton and Bunbury (Figs 3.10, 3.11).
Figure 3.10. Average allele frequencies for 5 diagnostic loci. The red symbols indicate alleles frequent in the northern end and the black symbols indicate alleles frequent in the southern end of the species range.
Figure 3.11a Allele Frequencies along the geographic distance for 4 Banksia ilicifolia loci. The different colours represent different alleles for each loci. Solid lines indicate diagnostic alleles showing presence in the intermediate contact zone and either the northern or southern region.
Figure 3.11b Allele Frequencies along the geographic distance for 4 *Banksia ilicifolia* loci. The different colours represent different alleles for each loci. Solid lines indicate diagnostic alleles showing presence in the intermediate contact zone and either the northern or southern region.
3.4 Discussion
The microsatellite data have revealed significant spatial genetic structure across the range of *B. ilicifolia*. There was an overall significant correlation between genetic distance and geographic distances indicated by the Mantel test. Moreover, there was a genetic disjunction between the northern and southern regions as shown by PCA analysis. Second, Bayesian clustering confirmed the grouping of two overarching regions and clinal variation within regions. Finally, Bayesian analysis and the spatial analysis of allele frequencies indicated a sharp transition zone between these two regions, indicative of a possible secondary contact zone between the two genetically differentiated regions.

Molecular clock and phylogenetic studies indicate that the *Isostylis* tribe in *Banksia* diverged from its ancestor around 19 million years ago (He et al., 2011). This group then diverged into three sister species as the climate became more arid. *Banksia cuneata* and *B. oligantha* are likely the result of speciation events related with improved adaptation to increasing aridity and diverging one at a time into the relic populations seen today as the drought-intolerant ancestor was driven to the more mesic coastline and becoming the drought-intolerant *B. ilicifolia*. This divergence indicates the inability of *B. ilicifolia* to cope with conditions of increasing aridity.

Analysis of spatial genetic structure with Bayesian analysis revealed that there was a hierarchical structure of population clustering. STRUCTURE and TESS both estimated an optimal K=7 for the number of clusters. However, assessing the STRUCTURE results with lnPD converted to ΔK and plotted (Evanno et al., 2005) revealed two overarching clusters that encompassed a northern and southern zone. This suggests that there is sub structuring within two larger regions. The PCA analysis was congruent with the Bayesian analysis and showed the split of north and south regions along axis 1, giving more confidence in the conclusions. This genetic disjunction in *B. ilicifolia* into northern and southern clusters has not been previously reported, as in the earlier genetic study, Broadhurst and Coates (2004) did not include populations from the southern range of the species.
The Mantel test showed a significant correlation indicating an overall isolation by distance effect. As expected, populations in the same region have smaller genetic distances than populations from different regions. It is interesting to note that Figure 2 shows the spread of genetic distance against geographic distance between the central populations and the other populations was sizeable with large and small genetic distances between populations with similar geographic distances.

The most interesting point about the two regions is the striking pattern of genetic change between them. This change occurred through the sampled intermediate populations of Bunbury and Busselton. The genetic signal that is seen in the admixture proportions between the two regions in the TESS analysis identified that the individuals in Bunbury and Busselton had admixture proportions assigned to both regions rather than assignment to one region. This genetic mixture of the two regions shows a clear transition zone between the two regions. This genetic signal is very similar to one seen in natural populations of *Arabidopsis thaliana*, where admixture was identified between two different lineages from glacial refugia in Europe using TESS (François et al., 2008).

Further evidence of a transition zone can be seen in the allele frequency distributions, the Mantel plot, as well as in the levels of admixture in the proposed contact zone. The sigmoidal shape of the curve in the allele frequency distribution plots is typically associated with hybrid and secondary contact zones in contrast to a linear plot which may indicate more isolation by distance (Barton and Hewitt, 1985, François and Durand, 2010). The sigmoidal curve can also be seen when PCA Eigen values are plotted with geographic distance. The zone where the curve drops corresponds to the Bunbury and Busselton populations, which, combined with the presence of private alleles that were unique to either the northern or southern region supports the conclusion of a secondary contact zone between two historically separated lineages.

The two clusters must be currently or historically separated lineages to produce the observed genetic differentiation. There are no large water bodies or mountain ranges in the study area that would form a physical barrier to gene
flow however smaller ones may exist but this was not tested. Since
microsatellites are neutral markers it is unlikely that this transition zone is
associated with current substrate or climate changes. The most likely
explanation for the transition zone is historical population demographics and
how past climates and landform influenced the species range.

Current evidence indicates that during the time that coincides with the Northern
Hemisphere Last Glacial Maximum (for simplicity referred to as LGM) for
simplicity LGM southwest Australia was cooler, drier and windier than currently
core studies, Pickett (1997) suggests that during the LGM (28000-11000 yrs
BP) the vegetation on the Swan Coastal Plain was dominated by Casuarina and
drought-tolerant Banksia species. The exposure of the continental shelf, which
at this time would have been 30 km west of the current coastline, also brought
drier conditions to the Swan Coastal Plain (Seddon, 1972).

The climate in southwest Australia is Mediterranean with rainfall predominantly
during the winter months and hot dry summers. Banksia ilicifolia relies heavily
on ground water in the summer months when soil moisture in the unsaturated
zone is depleted. It can only access groundwater at a depth of less than 8 m
(Zencich et al., 2002). A drop in groundwater levels of 2.2 m was shown to
affect the survival of B. ilicifolia (Groom et al., 2000). Drier conditions during the
LGM would presumably have caused loss of access to superficial aquifers due
to a drop in water level and reduced precipitation for recharge. The impact of
such a scenario on B. ilicifolia has been shown recently with B. ilicifolia mortality
caused by groundwater abstraction from Perth’s biggest aquifer (Groom et al.,
2000). The westward movement of the coastline may have also changed the
dynamics of groundwater and reduced water levels in areas inhabited by B.
ilicifolia; however, the degree is unknown (Pickett, 1997). The impacts of the
drying climate may have been more severe in the central to the southern Swan
Coastal Plain leading to local extinction of B. ilicifolia as the genetic data
support a scenario of persistence in and separation between northern and
southern refugia.
As the dry conditions of the LGM eased and sea levels rose again, *B. ilicifolia* would have undergone range expansion to its current distribution. Sea level rise would have caused increased precipitation, even to levels that were greater than present (Pickett, 1997). This wetter climate perhaps resulted in a rapid expansion of *B. ilicifolia* into its current range. With a rising sea level and greater precipitation following the LGM the groundwater levels would then return to a pre-LGM level and create more suitable habitat for *B. ilicifolia* to expand into the central region where it is currently distributed, leading to the secondary contact zone inferred from the genetic data. A similar scenario of past climate affecting contemporary genetic structure has been demonstrated in the species *Telopea speciosissma* occurring in the Eastern side of Australia where historical climatic conditions across an altitudinal cline established selective reproductive barriers that rescinded once the climatic conditions became favourable however still leaving a genetic signal (Rossetto et al., 2011).

Another historical driver of the observed genetic structure may have occurred during the Pleistocene (2.5-3 Mya) when the sea level was 25-30 m higher than present day (Dodson and Macphail, 2004). *Banksia ilicifolia* populations on the southern Swan Coastal Plain would have been severely impacted as a sea level rise of this magnitude inevitably resulted in a substantial loss of possible *B. ilicifolia* habitat, assuming that the species distribution was similar to the current distribution (Fig. 3.13). This loss coincides with the middle of the species range producing two potentially isolated regions to the north and south when the southern Swan Coastal Plain disappeared due to the rise in sea level.

The most likely scenario for the spatial genetic structure seen in *B. ilicifolia* is historical separation and consequent secondary contact of the species. However, because Bayesian analysis with admixture makes no assumptions on timing of events, the cause cannot be verified (François and Durand, 2010). Both the LGM drying climate and 3 Mya inundation are historical events that provide viable explanations for the current patterns of spatial genetic structure observed.

In future climate change scenarios southwest Australia is predicted to become warmer and drier (Yates et al., 2010). Range shifts for *B. ilicifolia* has been
modeled and under a low severity change in climate, the range is expected to contract by 20% (Fitzpatrick et al., 2008). If the climate change scenario modeled at the highest severity then the predicted range contraction is to about 54% of the current range (Fitzpatrick et al., 2008). The genetic structure has revealed the contraction of *B. ilicifolia* into two distinct areas that will likely have been refugia under stressful environmental conditions, if climate change does occur and there is range expansion then it is likely that this contraction will separate *B. ilicifolia* again. When the previous separation occurred there wasn’t enough time to produce distinct species but this may occur if future climate changes are more permanent and in the time frame of the separation between *B. ilicifolia* and the sister species *B. cuneata* and *B. oligantha*.

In conclusion, the spatial genetic structure seen in *B. ilicifolia* can be best explained as being driven by historical events, which have divided the species as can be seen by the legacy of two distinct genetic regions and a rapid zone of transition in the behaviour of genetic markers between these clusters. Interestingly, there is a secondary contact zone where the two regions have come together when conditions became favourable. While post-glacial secondary contact is a common occurrence in the northern hemisphere (Adams et al., 2006, Durand et al., 2009), this is the first time that a secondary contact zone has been identified in southwest Australia. Studies into other species will inform whether this is a species-specific phenomenon, or whether there is a pattern in the region. However, given the large-scale landscape impacts, I hypothesise that a similar genetic signal will be observed for at least some other co-occurring species.
Fig 3.13. Sea level inundation of southwestern Australia if level rose 30 m A) current sea level at 0 m B) proposed sea level at 30 m (Tingle, 2006)
Chapter 4

Urban fragmentation alters pollen-dispersal patterns in *Banksia ilicifolia* populations

**Abstract**

Fragmentation of natural bushland leads to reduced population size and increased isolation. This may lead to decreases in genetic diversity due to genetic drift and an increase in inbreeding. There may also be changes to pollen dispersal patterns due to disruptions to pollinator services by loss of pollinators or changes in pollinator composition. I explored the genetic consequences of fragmentation in the bird pollinated *Banksia ilicifolia* (Proteaceae) R. Br. I used 8 microsatellite markers to quantify genetic structure of adult and offspring cohorts in a small fragmented population, an intermediate fragmented population and a large unfragmented population. Using paternity analysis I was able to map the pollen dispersal patterns in these different populations. The small urban population had the same pollen input than the larger populations, however due to 45% of the pollen produced by the nearest neighbour there was a greater decrease in genetic diversity. The intermediate fragmented and large unfragmented populations had most pollen produced by individuals 10 to 20 m away. Despite having equivalent pollen input the small population was genetically compromised due to significantly greater nearest neighbour mating. This study has shown through genetic analysis of pollen flow that in small populations of fewer than 40 *B. ilicifolia* plants, pollinator behaviour or composition has been impacted leading to a negative genetic outcome for the offspring of these plants. From these results, it is recommended that in the management and future development planning, bushland remnants be large enough to ensure that pollinator services are not compromised.
4.1 Introduction

With a growing human population there is increasing pressure on biodiversity, as more land is needed for habitation. Habitat fragmentation from human actions is one of the major threats to biodiversity in the world today (Fahrig, 2003). Remnants of natural vegetation often contain rare or endemic species of high conservation value (Fahrig, 2003). Changes in the environmental factors within fragmented ecosystems have many consequences for plant populations (Saunders et al., 1991). Fragmented populations can experience reduction in population size, habitat loss, altered ecosystem processes, edge and Allee effects and decreased genetic connectivity (Murcia, 1995, Young et al., 1996, Laurance, 2008). Besides conservation of the existing remnants, it is important to understand how fragmentation affects ecosystem processes within and among remnants to be able to achieve population persistence into the future.

Geographic separation and small population size may have a negative affect on the genetic diversity of fragmented populations (Ellstrand and Elam, 1993). Fragmentation may decrease genetic diversity through genetic bottlenecks when plant numbers are reduced removing genetic diversity (Young et al., 1996). Further, these geographically separated populations are potentially subject to increased genetic drift, increased isolation and inbreeding (Young et al., 1996). These processes can lead to decreased genetic diversity and reduced fitness, limiting the viability of the population (Aguilar et al., 2008). The negative effects of inbreeding and genetic drift can be negated through long distance gene flow either by pollen or seed dispersal (Ellstrand and Elam, 1993). However, long distance gene flow depends on the permeability of the matrix surrounding the fragment, and it may not be sufficient enough to counter the negative consequences of small population size.

Mating systems of species in fragmented populations are affected by factors such as changes in pollinator services, mate limitation and increased correlated paternity (Coates et al., 2007). Pollinators may have decreased in number, have changed behaviour or there may be a change in the pollinator community composition leading to low pollination or increased correlated paternity (Hardy et al., 2004). Self-incompatible species may have mate-limitation issues with less plants available as sires in smaller populations (Aguilar et al., 2008).
Outcrossing species will be potentially more affected more than selfing species by increased inbreeding in these small fragments (Aguilar et al., 2008).

Urbanisation is one form of habitat fragmentation that is particularly harmful as it is responsible for large local extinctions and is often more permanent than other types of fragmentation (McKinney, 2002). Within urban areas, there are often remnant patches of bush land that are ecologically and taxonomically significant as well as important areas for recreation and aesthetics. Along with the usual threats of small population size and isolation associated with fragmentation, urban fragments are more often subject to an increase of invasive species, increased water stress and issues of pollution (Grimm et al., 2008, Williams et al., 2009). Because of these threats and the high societal value of urban fragments, it is important to understand how ecosystem processes are affected by fragmentation and, based on this knowledge, put in place strategies to circumvent the adverse effect of fragmentation to ensure the long-term viability of these populations.

One of the most important ecological processes that is affected by fragmentation is pollination. For most species, pollination is critical for reproductive fecundity and maintenance of genetic diversity levels (Kwak et al., 2009). It is important to understand pollination and pollen dispersal in fragmented habitats for long-term population survival. Much focus has been on wind- and insect-pollinated neotropical trees (Dick et al., 2003, Ghazoul, 2005) and many studies have focused on insect pollination in areas fragmented by agriculture (Klein et al., 2007, Steffan-Dewenter and Westphal, 2008). The biodiversity hotspot of southwest Australia is an international hotspot for vertebrate pollination, with 15% of the flora having birds or mammals as pollen vectors (Phillips et al., 2010). With this high number of vertebrate pollination there are surprisingly few studies on species with this pollination syndrome (Smith – Ramirez and Armesto, 2003, Byrne et al., 2007, Yates et al., 2007a). Furthermore, these studies are limited to agricultural regions, and there is a paucity of research on bird-pollinated systems situated in urban fragmented landscapes (Roberts et al., 2007).
To address the lack of knowledge on effects of urbanization on bird pollination the following hypotheses were tested. First, due to possible changes in pollinator abundance and behaviour in small populations I hypothesise that pollen dispersal in small populations will be altered in comparison to larger populations resulting in a decreased pollen dispersal and a smaller amount of pollen input from external sources. Second, because of the reduced pollen dispersal, smaller populations will be expected to have greater disparity in genetic diversity between generations. With these hypotheses this study aimed to quantify pollen dispersal of the bird-pollinated species *Banksia ilicifolia* R.Br. in urban vegetation fragments. This species occurs in *Banksia* woodland on the coastal plains of southwest Australia. Within the Perth metropolitan area, these communities have been extensively cleared for housing, with 80% of the vegetation cleared within the last 150 years (WAPC, 2000). Within this urban sprawl remain remnants of natural *Banksia* woodland containing stands of *B. ilicifolia*. Using this species, this study will provide an essential understanding of the permeability of the urban matrix as well as the resilience of small populations to withstand the effects of urbanization in the long term.
4.2 Methods

4.2.1 Study species

*Banksia ilicifolia* is an over-story species that occurs on the southwest coastal plain of Western Australia with a range of over 750 km. *Banksia ilicifolia* is associated with a water table at less than 10 m depth (Groom et al., 2000, Zencich et al., 2002). It is found in low-lying areas near swamps and lower dune slopes (Dodd and Bell, 1989). Flowering occurs year round with peak flowering around August to October (Collins, 2008). *Banksia ilicifolia* is not a serotinous species and seeds are released from the follicles in early autumn. The flowers are yellow when first opened changing to red or orange as they age, possibly a signal to pollinators of decreased nectar availability and stigma receptivity (Lamont and Collins, 1988). This species occurs in many urban bushland remnants and provides a source of nectar to birds and small marsupials (Ramsey, 1988b, Bradshaw et al., 2007). It is a preferentially outcrossing species and is primarily pollinated by birds (Lamont and Collins, 1988, Heliyanto et al., 2005).

4.2.2 Study sites and plant material

The study sites consisted of three different size stands of *B. ilicifolia* within the Perth metropolitan area, Western Australia. These stands have become fragments as the habitat around them has been cleared and reduced in size within the last 20 years. These stands will be called populations that have been defined arbitrarily by the boundary of the bushland remnant that they exist in. The small population consists of 37 individuals, the intermediate 97 plants and the large over 500 plants with more in neighbouring reserves. Leaf material was collected from every plant within the small and intermediate populations and a subset of 83 plants in the large reserve as time and cost prohibited sampling of the entire population. The intermediate population contains a large swamp in the centre. Each individual’s location was GPS (accuracy 5m) recorded and mapped in DIVA-GIS (Hijmans et al., 2004)(Figs 4.5,4.6,4.7). The leaf material was fresh frozen at -80 °C until used for DNA extraction. In addition, 30 seeds were collected from each of four trees, chosen at random in each population. Seeds were from different inflorescences. Seeds were extracted from follicles with pliers and stored at room temperature until DNA extraction.
Table 4.1. Population characteristics for the small, intermediate and large populations of Banksia ilicifolia used for pollen flow studies.

<table>
<thead>
<tr>
<th>Population parameter</th>
<th>Small</th>
<th>Intermediate</th>
<th>Large</th>
</tr>
</thead>
<tbody>
<tr>
<td>Population area (sqm)</td>
<td>24000</td>
<td>39000</td>
<td>2500000</td>
</tr>
<tr>
<td>Number of individuals</td>
<td>37</td>
<td>97</td>
<td>&gt;500</td>
</tr>
<tr>
<td>Density (n/100 sqm)</td>
<td>0.15</td>
<td>0.25</td>
<td>0.18</td>
</tr>
<tr>
<td>Distance to next closet population</td>
<td>1.9 km</td>
<td>1.7 km</td>
<td>0.26 km</td>
</tr>
<tr>
<td>Surrounding matrix</td>
<td>Urban</td>
<td>Urban</td>
<td>Natural habitat</td>
</tr>
</tbody>
</table>
Figure 4.1 Google earth images of the three populations of *Banksia ilicifolia* A) Where the populations are situated within the Perth metropolitan area, B) small population, C) intermediate population and D) the large population where the circle indicates the location of the subset of sampled trees.
4.2.3 DNA extraction and analysis

DNA was extracted from frozen leaf tissue using the procedure outlined by (Doyle and Doyle, 1990) with the additional purification steps of 5 M potassium acetate after the chloroform extraction and 5 M sodium chloride after precipitating in isopropanol and dissolving in water. DNA of offspring was extracted from seeds using the procedure outlined by Jobes et al. (1995). Before extraction, seed coats were removed to eliminate maternal tissue contamination. Extracted DNA was electrophoresed in a 2% (w/v) agarose gel to assess its quality and quantity. Eight microsatellite markers were used for genotyping. They were from *B. ilicifolia*: D3, B104, A3, B105, A110, C103 and from *B. attenuata*: C8, A3 (See Chapter 2 for annealing temperatures). Polymerase chain reactions (PCR) were carried out in 10 µL total volume containing 3-5 ng of template DNA, 2 µL of 5x buffer (Fisher Biotec: final concentration of 67 mM TRIS (pH 8.8) 16.6 mM (NH₄)₂SO₄, 0.45% Triton X-100, 0.2 mg/mL gelatin, 0.2 mM of each dNTP), 0.025 U/µL Taq DNA polymerase (Fisher Biotec), 1.5-3 mM MgCl₂ and 0.75 µM of forward and reverse primer. PCR reactions for each marker were conducted separately with initial activation of 94°C for 3 minutes followed by 35 cycles of denaturation at 94°C for 40 seconds, annealing at various temperatures for 40 seconds and extension at 72°C for 30 seconds with a final extension at 72°C for 15 minutes. The PCR products were pooled with three primers per mix and run on the Beckman Coulter 8800 Capillary sequencer with a 400 DNA size standard (Beckman Coulter Brea, California, USA). Amplified products were visualised using a CEQ 8800 Genetic Analysis System and CEQ fragment analysis software (Beckman Coulter, California, USA).

4.2.4 Data analysis

Population genetic diversity statistics were calculated to compare between the different size populations and between adults and offspring. Genetic dissimilarity, number of actual and effective alleles as well as observed and expected heterozygosity were assessed using Genalex 6.41 (Peakall and Smouse, 2006). Allelic richness was calculated using FSTAT (Goudet, 1995). A two-way Analysis of Variance (ANOVA) assessed the statistical significance of differences in genetic diversity statistics between populations and between
adults and offspring, with a Tukey post-hoc test, in SPSS (Version 17, IBM Corporation, New York, USA). Genetic dissimilarity for offspring was calculated between each cohort of each maternal and averaged. To account for the potential of creating a bottleneck within the sampling design of collecting from only four maternal lines the genetic dissimilarity for the adult population was calculated as the average of the genetic dissimilarity between the maternal tree and the other adult individuals in the population. The difference between the adult and offspring dissimilarity was then calculated and compared with a one-way ANOVA and Tukey post-hoc in SPSS.

Population genetic structure was assessed using Spatial Autocorrelation Analysis (SAA) in GENALEX 6.41 (Peakall and Smouse, 2006). The analysis was conducted using all loci and distances between trees were placed into 10 m interval classes. The correlation coefficient $r$ was calculated for each distance class with confidence limits. The correlation coefficient $r$ is a measure of genetic similarity between individuals within a specified distance class and becomes significant when the $r$ passes through the first confidence interval. The $r$ intercept is the distance at which there is no more genetic structure. Permutation testing allows for statistical testing of significant population structure (Peakall et al., 2003b). Correlograms were plotted using GENALEX 6.41.

Mating system parameters were estimated with the program MLTR for each adult and offspring population (Ritland, 2002). Estimates of multi- and single-locus outcrossing rates, biparental inbreeding and correlated paternity ($R_p$) were calculated with 500 bootstraps and the Expected-Maximization method using 500 bootstraps. Expected number of pollen donors was estimated from the inverse of $R_p$ (Ritland, 2002).

Realized pollen flow was determined by assigning paternity to the seeds collected from each population. Paternity assignment was calculated with the software CERVUS (Marshall et al., 1998, Kalinowski et al., 2007), which uses categorical allocation to assign offspring to the plant that has the highest likelihood of being the sire by calculating likelihood ratios (LOD). For a measure of statistical confidence, the difference between the LOD of the most likely candidate and second most likely candidate is calculated, which is the delta
score. Through simulations of parentage analysis the critical delta score is calculated at the 95% (strict) and 80% (relaxed) confidence levels and paternity was assigned when a sire’s comparative delta score was within these confidence levels. The total percentage of sires unsampled is unknown. Due to the nature of isolation in the small and intermediate populations CERVUS was set to have sampled at least 80% of the sires in the simulation because it is assumed that 100% of the local population has been sampled, however, because there is likely to be some pollen influx and to be comparative with the large population. In the case where all local plants were excluded as possible sires, it was concluded that the pollen had come from outside the sampled population.

Using the 80% confidence level a pollen-dispersal curve was constructed by graphing the frequency of successful pollination events against the distance between the assigned sire and the maternal tree, for distance classes of 10 m. Plant pair distances were defined as the distance of all individuals in the population to the maternal tree. The distance of the pollen origin was then compared with plant pair distances with a Kolmogorov-Smirnov two-sample test in SPSS to assess for random mating. Distances were calculated with the mapping program DIVA-GIS. Pollen-dispersal distance distributions among the different population sizes was also compared with a Kolmogorov-Smirnov two-sample test for effect of population size differences. The pollination events were then plotted as a function of rank distance where the nearest neighbour to the maternal plant was ranked 1, the next 2 and so forth. The relationship was assessed with linear regression analysis in SPSS.
4.3 Results

4.3.1 Spatial structure
The spatial autocorrelation analysis revealed different strength and scale of genetic structure between the three populations (Fig. 4.2). The r intercept for the small population indicated that there was significant genetic structure up to 20 m. In the intermediate there was significant genetic structure up to 40 m and in the large population up to 30 m.

4.3.2 Mating system parameters
Estimates of multi-locus outcrossing rates were not significantly different from one for all three populations indicating no selfing in any population (Table 4.2). This is confirmed by the observation that all offspring in all populations having at least one non-maternal allele (Fig. 4.3). MLTR showed low biparental inbreeding rates (0.001-0.051), and correlated paternity estimates were low for all populations, but there were large differences between the small and intermediate populations at 0.103 and 0.074 respectively, and the rate for the large population was 0.004 (Table 4.2).

4.3.3 Paternity assignment
Paternity was assigned to 65% of offspring across all populations within an 80% and above confidence when maximum likelihood was calculated, and to 6% of offspring with a 95% and above confidence. All known candidate sires were excluded as the true sire for 23% of the offspring using private alleles (See section 4.3.4). The large population had 34% unassigned offspring, the intermediate had 37% unassigned offspring and the small had 30%.

Within the large population, 36% of the pollination events resulted in full-sibs within all maternal plants, with an average of 10 full-sibs in each maternal. The intermediate population had 27% pollination events resulting in full sibs with an average of 8 full sibs in each maternal and the small population had 47% full-sibs with each maternal having an average of 17 full-sibs. The large and the intermediate populations each had up to ten different sires producing more than two pollination events on the same maternal tree. In contrast, the small population had six sires producing more than two pollination events on the same maternal tree, with one sire responsible for 11 events on one maternal
plant. There was no one direction evident from which the sires were from (Fig 4.6,4.7,4.8)

The small population had a maximum plant pair distance of 95 m and an average of 48.6 m. Considering paternity assignment with the 80% confidence interval, the maximum detected pollination event distance was 94 m with an average of 24.2 m. The greatest amount (41%) of the realised pollination events happened over a distance of 0-10 m, and 22% over 30-40 m (Fig. 4.4, 4.6). The intermediate population had a maximum plant pair distance of 276 m and an average parent pair distance of 109 m. The maximum detected pollination event distance was 258 m, and the average distance was 87.1 m (Fig. 4.4,4.7). The distance classes with the majority of pollination events were 100-110 m and 30-40 m with 14 and 13% of the total pollination events, respectively. The maximum distance of plant pairs in the large population was 360 m with an average of 67.4 m and the maximum detected distance for a pollination event was 340 m with an average of 63.1 m (Fig. 4.4, 4.8). The distance class with the majority of pollination events was 10-20 m with 25% of the total pollination followed by 20-30 m with 14%.

Pollen-dispersal distributions were significantly different from the plant pair distributions in the small and large populations (Kolmogorov-Smirnov two sample test, P<0.001) but not in the intermediate (Kolmogorov-Smirnov two sample test, P=0.54) (Fig. 4.4), indicating that there is a departure from random mating in the small and large populations and that this is significantly influenced by inter-tree distance. There was a significant difference in pollen distributions between all three populations (Kolmogorov-Smirnov two sample test, P<0.001). This difference occurred because of the greater amount of pollen coming from plants at a distance <10 m in the small population rather than >10 m, as well as the pollination in the intermediate population where there was an equivalently large amount of pollen coming from 30-40 m and 100-110 m.

Pollination as a function of ranked distance of plant pairs to the maternal highlight that in the small population the largest amount (nearly 40%) of the pollination was coming from the nearest neighbour (Fig. 4.5.). This analysis also showed the nature of the pollination in the intermediate population, where there
was a large amount of pollen coming from the 6\textsuperscript{th}, 14\textsuperscript{th}, 15\textsuperscript{th} and 16\textsuperscript{th} plant which was around 30-40 m away and at the 65\textsuperscript{th} and 77\textsuperscript{th} plants which were 110-120 m away (Figs 4.4, 4.5). The large population shows the greatest amount of pollen coming from the 3\textsuperscript{rd} plant with the rest having a uniform distribution.
Figure 4.2. Spatial autocorrelation correlogram for the three different sized populations of *Banksia ilicifolia*. The value of $r$ is bound by 95% confidence interval and 95% confidence error bars. A) Small population, B) Intermediate population and C) Large population.
Table 4.2. Mating system parameters of *Banksia ilicifolia* for the three different sized populations estimated using MLTR. Italicised numbers are standard error.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Small</th>
<th>Intermediate</th>
<th>Large</th>
</tr>
</thead>
<tbody>
<tr>
<td>Multi-locus outcrossing rate</td>
<td>0.93</td>
<td>0.97</td>
<td>1.00</td>
</tr>
<tr>
<td></td>
<td>0.02</td>
<td>0.114</td>
<td>0.00</td>
</tr>
<tr>
<td>Single-locus outcrossing rate</td>
<td>0.98</td>
<td>0.92</td>
<td>0.99</td>
</tr>
<tr>
<td></td>
<td>0.09</td>
<td>0.003</td>
<td>0.03</td>
</tr>
<tr>
<td>Biparental inbreeding</td>
<td>0.051</td>
<td>0.051</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td>0.103</td>
<td>0.026</td>
<td>0.03</td>
</tr>
<tr>
<td>Correlated paternity</td>
<td>0.103</td>
<td>0.074</td>
<td>0.004</td>
</tr>
<tr>
<td></td>
<td>0.033</td>
<td>0.032</td>
<td>0.002</td>
</tr>
<tr>
<td>Estimated number of pollen donors</td>
<td>9.7</td>
<td>13.5</td>
<td>250</td>
</tr>
</tbody>
</table>
Figure 4.3. Complete outcrossing shown by the percent of offspring having one or more non-maternal alleles of *Banksia ilicifolia* from eight microsatellite markers in three different sized populations. Small population (Red), intermediate population (Blue) and large population (Green).
Figure 4.4. Distribution of pollination events (red) and parent pair distances (possible sire from the maternal) (black) for 10 m distance classes across the 4 maternal plants. A) small population, B) intermediate population and C) large population of *Banksia ilicifolia*
Figure 4.5. Frequency of pollination events against ranked distance for all 4 maternal plants in different sized population. Ranked distance starts at the nearest neighbour A) small population, B) intermediate population and C) large population in Banksia ilicifolia.
Figure 4.6 Map of sampled plants of the small population of *Banksia ilicifolia* showing each maternal plant (red squares), sires (black triangles) and other genotyped individuals (grey dots). A, B, C, D are maps of each maternal plant sampled in the population.
Figure 4.7b. Map of sampled plants of the intermediate population of *Banksia illicifolia* showing each maternal plant (red squares), sires (black triangles) and other genotyped individuals (grey dots). A,B,C,D are maps of each maternal plant sampled in the population.
Figure 4.8b. Map of sampled plants of the large population of *Banksia ilicifolia* showing each maternal plant (red squares), sires (black triangles) and other genotyped individuals (grey dots) A,B,C,D are maps of each maternal plant sampled in the population
4.3.4 Private alleles
Of all the offspring in the small population, 17% contained unique alleles in four loci that were not present in the local genotyped population. The intermediate had 18% of offspring with unique alleles from outside the population in four loci and the large population had 16% of offspring with unique alleles from five loci. It can be inferred that these offspring were sired from outside the genotyped trees with longer-distance pollen flow at these levels.

4.3.5 Genetic consequences of population size
The eight microsatellite loci that were scored had between 4 and 11 alleles, with an average of 8 alleles per locus. Allelic richness ranged from 8.68 to 10.56 in the adult populations, and from 5.83 to 9.22 in the offspring populations (Table 4.3). There was no significant difference for the allelic richness between adults and offspring in the intermediate and large populations (Fig. 4.9). However, there was a significant difference in the allelic richness between adults and offspring in the small population (P=0.01; Fig. 4.9). There was no significant difference in observed or expected heterozygosity between the adults and offspring for each population (P<0.2), but there was a significantly lower (P=0.01) number of alleles in the small population offspring compared to adults (Table 4.2). The difference of genetic dissimilarity between the maternal plants and their offspring was significantly different between the large population and the intermediate and small populations (P<0.001) (Fig. 4.10)
Table 4.3. Genetic diversity parameters for adult and offspring *Banksia ilicifolia*. Large population, intermediate and small population. Average number of alleles per population (Na), Average number of effective alleles per population (Ne), Observed Heterozygosity (Ho), and Expected Heterozygosity (He). Italicized numbers are standard error.

<table>
<thead>
<tr>
<th>Population</th>
<th>Sample Size</th>
<th>Na</th>
<th>Ne</th>
<th>Ho</th>
<th>He</th>
</tr>
</thead>
<tbody>
<tr>
<td>Large Adult</td>
<td>83</td>
<td>10.13</td>
<td>4.68</td>
<td>0.74</td>
<td>0.76</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.22</td>
<td>0.63</td>
<td>0.04</td>
<td>0.03</td>
</tr>
<tr>
<td>Large Offspring</td>
<td>110</td>
<td>8.50</td>
<td>3.68</td>
<td>0.71</td>
<td>0.71</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.68</td>
<td>0.42</td>
<td>0.03</td>
<td>0.03</td>
</tr>
<tr>
<td>Intermediate Adult</td>
<td>94</td>
<td>10.83</td>
<td>4.52</td>
<td>0.80</td>
<td>0.76</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.11</td>
<td>0.56</td>
<td>0.04</td>
<td>0.04</td>
</tr>
<tr>
<td>Intermediate Offspring</td>
<td>82</td>
<td>9.67</td>
<td>4.62</td>
<td>0.70</td>
<td>0.77</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.88</td>
<td>0.42</td>
<td>0.03</td>
<td>0.02</td>
</tr>
<tr>
<td>Small Adult</td>
<td>30</td>
<td>8.71</td>
<td>3.68</td>
<td>0.78</td>
<td>0.72</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.81</td>
<td>0.25</td>
<td>0.04</td>
<td>0.02</td>
</tr>
<tr>
<td>Small Offspring</td>
<td>68</td>
<td>6.14</td>
<td>2.72</td>
<td>0.73</td>
<td>0.60</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.55</td>
<td>0.32</td>
<td>0.07</td>
<td>0.05</td>
</tr>
</tbody>
</table>
Figure 4.9. Allelic richness for adults and offspring in three different sized populations of *Banksia ilicifolia* for adults (Black) and offspring (Blue). Letters denote significant differences.
Figure 4.10. The difference in genetic dissimilarity between adults and offspring of the three different sized populations of Banksia ilicifolia. Letters denote significant differences.
4.4 Discussion

The two proposed hypotheses were supported in this study. First, the small population did have an altered pollen dispersal compared to the larger populations with a greater proportion of the pollination occurring between nearest neighbours. However, the small population had a small amount of pollen dispersal to the maximum possible distance of 90 m, and the same amount of pollen input from external sources as the larger populations. Due to the high amount of nearest neighbour mating, the second hypothesis was supported with the small population having a larger decrease in genetic diversity and allelic richness. This result demonstrates a change in realised pollen dispersal can be detected through understanding genetic signals that fragmentation has on species and its relationship to the pollinator community.

Pollen dispersal patterns differed in the three populations partly due to population size and partly due to features of the habitat. The large population displayed a realised pollen dispersal pattern that was similar to other relatively undisturbed *Banksia* populations, with more pollen coming from individuals that are not the closest to the maternal and is a departure for typically insect and wind pollinated species (Omodei, 2008, Krauss et al., 2009). The intermediate population has a pollen dispersal that can be explained by the habitat perhaps acting like two sub-populations. The middle of the population is a large swamp with low scrub allowing access of pollinators from one side of the swamp to the other. The small population had predominately nearest-neighbour mating which is atypical of bird pollination but similar to that found for insect pollinated species (Levin, 1981).

Pollen flow in the small population was dominated by near-neighbour mating. Of all realised pollen movements, 41% were less than 10 m. This indicates that either the current pollinators have different local behaviour patterns in the small population or that there has been a change in pollinator composition leading to different local behaviour of the whole community. Generally, a large percentage of nectar-feeding birds will move from the foraging tree to a distant one (Ramsey, 1989). However, as resources may be more limited in small fragmented populations, birds may be visiting more inflorescences on individual
trees and moving onto neighbouring plants instead of distant individuals (Yates et al., 2007a).

Pollinator composition changes with the size of the fragment, such as introduced feral bees, where the new pollinators are less efficient in delivering pollen to the stigma (Ramsey, 1988a, Paton, 1993, Elliott et al., 2012). Pollinator compositional changes in the urban system are not well understood and more research needs to be conducted into assemblages in urban remnants compared to natural populations to fully understand the effects caused by habitat fragmentation. For example, Elliot et al. (2012) demonstrated small linear remnants contain a greater number of large honeyeater species than larger remnants, which in turn reduced the reproductive success of *Eremophila glabra*.

The microsatellite markers that were used in this study may have underestimated the assigned paternity when there is an actual match due to a lack of power. Therefore, to be confident in the calculation of pollen input from outside the population only offspring with private alleles were assumed to have a sire outside the genotyped adult population. This means that pollen input could be much higher than reported here with some of the unassigned offspring resulting from matings with sires outside the population, and when comparing with other populations this needs to be considered.

There has been a negative effect of habitat fragmentation on pollen flow in *B. ilicifolia* even though there were equal amounts of pollen input as in other populations. There was an increase in near-neighbour mating and therefore a reduction in the number of sires from the small population. Similar patterns were reported for *B. sphaerocarpa*, which also had a reduction in local sires and an increase in selfing in smaller populations (Llorens et al., 2012). Unlike *B. sphaerocarpa*, in which a large connected population had greater influx of pollen (10.1% in the smaller populations and 37% in the large population), the input of pollen in the large and small populations of *B. ilicifolia* was similar at around 16%. The quantifiable pollen immigration in the small population of *B. ilicifolia* is similar to that of *B. hookeriana*, which also showed 16% pollen input from outside the population fragment across a fragmented landscape (Krauss et
al., 2009, Llorens et al., 2012); however, it could be larger due to the unassigned offspring.

The equal amounts of pollen influx across the three populations indicate the equality in the permeability of the surrounding matrix around all three populations with the large population being surrounded by intact bushland while the other two surrounded by urban development. Urban matrices are thought to be more complex and restrictive to pollinator movement (Culley et al., 2007). However, my data showed that there is substantial connectivity of a similar level among all sampled populations. The pollen dispersal observed indicates that the urban matrix is permeable to the highly mobile bird movements. The urban matrix possibly allows for greater movement through the presence of bird-attracting flowering plants in suburban gardens, rather than just crop fields as seen in an agricultural matrix. While the distance of the outside pollen source is unknown, the nearest population to the small fragment was 2 km and it is known that honeyeaters travel distances up to 9 km (Saunders and De Rebeira, 1991). This indicates that pollinators are travelling up to 2 km in these urban landscapes, which was previously unknown.

This study showed that all populations assessed were completely out-crossing, as all offspring contained at least one non-maternal allele. Previous assessment of the breeding system in this species indicated that selfing is possible, but that there is preferential outcrossing with post-zygotic abortion, because of strong early acting inbreeding depression (Heliyanto et al., 2005). Therefore, self-pollination may be much higher than suggested in this study, and as a consequence of the abortion of selfed seed there may be fewer viable seed available. Indeed, the number of aborted seed was higher in small populations (Chapter 5). Therefore, these new data suggest that small population sizes of around 40 plants, result in not only fewer seed, but also more inbred seed as our elevated correlated paternity measures showed.

Correlated paternity was highest in the smallest population due to the high near-neighbour mating or the low number of sires. This relationship between correlated paternity, population size and fragmentation effects is seen in many other species in Australia with the smallest populations typically having higher
correlated paternity (Wells and Young, 2002, Coates et al., 2007). Mimura et al. (2009) demonstrate in *Eucalyptus globulus* that small fragments have higher correlated paternity but not lower genetic diversity contrasting with the results of this study where genetic diversity of offspring is also lower. Higher correlated paternity in small populations has been attributed to changes in pollinator behaviour, as birds are more likely to focus on one plant leading to higher correlated paternity in small fragments, whereas movement is more likely to be between plants as fragment size increases (Yates et al., 2007a).

Reduced genetic diversity in small populations is common in many systems where it is shown that genetic diversity measures (e.g., allelic richness, heterozygosity) are correlated with population size (Buza et al., 2000, Luijten et al., 2000, Vergeer et al., 2003). While my results show that there is no difference in genetic diversity measures between adult population sizes, there is evidence of genetic erosion between generations which can lead to reduced genetic diversity of adult populations in the future (Van Geert et al., 2008). If the nearest-neighbour and correlated paternity pattern of pollen dispersal were to continue, it could lead to an ever-increasing amount of inbreeding and reduction of heterozygosity as the high correlated paternity indicates a high proportion of full-sibs in the next generation and the likelihood of the next generation nearest neighbour being more closely related. A feedback loop may form where small population size leads to inbreeding and loss of genetic diversity, which will in turn lead to populations becoming demographically unstable, keeping population size small and hence more inbreeding and a continuing loss of genetic diversity (Frankham et al., 2002). While the data from the comparison of adults to the first generation indicate genetic erosion between them, only further study on future generations of the small populations of *B. ilicifolia* will help resolve whether there really is continued inbreeding that may be reducing genetic diversity, and hence understand whether there is the possibility of these small populations heading into an extinction vortex (Frankham et al., 2002).

Levels of gene flow via pollen from outside the population in the smallest fragmented population were similar to that in the other larger populations. Despite this evidence for gene flow through pollen from outside the local fragmented population, this may not be enough to prevent genetic erosion and
bottlenecks (Sork and Smouse, 2006). A population size of around 40 plants has led to greater nearest-neighbour mating, substantially higher level of full-sibs and genetic erosion that has not been counteracted by the observed gene flow. The intermediate population of around 100 plants does have relatively high nearest neighbour mating but has a long tail of other local mating events that is absent in the small population and has not shown a large reduction in genetic diversity in the next generation. From these results it seems that there is a threshold number of individuals that is the minimum number for a viable population somewhere between 40 and 100. A population that contains less than 100 plants may lose the key pollination processes and unaltered pollinator behaviour to ensure that there is no loss of genetic variation and increased inbreeding. In future planning of habitat clearing and restoration it would be prudent to make sure that the population size be above 100 individuals to ensure key population processes continue.

This study is one of the first that delves into the effect of fragmentation, in an urban situation, on pollination of a predominantly bird-pollinated species. It has provided information for the future management of current urban fragments as well as for planning future development. This study has shown that population size has minimal effect for inter-population gene flow as this is similar at all sites. It appears that there may be a threshold population size for this species in urban fragments, below which genetic diversity decreases in the next generation due to increased nearest-neighbour mating and correlated paternity. The intermediate population appears to be large enough to sustain a viable pollen dispersal pattern; however, the small population will need intervention to ensure survival. Further research needs to be conducted on the consequences of intervention, such as supplementing the population with seedlings from larger populations. When planning future developments containing natural remnants, a population size of at least 100 should be aimed for. However, more research is needed to identify the exact size as well as the nature of the fragment, and more species should be considered as different mating systems and pollinators will lead to different requirements for population sizes and isolation tolerances.

This study highlights the permeability of the urban matrix to the movement of bird pollinators and more effort should be made to understand the role of
suburban gardens to facilitate this movement. This study has also shown that population size is irrelevant for inter-population gene flow as this is similar at all sites. If pollinator services can be maintained or restored within the urban matrix then there may be enough gene flow to negate the negative effects of fragmentation and small population sizes. If pollinator restoration is not understood or not possible, genetic rescue of populations under the threshold may be required to stop the cycle of inbreeding and genetic erosion.
Chapter 5
Maternal population size affects seedling health and survival under drought stress

Abstract
The objective of this chapter was to assess the effect of population size on the fitness and adaptability to environmental change of progeny from small and large populations. This was done by evaluating the reproductive success of four small and four large populations of *Banksia ilicifolia*, and then subjecting seedlings from these populations to drought stress in a glasshouse trial. The small populations had a lower reproductive success due to a greater number of aborted seeds. Germination of the seeds of small populations, however, was 100%, which was the same as that of the larger populations. When water was withheld, five of the seedlings of the larger populations survived 15 days longer than those from smaller populations. This was associated with a greater proportion of roots in the top 20 cm of the soil profile compared with those from smaller populations. A greater amount of roots in the faster drying top soil may produce greater amount of Absisic acid and signal a tighter stomatal control. Based on this study it is recommended that for ecological restoration, seeds sourced from larger populations may harbour greater resilience to a changing climate, leading to improved restoration success.

5.1 Introduction

With the increase of the human population has come the degradation of many natural ecosystems. The scale of the degradation varies from the introduction and proliferation of invasive species to complete destruction of habitats for mining, agriculture or urban development. The rehabilitation of these sites also varies, ranging from removal of invasive species to replacement of entire ecosystems, all of which provides challenges to the restoration practitioner. There is increasing effort in the restoration of these habitats and the science that needs to be conducted to make restoration successful (Young et al., 2005).
A key requirement for restoring a plant community on a degraded site is seed material to increase population numbers. Current guidelines in the literature suggest that seed should be sourced from an area as local as possible to the restoration site (Mckay et al., 2005, Vander Mijnsbrugge et al., 2010). A key rationale for this to ensure that there are no maladapted genotypes or a contribution to outbreeding depression. (Hufford and Mazer, 2003). There is, however, debate around the current guidelines prescribing locally sourced seed when local population sizes are small and the seed produced has a high chance of being inbred and low in genetic diversity (Aguilar et al., 2008, Broadhurst et al., 2008). The genetic consequences of small population size have been well documented (Ellstrand and Elam, 1993, Oostermeijer et al., 1994a, Oostermeijer et al., 1994b, Mavraganis and Eckert, 2003, Hensen and Wesche, 2006) and small populations may display inbreeding depression resulting in offspring that are less fit than the adult population or outcrossed offspring (Reed and Frankham, 2003). It has also been argued that collecting from a small population will reduce the genetic diversity of the seed cohort and limit the evolutionary potential for adaptation to any changes in environmental conditions (Broadhurst et al., 2008). Offspring that are inbred may also be more prone to inbreeding depression. Collecting seed from small populations may be detrimental to restoration efforts if the quality of seed is compromised.

Conventionally, fitness of populations and offspring has been measured by reproductive fitness measures, such as flower production and number of fruits or seeds, and seedling fitness measures including survival, biomass and vigour (Oostermeijer et al., 1994a, Leimu et al., 2006, Krauss et al., 2007). These fitness measures have then been used to display the effects of small population size and inbreeding depression (Heschel and Paige, 1995, Teixeira et al., 2008). However inbreeding depression under stressful environments may be exacerbated. Growth, reproduction and vigour all depend on the ability to acquire resources such as water and nutrients, and to fix carbon by photosynthesis. In the current study, it is assumed that healthy plants have faster rates of photosynthesis and transpiration than unhealthy or genetically impoverished plants, and particularly so in drought-stressed conditions. High stomatal conductance correlates with high transpiration and allows high photosynthesis, and is therefore used in this experiment as a proxy for high
physiological activity underlying high fitness. However, in hot dry environments it can be a disadvantage to have high transpiration rates.

The effects of stressful conditions, such as drought, may be exacerbated in small populations that experience inbreeding depression. While in some cases it has been shown that inbreeding depression is not increased under mild stress, Fox and Reed (2011) demonstrate with a meta-analysis that under severe forms of stress higher magnitudes of inbreeding depression may be expressed. Deleterious alleles may not be expressed until specific environmental conditions are experienced (Armbruster and Reed, 2005) therefore assessing inbreeding depression in small populations with common garden experiments under benign conditions may not give a true representation of the effects of inbreeding depression on fitness.

In the context of the debate in the literature about the collection zones of seed for ecological restoration, it is important to understand the real effects of population size on seed quality and offspring fitness. General population genetic theory indicates that small populations have a lower reproductive fitness than larger ones (Ellstrand and Elam, 1993). Therefore the aim of this study is to examine the relationship between population size and seedling vigour under environmental stress. If the seedlings from small populations are less fit this will have implications for ecological restoration if seed is sourced from small populations. To examine this concept the species *Banksia ilicifolia* R.Br. will be used as a model. *Banksia ilicifolia* is an over-story tree species that occurs on the coastal plain of Western Australia where the water table is relatively close to the surface (Zencich et al. 2002). It has a high dependency on groundwater that is above 4m in the water table. Previous studies have indicated that *B. ilicifolia* derive over 50 percent of water from groundwater sources which increases over dry summer months and deaths have occurred in areas where groundwater levels the root zone (Groom et al. 2000, Zencich et al. 2002) Because of this sensitivity to water, drought was the stress chosen to test the following hypotheses: firstly, I hypothesise that populations with less than 100 individuals will produce less seed per plant than larger populations of over 500 individuals due to increased inbreeding depression through late acting seed abortion (Heliyanto et al., 2005). Population genetics theory also indicates that the
offspring themselves may be less fit if small populations are more inbred compared with larger ones, therefore I hypothesise that the smaller populations will have lower plant emergence, survival, biomass and general plant health than plants from larger populations. Evolutionary potential is important in the face of a changing climate and greater habitat fragmentation. Having reduced genetic variation from isolation and inbreeding may reduce the capacity of small fragmented populations to cope with environmental stresses such as drying climates. I therefore hypothesise that the offspring from smaller populations will show greater inbreeding depression under drought conditions than larger populations.
5.2 Methods

5.2.1 Study species

*Banksia ilicifolia* R.Br. is an over-story tree species that occurs on the coastal plain of Western Australia where the water table is relatively close to the surface (Zencich et al. 2002). It is found in low-lying areas near swamps and lower dune slopes (Dodd and Bell, 1989). This dependency on groundwater is the reason for a fragmented natural distribution of the species with population size ranging from 300 to greater than 1000 individuals. Flowering is from late August to September, and the flowers are yellow when first opened, changing to red or orange as they age (Lamont and Collins, 1988). It is a preferentially outcrossing species (Heliyanto et al., 2005) primarily pollinated by birds (Lamont and Collins, 1988). *Banksia ilicifolia* is not a serotinous species and seeds are released from the follicles in early autumn. The seeds do not have any dormancy and recruitment is very low.

5.2.2 Study sites

To assess the effects of population size on *B. ilicifolia*, four large and four small populations were chosen within the Perth metropolitan area. These sites all occur on the Swan Coastal Plain and were chosen because the surrounding areas had intense urban development within the last twenty years. Each small population had to consist of less than 100 trees, while each large population consists of over 300 trees (Table 5.1).

5.2.3 Field studies

Reproductive output was assessed in all populations by measuring inflorescence to fruit ratio, number of seeds per fruit and percentage seed abortion. Inflorescence to fruit ratio was measured in each population on 10 individual plants. Fruits were collected from these trees and the seeds extracted by cracking the fruit open with pliers. Number of seeds per follicle was recorded as well as the number of aborted seeds.
Table 5.1. Location and size of populations of *Banksia ilicifolia* used for population size effects studies around the Perth metropolitan area.

<table>
<thead>
<tr>
<th>Population</th>
<th>Latitude</th>
<th>Longitude</th>
<th>Size Class</th>
<th>No. of trees</th>
</tr>
</thead>
<tbody>
<tr>
<td>Harry Waring</td>
<td>32° 9'42.90&quot;S</td>
<td>115°50'27.06&quot;E</td>
<td>Large</td>
<td>&gt;500</td>
</tr>
<tr>
<td>Baldivis</td>
<td>32°20'58.50&quot;S</td>
<td>115°54'33.41&quot;E</td>
<td>Large</td>
<td>&gt;500</td>
</tr>
<tr>
<td>Jandakot</td>
<td>32° 6'20.61&quot;S</td>
<td>115°54'15.04&quot;E</td>
<td>Large</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>Pinjar Park</td>
<td>31°40'31.57&quot;S</td>
<td>115°48'53.30&quot;E</td>
<td>Large</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>Ellenbrook</td>
<td>31°46'8.69&quot;S</td>
<td>115°57'42.65&quot;E</td>
<td>Small</td>
<td>&lt;50</td>
</tr>
<tr>
<td>Caporn Park</td>
<td>31°43'55.18&quot;S</td>
<td>115°48'31.84&quot;E</td>
<td>Small</td>
<td>37</td>
</tr>
<tr>
<td>Caladenia Reserve</td>
<td>32° 4'34.40&quot;S</td>
<td>115°54'0.50&quot;E</td>
<td>Small</td>
<td>97</td>
</tr>
<tr>
<td>Banksia Grove</td>
<td>31°42'53.21&quot;S</td>
<td>115°48'15.23&quot;E</td>
<td>Small</td>
<td>74</td>
</tr>
</tbody>
</table>
5.2.4 Glasshouse trial

To test effects of stress on seedlings from different population sizes, an experiment that included a drought trial was conducted. Drought was chosen as the stress due to the association of *B. ilicifolia* with a shallow water table and because drought poses the most imminent threat to this species, due to increased groundwater extraction as a result of the growing human population and decreasing rainfall as a result of climate change. A total of 42 seeds from seven maternal plants per population were collected and extracted from follicles with pliers. The seeds were then sown into 100 mm diameter, 1 m tall pots filled with washed white river sand. The plants were given a 5 ml nutrient solution as per the recipe in Shane et al. (2003) every month. Emergence was scored and the plants were grown for two years. After two years the surviving plants were subjected to the water-stress experiment.

Twelve plants from each population were subjected to the drought experiment. Plants were well watered to field capacity for a week, and then watering was stopped. Water use was calculated by weighing pots after watering and then every three days after water was withheld. Stomatal conductance was measured to assess the level of stress, at the beginning of the experiment under well-watered conditions, and then every three days. Stomatal conductance was measured using the SC-1 Leaf Porometer (Decagon Devices Inc., Pullman, USA). However, this machine sometimes does not function due to high ambient humidity and some readings could not be made on scheduled days, and were taken the next day.

The water-stress experiment was continued until all the plants had died, and time of death was recorded for each plant. After death, plants were harvested and separated into four sections: The bottom 10 cm as all plants had reached the bottom of the pot and then evenly with middle 40-90 cm of roots, top 40 cm of roots and shoots. The roots were washed to remove traces of sand, and all sections were dried at 60 °C for seven days before recording dry weights.
Figure 5.1 Glasshouse experiment comparing large and small populations of *Banksia ilicifolia*. Populations are randomly assigned in 1 m pots. A) and B) 1 week after emergence. C) 14 weeks after emergence.
5.2.5 Data analysis
The data for inflorescence to fruit conversion were assessed for normality with a q-q plot and subsequently analysed with a Mann-Whitney U non-parametric test as the data were not normally distributed. Seed count data were also assessed for normality and transformed with an arcsine transformation. Differences between large and small populations were analysed with a t-test in SPSS (Version 17, IBM Corporation, New York, USA).

Water use was plotted against time for the first 24 days and analysed with a repeated measures Generalised Linear Model (GLM) Stomatal conductance were plotted against time and the difference between the large and small populations was analysed with GLM with repeated measures in SPSS. Time until death was analysed with a Kaplan-Meier survival analysis in SPSS, and the dry weights were compared with a Mann-Whitney U test also in SPSS. These tests were carried out first by comparing each population within each size class (four in the large and four in the small size class) and no significant differences were found, therefore populations were pooled into the two size classes and these results are presented.
5.3 Results

5.3.1 Field studies
There was no significant difference between large and small populations in the percentage of inflorescences converting to fruit (Z=-0.227, P=0.853) with an average of 19.2% conversion of inflorescence to fruit. There was a wide range of percent conversion among the populations, with the large populations showing a range from 11% to 30% and the small populations exhibiting a range of 11% to 34%. There was no significant difference in the average number of seeds per follicle between the two population size classes (t=1.37, P=0.1). There was, however, a significant difference in the percentage of aborted seeds, with the small populations exhibiting more aborted seeds, 9 ± 1.8%, than the larger populations, 4 ± 1.3% (t=-3.03, P=0.003) (Fig 5.2).

5.3.2 Glasshouse trial
Water use of seedlings from large and small populations was not significantly different (F=1.45 P=0.214, Fig. 5.3). Stomatal conductance for across all individuals had a mean of 99 m$^{-2}$ s$^{-1}$ (Fig. 5.4). Towards the end of the experiment (after 113 days of water stress), the stomatal conductance was 20 mmol m$^{-2}$ s$^{-1}$ for both large and small populations, with a significant effect of time since last watering (P<0.01). The GLM analysis found no significant difference in stomatal conductance between the population sizes in the response to withdrawal of water over time (F=1.089, P=0.299).
Table 5.2 Percent of total seed per population size class for small and large populations of *Banksia ilicifolia*. Aborted seed includes those aborted within follicles with one viable seed and one aborted and follicles in which both seed were aborted. SE is standard error, n=4 for each size class.

<table>
<thead>
<tr>
<th>Population Size</th>
<th>Two seeds per follicle</th>
<th>One seed per follicle</th>
<th>Aborted seed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Large</td>
<td>42.2</td>
<td>45.9</td>
<td>4.5</td>
</tr>
<tr>
<td>SE</td>
<td>3.9</td>
<td>3.2</td>
<td>1.3</td>
</tr>
<tr>
<td>Small</td>
<td>31.9</td>
<td>45.8</td>
<td>11.5</td>
</tr>
<tr>
<td>SE</td>
<td>3.4</td>
<td>3.8</td>
<td>2.4</td>
</tr>
</tbody>
</table>
Figure 5.3. Water loss from pots planted with *Banksia ilicifolia* for the first 24 days after water was withheld. Large populations (grey) and small populations (red). Bars represent standard error, n=48
Figure 5.4. Stomatal conductance over 113 days for large (grey line) and small (red line) populations of *Banksia ilicifolia* since time of withdrawal of the water supply (Day 0). Bars represent standard error n=48. Once plants had died they were removed from the analysis.
There was no difference in the time to emergence of seedlings from the large and small populations (Z=-1.5, P=0.9). Survival of the individual seedlings was measured for 140 days after withholding water, showing a significant difference between large and small populations ($\chi^2=7.85$, P=0.005, Fig. 5.4). The last of the small population plants died 125 days after water was withdrawn, with five plants of the large populations still alive at this time. The last of the large population plants died 140 days after water was withheld.

There was no significant difference in the shoot dry mass between the two population size classes (Z=-0.34, P=0.73), with large populations showing a mean of 3.9 ±0.5 g shoot$^{-1}$ and the small populations showing a mean of 3.9 ±0.6 g shoot$^{-1}$, nor was there a significant difference in the shoot to root ratio (Z=-0.39, 0.69). No significant difference was found in the total root dry mass (Z=-0.33, P=0.74), with the large population showing a mean of 8.7 ± 0.7 g root plant$^{-1}$, and the small population showing a mean of 8.7 ± 0.6 g root plant$^{-1}$. All plants had roots reaching the bottom of the pots, but there were differences in the distribution of root mass down the soil profile. Both population size classes showed the same percentage of root mass in the bottom 10 cm of the soil profile (Z=-0.6, P=0.55), but they differed in the percentages of roots in the top and middle sections. The large population plants had more roots in the top 40 cm of the soil profile than the smaller population plants (Z=-2.79, P<0.01, Fig. 5.5), with 55.4% and 45.1% of the total root mass in this section respectively. The smaller population plants had a greater percentage of the root mass in the middle 40-90 cm of the soil profile: 32.7% compared with 23.3% for the large population plants.
Figure 5.5. Percent survival of plants from the large (grey line) and small (red line) populations of *Banksia ilicifolia* measured since the time water was withheld (time 0). Significant difference between the two lines ($\chi^2=7.85$, $P=0.005$).
Figure 5.6. Dry weight of roots for plants from large (grey) and small (red) populations of *Banksia ilicifolia*. Top 40 cm measured from root-shoot junction. Letters indicate significant differences for depth. Total root mass showed no significant difference between population sizes.
5.4 Discussion

This study aimed to assess the reproductive fitness and vigor of seed collected in small populations compared to large populations of *Banksia ilicifolia* for its use in restoration. The first hypothesis that reproductive fitness would be lower in small populations than larger ones was supported, with small populations of *B. ilicifolia* having a lower reproductive fitness than the larger populations. However, the second hypothesis that offspring from small populations would have lower vigor than those from larger populations was not supported, as the small populations had the same emergence, biomass and stomatal conductance as the larger populations. The third hypothesis that offspring from smaller populations would show greater stress under drought conditions was supported as the plants from the larger populations survived longer under drought conditions, suggesting an overall difference in ability to respond to increased drought under climate change.

Reproductive fitness was reduced in the small populations due to the increased proportion of aborted seeds. This result is consistent with many other studies that have found correlations between population size and seed set (Luijten et al., 2000). Remarkably, this measure was the only one to show a difference. Flower to fruit conversion was not different between populations, and personal observations on the number of flowers per plant suggested no difference between populations; however plants within single populations varied widely in their flowering number and the same pattern was seen in all populations. Plasticity of flower output in response to population size seems to be species-specific. Like *B. ilicifolia* here, studies on *Aquilegia canadensis* showed no correlation between flowering and population size (Mavraganis and Eckert, 2003). In contrast, *Primula vulgaris* did show reduced flowering in smaller populations (Brys et al., 2004). Similarity, fruit production indicates that there was no pollen limitation difference between the populations. The increase in seed abortion is likely to be late-acting selection on selfed seeds (Heliyanto et al. 2005).

The seedlings of both population classes emerged at the same time and grew to similar sizes. Stomatal control was similar at the start of the drought experiment. These results are supported by those of Krauss et al. (2007) in
Eucalyptus salubris, Lammi et al. (1999) in Lychnis viscaria, and Fischer and Matthies (1997) in Gentianella germanica which showed that seedling vigour was independent of population size. This is in contrast with other studies that have found a negative effect of population size on plant fitness (Kery et al., 2001) and demonstrates that population size has a variable effect on plant fitness according to species. Aguilar et al. (2012) demonstrated that even in the same species different stages and fitness measures may not respond in the same way in Prosopis caldenia when population size had no effect on seed mass or germination, but there was a negative effect on seedling growth.

Conclusions could be made at this point that collecting from small populations will have no detrimental effect on restoration, as small and large populations apparently have the same quality seed and same seedling vigour. However, this is only a snapshot in time under ideal conditions, and it is important to understand how progeny respond to environmental stress and hence the B. illicifolia seedlings were subjected to drought stress. From these results it can then be seen whether population size influences the seedlings’ ability to respond to changing environments.

The root distribution differed between plants of small and large populations with plants from the larger populations having more roots in the upper layer of the soil column. Proliferation of roots at the soil surface is generally explained by the higher concentrations of nutrients in the topsoil (Jobbágy and Jackson, 2001, Lynch and K.M, 2001). In the current experiments, nutrients were also applied to the surface. These results indicate that seedlings from large populations may have a competitive advantage in nutrient-poor soil as the larger proportion of roots in the topsoil would provide better access to the higher concentrations of nutrients (particularly phosphorus) (Jobbágy and Jackson, 2001). On the other hand, a tendency to grow deeper roots may be an advantage when access to soil moisture is more critical. A similar experiment to this study, combining levels of nutrients and levels of soil moisture, will provide more information on this potential trade-off in root architecture and differences between large and small populations.
The observed differences in root distribution seem to have had little effect on patterns of water use. It might be expected that concentration of roots in the upper part of the soil profile would make plants from the large population more susceptible to drought as this part of the soil profile would dry out first. However, this may be an advantage as roots in contact with drying soil release Abscisic acid (ABA) (Dodd, 2005), which induces stomatal closure. In the current study stomatal conductance stayed at a steady rate in plants from large and small populations, suggesting that root distribution did not affect water availability and stomatal regulation and that the leaves conducted right until death. Further the stomatal conductance between short, medium and long-lived plants was not significantly different indicating that perhaps in B. ilicifolia stomatal conductance is not a good measure of drought stress. However, the use of the porometer to measure stomatal conductance was hampered due to high humidity and the readings taken may not have been accurate. It would be advantageous to repeat the experiment with an instrument that can negate the ambient humidity.

Survival of the seedlings was different in the last two weeks of the experiment when the conditions were the most extreme with 140 days of no additional water. The large population seedlings out-lived the small population seedlings by 15 days. This indicates that if conditions were harsh in the natural environment any extra time plants survive there is a greater chance of encountering a period of rain and milder environmental conditions that will allow them to recover and survive until the next harsh period. It has previously been shown that B. ilicifolia preferentially takes up water from subsurface soil during rainfall events rather than groundwater (Zencich et al., 2002). The seedlings in the large population with roots at the surface are better positioned to take advantage of rainfall events than seedlings from the small population. This may be a consequence of reduced genetic diversity in the smaller populations and reflecting reduced evolutionary potential.

Management implications

For ecological restoration, collection of seed from small populations may be limited due to the higher abortion rate. This may increasingly become a problem
as the smaller populations become more inbred leading to more aborted seed. Efforts to retain connectivity between populations to maintain gene flow should be made to try and circumvent this problem. Ideally, long-term trials should be established assessing the effects of inbreeding on the reproductive fitness of future generations including flower production and seed set.

Use of local seed from a small population may be detrimental to restoration if the population experiences drought conditions as the seedlings may not survive. The additional use of seed from the nearest large population may circumvent restoration failure, especially in the context of climate change. Previous pollination studies on *B. ilicifolia* indicate that there is increased reproductive success with pollen sourced from 30 km away (Heliyanto et al., 2006) indicating that outbreeding depression may not be an issue in the first generation; however, further study into whether outbreeding depression will have an effect on the population should be made before definite recommendations are made.

In conclusion, reproductive success was impaired in the small populations, because of the increase in aborted seed, and seed collection in small populations may be limited in the future. Plants from small populations had the same emergence and total biomass as the plants from larger populations as well as the same stomatal response to drought stress; however, when placed under stress, larger populations endured longer than the smaller populations. However, these are only minor differences and may not be of real consequence. It is recommended that for restoration of small populations seed be used from the local population and the nearest large population to reduce inbreeding and to provide for the potential to survive increased temperature and decreased rainfall that might occur due to climate change.
Chapter 6
General Discussion

6.1 Introduction

Understanding how to respond to the effects of habitat fragmentation on remnant populations is one of the most important areas of research for conservation biology. In this thesis, I set out to answer some important questions and address knowledge gaps in conservation genetics of a bird-pollinated plant species. I have successfully answered these questions with the use of genetic tools and the incorporation of ecophysiology, in a novel integration of disciplines in the study of conservation and restoration genetics.

I have developed microsatellite markers and undertaken a landscape scale approach to understand historical gene flow and how this shapes current genetic spatial structure. My study has also used an understanding of past responses to climate change to predict how *Banksia ilicifolia* might respond to future climate change. From a landscape scale I then focused down to a population scale by assessing the affect population size has on pollen dispersal and consequences for genetic diversity. For ecological restoration, seed quality and evolutionary potential are important considerations and with this view I tested the capacity of seedlings from small and large populations to cope with drought stress. The key findings were:

- Microsatellite markers for use on *B. ilicifolia* were found by cross transfer from other species and development of species specific markers. Cross-transfer of microsatellite markers from *B. attenuata, B. hookeriana* and *B. sphaerocarpa* was limited with 6 out of 56 loci being polymorphic and scorable. Because of this limited number, 8 novel polymorphic microsatellite markers were developed for *B. ilicifolia*. These specifically developed primers and the successfully cross-transferred primers were used to explore spatial genetic structure and pollen dispersal.
- Spatial structure across the range was found to comprise of two genetically distinct clusters. Evidence for a secondary contact zone at the
boundary between the two clusters was also discovered. The likely explanation for the secondary contact zone is that during the Last Glacial Maximum (LGM) the climate became drier restricting *B. ilicifolia* into refuges in the north and south of the range. As conditions became favourable after the LGM, the range of *B. ilicifolia* expanded again and came into contact as can be seen by the current distribution. Evidence of secondary contact can be seen by sigmoidal shape of the spatial frequency of alleles.

- Pollen flow patterns were altered in a small and fragmented population, with increased nearest neighbour mating and correlated paternity. This has lead to genetic erosion compared to larger populations despite the equivalent amount of pollen input from outside the sampled population.
- Reproductive success was reduced in small populations compared to larger populations due to the increased amount of aborted seed. However, reproductive output, emergence and dry biomass of plants in small and large populations was similar.
- When placed under drought stress, some plants sourced as seed from larger populations survived longer than all the plants from smaller populations. This is despite an equivalent decline in stomatal conductance. The root architecture was different between the population sizes with small population plants partitioning most root mass in the middle of the soil profile while the plants from the large population partitioning roots in the top of the soil profile.

6.2 Significance of findings

In facing a future with a changing climate and landscape it is important to understand how species respond to these changes in order to make informed decisions about their conservation. The research presented in this thesis provides some extra knowledge in this area. By demonstrating that *Banksia ilicifolia* has possibly shown a past range retraction into refugia against a drier historical climate indicates what may happen if the current climate becomes hotter and drier. However, these refugia may no longer be habitable due to human driven landscape change and management of small populations within these refugial areas becomes critical. My research has demonstrated, though
smaller populations have equal inputs of pollen flow, there is a decrease in genetic diversity from one generation to the next due to the higher amount of nearest neighbour mating. While not conclusive, a slightly longer survival time of large population seedlings indicates that when managing these small population in possible refugial areas the use of seed sourced from larger populations with greater genetic diversity and less nearest neighbour mating may be beneficial for a drying climate. Further significance of the findings is summarised under each of the main research questions posed in the introduction:

Q1 *Can microsatellite markers be developed for B. ilicifolia to use in landscape and pollen dispersal studies?*
Microsatellite markers were successfully developed and were sufficiently powerful for landscape studies. However, due to low assignment of paternity may not be polymorphic enough for pollen dispersal studies. It is recommended that more markers are used for pollen dispersal studies.

Q2 *What are the possible drivers of current spatial genetic structure?*
The discovery of a secondary contact zone is a significant one as this is the first time it has been shown in the southwest Australia. It also demonstrates historical range shifts in a landscape that has not undergone glaciation and therefore demonstrates more subtle yet no less meaningful changes in genetic structure than are seen in the northern hemisphere. I have also shown that past historical distributions can be inferred by current genetic structure. This study has also shown how past demographic changes revealed by genetic structure can possibly infer future range modification if climate change occurs as predicted.

Q3 *How is pollen dispersal affected by habitat fragmentation and small population size* 
There is a paucity of studies on bird pollination and how it is affected by population size in urban landscapes. This is an important gap in knowledge because pollination is a key function for reproductive success and gene flow (Ellstrand, 1992). This is especially the case for a biodiversity hotspot such as southwest Australia, which has the highest occurrence of bird pollination world-
wide (Dixon, 2009). I found a population size effect on pollen dispersal in a bird-pollinated plant species that resulted in greater nearest neighbour mating in a small population. This suggests that populations may require a minimum size to maintain key ecosystem services (Schemske et al., 1994). Changes in pollen flow gives insights into the changes in the pollinator guild or behaviour which has been seen in other bird pollinated systems (Elliott et al., 2012, Llorens et al., 2012). This is an important finding as it gives an indication of how pollination changes may affect restoration efforts. If pollinators are not present in a restored habitat then the restoration effort may not be successful due to limited poor quality seed production. The introduction of framework plant that will attract pollinators may be beneficial (Menz et al., 2011). It has been shown though that in some restored systems that pollinators are not limited such as the restoration of Banksia woodland and the bird pollinated Banksia attenuata in the restoration site had similar outcrossing rates and low correlated paternity as the natural population (Ritchie and Krauss, 2011).

It has been shown in the shrub species Calothamnus quadrifidus and the tree species Eucalyptus wandoow growing in bushland remnants in the Western Australian agricultural region, pollen immigration was not related to population size or isolation (Byrne et al., 2007, Byrne et al., 2008). This shows that there is permeability in fragmented natural habitats in an agricultural matrix for the movement of birds which is be understandable as it has been shown that honeyeaters can travel large distances and that cleared fields with isolated paddock trees may not pose a barrier (Byrne et al., 2008, Ottewell et al., 2009). A high concentration of physical barriers such as buildings and roads within an urban matrix may not be very permeable to pollinators, as has been shown in some insect-pollinated plants (Van Rossum, 2008). In contrast, my research has shown that there is pollen flow into urban populations with bird pollination and this important finding suggests that the urban matrix is permeable to bird pollinator movement.

Q4 Does habitat fragmentation affect reproductive success, germination and plant growth in bird pollinated species?

I have shown that while flower production and conversion from flowers to fruit was not reduced in small populations compared to large populations, there was
an increase in the percentage of aborted seed in the small populations. This supports other studies that have found that population size negatively affects seed production – however, many of these are wind or insect pollinated species (Pauw, 2007, Johnson et al., 2009, Gao et al., 2010, Wang et al., 2010). Other studies on bird pollinated species have however demonstrated that population size can have a negative effect on seed set (Yates et al., 2007b).

Q5 *How is population size going to affect plants under environmental stress?* While there is little knowledge about the effect of population size on seedlings under stress, there are many studies that measure inbreeding depression under stressful environments (Cheptou et al., 2000, Waller et al., 2008). These studies tend to indicate that stressed inbred plants have lower survival than non-inbred plants, although results are not strong and are quite variable among the studies (Hauser and Loeschcke, 1996, Cheptou et al., 2000, Waller et al., 2008). In a meta-analysis of the literature, Armbruster and Reed (2005) found that stressful situations do amplify inbreeding depression, however there was still large variation and a large number of studies that showed no, or a decrease in, inbreeding depression under stress. This meta-analysis was conducted on only 21 studies, indicating the importance of more research into the way stress affects inbreeding depression. My research has added to this gap and shown that the offspring of small populations (that are possibly more inbred) may not be as well suited to stress conditions as seedlings from large populations. It is also a novel combination of population genetics theory and ecophysiology in the way that I measured the fitness of the seedlings as many studies that measure fitness are limited to survival, fecundity and biomass (Armbruster and Reed, 2005). It emphasizes the benefits of a greater amalgamation between these two disciplines.

Indeed, it would be beneficial in many aspects of restoration to use eco-physiological information. Cooke and Suski (2008) argue that 21 out of 51 guidelines for developing restoration projects outlined by the Society of Ecological Restoration could be enhanced with the addition of physiological studies. For example, establishing a reference ecosystem, physiological measures can provide useful basis for comparison. These authors fail to mention how physiology can benefit our understanding of conservation genetics.
in the areas of provenance and local adaptation as well as issues associated with isolation and small population size as I have demonstrated here. Understanding physiological differences between individuals from different locations may provide an insight into choosing seed source sites for restoration. The improvement in portable gas-exchange systems and data loggers has made field-based research much more accessible (Ehleringer and Sandquist, 2006).

**Q6 How can this study be used for practical application?**

One of the most important outcomes from this study is to provide real management applications and recommendations to assist practitioners in conservation and restoration. Recommendations for conserving and restoring habitat fragments include:

1. **Maintain a minimum population size to ensure pollen dispersal and minimal genetic erosion.**
   With *Banksia ilicifolia* a minimum population size of 100 individuals may prevent loss of genetic diversity. This is despite complete outcrossing within the seed cohort. It is also advisable to maintain this minimum population number even if there are links between populations as equivalent gene flow may not be enough to counteract the genetic erosion that is caused by altered pollen dispersal.

2. **Seeds should not be sourced solely from small degraded populations**
   Through this study it can be seen that the best practice guidelines of only collecting local seed for use in restoration may not be beneficial if local populations are small. The pollen dispersal pattern in a fragmented habitat may be altered leading to a greater amount of inbred progeny and genetic erosion. This study has also shown that progeny from small populations may be less likely to survive under environmental stress. Even though differences between small and large populations may only be minor these differences may become major over several generations. To ensure that future generations are not compromised it is recommended that in addition to the local seed, seed collection zones be extended to the nearest large population to introduce higher quality of
seed as well as maintaining gene flow essential to counteract the negative consequences of genetic erosion. There are concerns that collecting and using non-local seed in restoration may be problematic due to maladaptation and outbreeding depression (Mckay et al., 2005). Procaccini and Piazzì (2001) suggest that heterozygosity and genetic plasticity are more important than provenance for success and survival under new conditions. However, outbreeding depression is a genuine problem and is not well understood (Vander Mijnsbrugge et al., 2010). The risks of inbreeding and low genetic diversity need to be outweighed against the risks of outbreeding depression and maladaptation before restoration is undertaken.

3. **Response to climate change should be understood and taken into consideration**

With climate change scientists predicting that the southwest Australia becoming drier and hotter (Yates et al., 2010) it would be wise to use seed in restoration that is more drought resistant like the seed found in larger populations or those found in drier conditions such as in the northern end of the range. Also the pattern of historical range contraction in response to climate change may provide an insight into where the range of *B. ilicifolia* may contract to as it may retract starting from the secondary contact zone.

6.3 **Further research**

From the conclusions drawn in this study there are further questions raised and the potential for further research to be conducted on the landscape scale as well as within populations that are affected by habitat fragmentation. The further research is outlined in three sections:

Section 1: Spatial Genetic Structure

- It would be pertinent to sample more populations around the area of the secondary contact zone to pinpoint the boundary.
• The study should be expanded to other species with the same distribution range to see if there are similar landscape scale patterns and drivers of genetic diversity.

• Finally, a greater understanding of historical climate change in southwest Australia is needed as there is not much information known. This will provide more concrete evidence to the drivers of the spatial genetic structure observed.

Section 2: Population size affects on pollen dispersal

• My study only incorporated one population of each size class. It is important to see if the patterns I observed are found in other small populations to make more solid recommendations. It would have provided a stronger comparison and determined if the nearest neighbour mating found in the small population was a common occurrence or specific to that population.

• To complement the realized pattern of pollen dispersal assessed through paternity assignment, pollinator observations can be conducted. This will confirm if the behavior of honeyeaters changes or the composition of the pollinator guild is the reason for differences in the observed patterns in pollen dispersal.

• Increase the range that the study is conducted. This will incorporate the response of pollinators to other matrix’s besides the urban as well as pollinators lost in the urban areas such as the honey possum.

• Examine other bird pollinated species to see if there is a common pattern as well as a comparison to insect pollinated species would provide an insight into restoration of a whole community.

Section 3: Affect of population size on progeny

• The major limitation of using the porometer to measure stomatal conductance was its reduced ability when the air is humid. During the experiment the weather was unseasonably hot and humid due to some summer storms. Using an instrument that can create a desired humidity such as a Licor portable photosynthesis measuring system would perhaps enhance the study given the difficulty of using the porometer.

• Extend the study to include other stresses such as nutrient limitation/excess. This will be useful to understand what other important factors influence the survival of B. ilicifolia. It will be beneficial in
understanding recruitment in areas that have a greater amount of nutrient input such as phosphorous in human impacted site.

- Test recovery of stressed plants by including a rewetting phase that would mimic real conditions.
- Test seedling fitness from small and large populations under field conditions. Glasshouse trials are a good guide to what might happen in the field but a field trial is still needed to confirm the findings.

6.4 Conclusion

With the extent of human caused damage to the natural environment by climate change, pollution and direct habitat destruction more now than ever there needs to be research to help understand how to halt and reverse impacts on population processes. Understanding how vegetation remnants and the plants within them function is essential for providing information to retain them for the future. It is also imperative to understand how these remnants fit into the landscape as a whole and how the surrounding matrix influences the ecosystem processes within (Hadley and Betts, 2012). The use of gardens as corridors could be very important in urban areas and public education on the conservation role of native gardens should be encouraged (Doody et al., 2010).

My study has shown that understanding genetic structure on a landscape scale informs on how past historical demographic changes can help predict what will happen with future climate changes. It also has shown that populations in native vegetation remnants need to be of a minimum size to ensure that ecosystem processes that drive genetic diversity and hence evolutionary potential still function. In the creation and conservation of vegetation remnants and the plants within population viability must be a major criterion (Soule and Simberloff, 1986). Without understanding how the small pieces interlock with the wider functioning landscape it will not be possible to conserve key ecosystem function. Scientists and land practitioners need to look at the landscape as whole and understand how an individual population functions within the landscape to be able to protect biodiversity for future generations.
References


SEDDON, G. 1972. Sense of place: a response to an environment, the Swan Coastal Plain, Western Australia, University of Western Australia Press Perth.


