Plant density effects on genetic variation and dispersal for two co-occurring *Persoonia* species

Jessica Anne Stingemore
BSc (Hons)

This thesis is presented for the degree of Doctor of Philosophy of The University of Western Australia
School of Plant Biology
2014
Cartoon by Joel Pett published in USA Today (2009)

ABSTRACT

Genetic variation is spatially structured at different scales, such as population, subpopulation or among neighbouring individuals. This spatial genetic structure is typically a product of environmental influences and ecological factors affecting reproduction and dispersal are likely to be particularly important in determining genetic structure. Consequently, information about ecological factors such as plant density, pollen dispersal and seed dispersal represent important processes for conservation managers. In this thesis, molecular markers are applied to better understand patterns and processes effecting genetic variation in two co-occurring tree congeners, chosen because they have densities that are typically three orders of magnitude different, yet near identical life-history parameters. *Persoonia longifolia* occurs in the southwest jarrah (*Eucalyptus marginata*) forest of Western Australia in relatively high densities, while *P. elliptica* occurs largely at very low densities.

To develop molecular markers for these two broadly distributed tree species, microsatellite markers developed for the southeast Australian *P. mollis* were initially screened for amplification in individuals of both *P. elliptica* and *P. longifolia*. This cross amplification of primers was unsuccessful for both study species. Therefore, microsatellite enriched libraries and 454 GS-FLX shotgun sequencing were employed to identify nine microsatellite loci for *P. elliptica* (one 454; eight cloning) and six loci for *P. longifolia* (three 454; three cloning). The microsatellites identified in this study enabled the examination of spatial structuring of genetic diversity and pollen and seed dispersal characteristics in *P. elliptica* and *P. longifolia*.

To identify the effects of plant density on genetic variation and its structuring, microsatellite markers were used to quantify genetic diversity, within population structure and among population structure for 20 and 22 (respectively) populations of *P. elliptica* and *P. longifolia*. Despite the marked contrast in density, the two species were found to have similarly high levels of genetic diversity. However, this study did reveal a significant negative relationship between density and both number of alleles and fixation indices.
These relationships were driven by very low density *P. elliptica* populations, indicating that density only begins to have an effect on number of alleles and fixation indices at a threshold below 0.01 plants per ha. While both species were found to display moderate genetic differentiation among geographically proximate populations (*F*<sub>ST</sub> = 0.18 ± 0.02 for *P. longifolia* and 0.30 ± 0.01 for *P. elliptica*), the high density species *P. longifolia* displayed significantly stronger within population genetic structure as measured by spatial autocorrelation. On average, *P. longifolia* populations displayed significant positive genetic structure at inter plant distances of up to 25 m, presumably reflecting the effects of plant density on pollinator behaviour, where the high density of neighbouring trees causes pollinators to travel shorter distances among more related trees, acting to produce seeds that have higher kinship than those of plants in less dense populations.

To comprehensively assess patterns of pollen dispersal in a geographically isolated study population of ca. 1,300 ha of *P. elliptica*, every adult tree (n = 450) was mapped and genotyped, together with 343 canopy seeds from ten maternal trees over two flowering seasons. This study revealed that *P. elliptica* has a realised mating system that is predominantly outcrossing, which is consistent with other investigations on mating systems for members of the Proteaceae family. Paternity assignment revealed that effective pollen dispersal in *P. elliptica* showed a departure from predominantly nearest neighbour mating, with long-distance pollen dispersal (0.00 – 5.01 km, with a mean of 1.59 km over the two flowering years) and high multiplicity of detected paternity (in 2010 84% and in 2012 86% of pollination events had a unique sire). Population genetic structure was detected up to 500 m and trending towards a pattern of isolation by distance. These results demonstrate an ability of pollinator bee species to travel long distances. The adult trees and canopy seed displayed similarly high levels of genetic diversity and little evidence for inbreeding.

To identify whether *Dromaius novaehollandiae* (emus) were a major seed dispersal vector for *P. elliptica*, motion sensor cameras were employed in the same geographically isolated study population of *P. elliptica*. *Dromaius novaehollandiae* was not observed interacting with the fruits, however two macropod species (*Macropus fuliginous* and *M. irma*) were identified as effective seed dispersal vectors. To thoroughly examine patterns of endozoochory-meditated seed dispersal, macropod scat in the same study population was systematically searched for *P. elliptica* endocarps. Over the two study years 115 macropod
scats containing 127 endocarps with seed were mapped and genotyped, together with every adult tree (n = 450) in the study site. Seed dispersal distances estimated from parentage assignments suggest that macropods moved seeds away from the parent tree and over long distances (mean 1.57 km and up to 4.40 km). Again, the dispersed seed displayed high levels of genetic diversity and little evidence for inbreeding.

This thesis has produced major new insights into the drivers of population genetic structure and gene flow and the importance of dispersal vectors facilitating long distance dispersal of pollen and seed at a scale of kilometres. Furthermore, this work represents one of the most intensive genetic studies of the *Persoonia* genus and is one of the first to apply molecular markers to understand the effects of plant density, pollen dispersal and seed dispersal in natural populations of southwest Australia *Persoonia* species. Given the remarkably high levels of dispersal observed in this study, the loss of dispersal vectors could have significant negative impacts on *Persoonia*. Overall, this work highlights the need for quantification of factors affecting species’ dispersal capacities in order to accurately inform management and conservation efforts.
# TABLE OF CONTENTS

Abstract ........................................................................................................ iii
Table of Contents .......................................................................................... vi
Acknowledgements ...................................................................................... x
Declaration of Candidate Contribution ....................................................... xi
List of Figures ............................................................................................... xii
List of Tables ................................................................................................. xvi

## CHAPTER ONE: General Introduction

1.1 Introduction .............................................................................................. 1
1.2 Ecological Determinants of Genetic Variation .......................................... 2
   1.2.1 Plant Density .................................................................................. 2
   1.2.2 Gene Dispersal .............................................................................. 3
      1.2.2.1 Pollen Dispersal ................................................................... 4
      1.2.2.2 Seed Dispersal ...................................................................... 5
      1.2.2.3 Measuring Gene Dispersal ..................................................... 6
1.3 Persoonia .................................................................................................. 8
   1.3.1 Persoonia in Australia .................................................................... 8
   1.3.2 Previous Studies on Persoonia ...................................................... 8
1.4 Study Species ......................................................................................... 12
   1.4.1 Persoonia elliptica ....................................................................... 12
   1.4.2 Persoonia longifolia ..................................................................... 15
1.5 Thesis Aims and Outline ........................................................................ 18

## CHAPTER TWO: Isolation and characterisation of microsatellite markers for two Australian Persoonia species using cloning of microsatellite enriched libraries and 454 GS-FLX shotgun sequencing

2.1 Abstract ................................................................................................. 21
2.2 Introduction ............................................................................................ 22
2.3 Methods and Materials .......................................................................... 24
   2.3.1 DNA Extraction .......................................................................... 24
   2.3.2 Cross Amplification from P. mollis Microsatellites ...................... 25
   2.3.3 Isolation and Characterisation of Microsatellite Markers .............. 25
      2.3.3.1 Microsatellite Enriched Libraries ........................................ 25
      2.3.3.2 454 GS-FLX Shotgun Sequencing ...................................... 27
   2.3.4 Genetic Diversity ........................................................................ 28
2.4 Results .................................................................................................................. 29
  2.4.1 Cross Amplification from *P. mollis* Microsatellites ........................................ 29
  2.4.2 Isolation and Characterisation of Microsatellite Markers .................................. 29
  2.4.3 Genetic Diversity .......................................................................................... 29
2.5 Discussion ........................................................................................................... 32

CHAPTER THREE: Plant density effects on genetic variation and its spatial structuring in
two co-occurring *Persoonia* species
3.1 Abstract .............................................................................................................. 33
3.2 Introduction ......................................................................................................... 34
3.3 Methods and Materials ....................................................................................... 39
  3.3.1 Study Species ............................................................................................... 39
  3.3.2 Sample Collections ...................................................................................... 39
  3.3.3 Population Density ....................................................................................... 44
  3.3.4 DNA Extraction ........................................................................................... 44
  3.3.5 Microsatellite Procedure .............................................................................. 44
  3.3.6 Data Analysis ............................................................................................... 45
    3.3.6.1 Genetic Diversity .................................................................................... 45
    3.3.6.2 Genetic Structure .................................................................................. 46
3.4 Results ................................................................................................................ 48
  3.4.1 Population Density ....................................................................................... 48
  3.4.2 Data Analysis ............................................................................................... 48
    3.4.2.1 Genetic Diversity .................................................................................... 48
    3.4.2.2 Genetic Diversity and Population Density .............................................. 53
    3.4.2.3 Genetic Structure .................................................................................. 53
3.5 Discussion ........................................................................................................... 63
  3.5.1 Effects of Density on Genetic Diversity ....................................................... 63
  3.5.2 Genetic Structure Within Populations ......................................................... 65
  3.5.3 Genetic Structure Among Populations ......................................................... 66
3.6 Conclusions ......................................................................................................... 68

CHAPTER FOUR: Paternity assignment reveals long distance dispersal of pollen in
*Persoonia elliptica*
4.1 Abstract .............................................................................................................. 71
4.2 Introduction ......................................................................................................... 72
4.3 Methods and Materials ....................................................................................... 76
  4.3.1 Study Species ............................................................................................... 76
  4.3.2 Study Site and Sample Collections ................................................................ 76
4.3.3 DNA Extraction ............................................................................. 79
4.3.4 Microsatellite Genotyping ............................................................... 79
4.3.5 Data Analysis .................................................................................. 79
  4.3.5.1 Genetic Diversity .................................................................... 79
  4.3.5.2 Adult Population Genetic Structure ........................................... 80
  4.3.5.3 Mating Systems Analysis .......................................................... 80
  4.3.5.4 Paternity Analysis ................................................................... 81
4.4 Results ............................................................................................... 82
  4.4.1 Genetic Diversity ........................................................................ 82
  4.4.2 Adult Population Genetic Structure .............................................. 86
  4.4.3 Realised Mating Patterns .............................................................. 89
  4.4.4 Paternity Analysis ...................................................................... 92
4.5 Discussion ......................................................................................... 103
  4.5.1 Realised Mating Patterns .............................................................. 103
  4.5.2 Realised Pollen Dispersal .............................................................. 104
  4.5.3 Long Distance Pollen Dispersal by Bees ....................................... 105
  4.5.4 Genetic Diversity ....................................................................... 107
  4.5.5 Adult Population Genetic Structure ............................................. 108
  4.5.6 Core Versus Edge Effects ......................................................... 109
4.6 Conclusions ....................................................................................... 110

CHAPTER FIVE: Long distance dispersal of Persoonia elliptica seed by macropods, inferred from parentage analysis

5.1 Abstract ........................................................................................... 111
5.2 Introduction ...................................................................................... 112
5.3 Methods and Materials .................................................................... 116
  5.3.1 Study Species ........................................................................... 116
  5.3.2 Study Site ................................................................................ 116
  5.3.3 Identification of Seed Dispersal Vectors ...................................... 116
  5.3.4 Sample Collection .................................................................... 117
  5.3.5 DNA Extraction ......................................................................... 117
  5.3.6 Microsatellite Genotyping .......................................................... 117
  5.3.7 Data Analysis ............................................................................ 118
    5.3.7.1 Genetic Diversity ................................................................. 118
    5.3.7.2 Seed Cohort Genetic Structure ............................................... 118
    5.3.7.3 Paternity Analysis ................................................................. 119
5.4 Results .............................................................................................. 120
  5.4.1 Identification of Seed Dispersal Vectors ...................................... 120
  5.4.2 Scat Collection .......................................................................... 120
  5.4.3 Genetic Diversity ...................................................................... 124
5.4.4 Seed Cohort Genetic Structure ........................................................................ 124
5.4.5 Parentage Assignment ..................................................................................... 127

5.5 Discussion ............................................................................................................ 133
  5.5.1 Identification of Seed Dispersal Vectors ....................................................... 133
  5.5.2 Realised Seed Dispersal ............................................................................... 133
  5.5.3 Implications of Macropod-Mediated Seed Dispersal ................................. 134
  5.5.4 Seed Versus Pollen Dispersal ...................................................................... 136
  5.5.5 Seed Immigration ......................................................................................... 137
  5.5.6 Genetic Diversity ......................................................................................... 137
  5.5.7 Seed Cohort Genetic Structure ................................................................... 137

5.6 Conclusions ......................................................................................................... 138

CHAPTER SIX: Closing Remarks

6.1 Introduction .......................................................................................................... 139

6.2 Key Findings ........................................................................................................ 140
  6.2.1 Successful cross amplification of microsatellites ........................................ 140
  6.2.2 High levels of genetic diversity are maintained in low density species .......... 140
  6.2.3 High density populations show greater spatial genetic structure within populations than less dense populations .................................................................................. 141
  6.2.4 Low densities species show greater spatial genetic structure among populations than more dense populations .......................................................................................... 141
  6.2.5 Native bees facilitate near random mating at a large spatial scale ............... 142
  6.2.6 Pollen mediated gene flow maintains high levels of genetic diversity and outcrossing rates on the periphery of the study population .................................................................................. 142
  6.2.7 Native macropods contribute to moving P. elliptica seed away from the parent tree and over long distances ........................................................................................................ 143
  6.2.8 Long distance dispersal in southwest Australia ............................................ 144

6.3 Future Directions ................................................................................................ 145

REFERENCES ............................................................................................................. 149
ACKNOWLEDGEMENTS

There are many people whom I wish to thank for making this project possible and for their valuable contributions throughout this research. First I would like to thank my supervisors Siegy Krauss, Paul Nevill and Michael Moody for the countless suggestions, corrections and encouragements throughout this PhD process. This thesis was improved by their ongoing investment, knowledge and valuable experience.

This research was funded by the Australian Research Council through a Discovery Scheme Grant (DP1095297), the School of Plant Biology, The University of Western Australia Postgraduate Travel Award, Botanic Gardens and Parks Authority and the Mary Janet Lindsay of Yanchep Memorial Fund.

To the staff and students in the Kings Park Science lab, in particular the genetics group, thank you for the advice, the laughs and the opportunity to be part of your research. Thanks especially go to Donna Bradbury, Michalie Foley, Alison Ritchie, Tanya Hevroy, Todd Erickson and Janet Anthony.

A huge thank you to my family, particularly my Dad, who have always supported my goals. Without their tireless help, whether it was collecting leaves or searching through scat, this thesis would not exist. I would also like to thank my little niece Olivia, I look forward to sharing my passion for Australia’s native flora and fauna with you, especially my love for snottygobble. Finally to Dingo, thank you for your love and encouragement over the past few years and for always letting me have the last glass of wine!
DECLARATION

This thesis contains published work and/or work prepared for publication, some of which has been co-authored. The bibliographical details of the work and where it appears in the thesis are outlined below.

One of the chapters of this thesis has been submitted and accepted for publication. The text in this chapter is an edited version of what appears in the paper.

CHAPTER TWO

Author contributions: JS 70%, PN 10%, MG 10% and SK 10%
LIST OF FIGURES

Figure 1.1 ................................................................................................................................. 13
Natural distribution of Persoonia elliptica in Western Australia. Maps sourced from Nature Map, Department of Environment and Conservation.

Figure 1.2 ................................................................................................................................. 14
Photographs of a) Persoonia elliptica tree in its natural habitat, b) bark characteristics, c) leaf shape, d) c.a. thirty-year old P. elliptica, e) seedling, f) P. elliptica tree in its natural habitat, g) fruit fall and h) introduced honeybee (Apis mellifera) collecting nectar from flower. All photographs taken by author.

Figure 1.3 ................................................................................................................................. 16
Natural distribution of Persoonia longifolia in Western Australia. Maps sourced from Nature Map, Department of Environment and Conservation.

Figure 1.4 ................................................................................................................................. 17
Photographs of a) Persoonia longifolia in natural habitat, b) unripe fruit, c) flowers, tree, d) bark characteristics, e) leaf shape, f) P. longifolia resprouting after fire, g) seedling and h) P. longifolia occurring near a mine site. All photographs taken by author.

Figure 3.1 ................................................................................................................................. 40
Geographic location of 22 sampled populations of Persoonia longifolia in southwest Western Australia. Population codes correspond to those used in Table 3.1. The grey dots indicate herbarium collections and show the range of the species. Maps sourced from Nature Map, Department of Environment and Conservation.

Figure 3.2 ................................................................................................................................. 41
Geographic location of 20 sampled populations of Persoonia elliptica in southwest Western Australia. Population codes correspond to those used in Table 3.2. The grey dots indicate herbarium collections and show the range of the species. Maps sourced from Nature Map, Department of Environment and Conservation.

Figure 3.3 ................................................................................................................................. 55
Relationship between (a) number of alleles ($r^2 = 0.39$) and (b) fixation index with log density ($r^2 = 0.21$) for populations of Persoonia longifolia (pink) and P. elliptica (blue). Solid line represents logarithmic line of best line.
Figure 3.4. Spatial autocorrelation analysis correlograms for populations of (a) *Persoonia longifolia* and (b) *P. elliptica* showing the genetic correlation coefficient ($r$) for increasing distance class sizes, with 95% confidence intervals about $r$ as determined by bootstrapping.

Figure 3.5. nMDS ordination of Nei’s genetic distance for 22 populations of *Persoonia longifolia*. Population symbols correspond to geographic region (northern: open triangle, southern: closed triangle).

Figure 3.6. nMDS ordination of Nei’s genetic distance for 20 populations of *Persoonia elliptica*. Population symbols correspond to geographic region (northern: open triangle, southern: closed triangle).

Figure 3.7. nMDS ordination of genetic distance for 568 individuals of *Persoonia longifolia*. Individuals are labelled according to population.

Figure 3.8. nMDS ordination of genetic distance for 182 individuals of *Persoonia elliptica*. Individuals are labelled according to population.

Figure 3.9. STRUCTURE results for *Persoonia longifolia*, a) delta K peaks at K = 2, b) plot of lnP (K) and c) bar chart of relative proportional membership of each individual (single bars) within each sampling location/population (solid lines) to one of two genetic clusters (red, green).

Figure 3.10. STRUCTURE results for *Persoonia elliptica* a) delta K peaks at K = 2, b) plot of lnP (K) and c) bar chart of relative proportional membership of each individual (single bars) within each sampling location/population (solid lines) to one of two genetic clusters (red, green).

Figure 4.1. Geographic location of all 450 *Persoonia elliptica* trees in the study area within Avon Valley National Park, Western Australia. The pink dots indicate core maternal trees and the blue dots edge maternal trees from which fruit were sampled in 2010 and 2012. Numbers correspond to Table 4.1. Maternal 8 was only sampled in 2010, due to tree death.
Figure 4.2. Number of non maternal alleles scored in the seed cohorts of *Persoonia elliptica* in Avon Valley National Park, Western Australia from 2010 (blue bars) and 2012 (green bars) from the nine microsatellite markers.

Figure 4.3. Spatial autocorrelation analysis correlogram for the study population of *Persoonia elliptica* in Avon Valley National Park, Western Australia showing the genetic correlation coefficient ($r$) for increasing distance class sizes, with 95% confidence intervals about $r$ as determined by bootstrapping.

Figure 4.4. Frequency distribution of observed pollination distances for all *Persoonia elliptica* seeds screened in Avon Valley National Park, Western Australia sampled in 2010 (blue bars) and 2012 (green bars); and frequency distribution of all plants within the population to the maternal trees (pink bars).

Figure 4.5. Frequency distribution of observed pollination distances for maternal trees in the core of the study plot in Avon Valley National Park, Western Australia, for seeds sampled in 2010 (blue bars) and 2012 (green bars); and frequency distribution of all plants within the population to the maternal trees (pink bars). Maternal tree number is displayed on the top right of each graph. Combined refers to the average frequency distribution for all maternal trees on the edge of the population.

Figure 4.6. Frequency distribution of observed pollination distances for maternal trees on the edge of the study plot in Avon Valley National Park, Western Australia, for seeds sampled in 2010 (blue bars) and 2012 (green bars); and frequency distribution of all plants within the population to the maternal trees (pink bars). Maternal tree number is displayed on the top right of each graph. Combined refers to the average frequency distribution for all maternal trees on the edge of the population.

Figure 4.7. Map of sampled plants from the study site in Avon Valley National Park, Western Australia showing each maternal plant (pink dot), 2010 sires (blue dots), 2012 sires (green dots), 2010 and 2012 sires (orange dots) and other genotyped individuals (black dots) for each maternal plant. Dot size corresponds to number of seeds sired. Maternal tree number is displayed on the top right of each figure.

Figure 5.1. a) Photograph of *Macropus fuliginosus* (western grey kangaroo); and b) *M. irma* (brush tail wallaby) consuming fruits of *Persoonia elliptica* in Avon Valley National Park, Western Australia taken by a Bushnell Trophy Cam Trail camera; c) photograph, d) x-ray photograph of *P. elliptica* seed in macropod scat from Avon Valley National Park, Western Australia and e) photograph of *Dromaius novaehollandiae* (emu)
passing but not interacting with fruits in Avon Valley National Park, Western Australia taken by a Bushnell Trophy Cam Trail camera.

Figure 5.2 .......................................................................................................................... 122
Map of sampled sites searched for macropod scat in 2010, in Avon Valley National Park, Western Australia. Blue dot represents sites were *Persoonia elliptica* seed was found in scat and red dot represents sites were no *P. elliptica* seed was found in scat. Dot sizes correspond to the number of seed found.

Figure 5.3 .......................................................................................................................... 123
Map of sampled sites searched for macropod scat in 2012, in Avon Valley National Park, Western Australia. Green dot represents sites were *Persoonia elliptica* seed was found in scat and red dot represents sites were no *P. elliptica* seed was found in scat. Dot sizes correspond to the number of seed found.

Figure 5.4 .......................................................................................................................... 126
a) Spatial autocorrelation analysis correlogram for a) the dispersed seed and b) adult population of *Persoonia elliptica* in Avon Valley National Park, Western Australia showing the genetic correlation coefficient (r) for increasing distance class sizes, with 95% confidence intervals about r as determined by bootstrapping.

Figure 5.5 .......................................................................................................................... 129
Map of *Persoonia elliptica* seed assigned parentage at 80% confidence that were collected in 2010 from macropod scat in Avon Valley National Park, Western Australia. Triangles represent seed and the circles of the corresponding colour represent parent pairs.

Figure 5.6 .......................................................................................................................... 130
Map of *Persoonia elliptica* seed assigned parentage at 80% confidence that were collected in 2012 from macropod scat in Avon Valley National Park, Western Australia. Triangles represent seed and the circles of the corresponding colour represent parent pairs.

Figure 5.7 .......................................................................................................................... 131
Frequency distribution of observed seed dispersal distances at 80% confidence for *Persoonia elliptica* seeds screened in Avon Valley National Park, Western Australia. Seed dispersal (blue bars), pollen dispersal (orange bars) and frequency distribution of all plants pairs within the population (green bars).

Figure 5.8 .......................................................................................................................... 132
Frequency distribution of observed seed dispersal distances at 50% confidence for *Persoonia elliptica* seeds screened in Avon Valley National Park, Western Australia. Seed dispersal (blue bars), pollen dispersal (orange bars) and frequency distribution of all plants pairs within the population (green bars).
LIST OF TABLES

Table 2.1........................................................................................................................................30
Microsatellite primers developed in *Persoonia elliptica* (listed first where relevant) and *P. longifolia*. Shown for each primer are locus name, development method, forward primer sequence, reverse primer sequence, repeat motif, size range, WellRED dye label (multiplex marker set) and GenBank Accession Number.

Table 2.2........................................................................................................................................31
Results of primer screening in two populations each of *Persoonia elliptica* and *P. longifolia*. For each primer pair the locus name, number of individuals sampled (n), number of alleles (*Nₐ*), observed (*Hₒ*) and expected heterozygosities (*Hₑ*) and deviation from Hardy Weinberg equilibrium (HWE) (*P*< 0.05; **P**< 0.01; ***P***< 0.001; ns. = not significant) are given. All values are based on samples from populations in southwest Australia.

Table 3.1........................................................................................................................................42
Geographic location, population code, latitude, longitude, number of samples (N) and population density for 22 populations of *Persoonia longifolia*. * At least 30 individuals were sampled for each population, however due to DNA extraction and genotyping difficulties the number of genotyped accessions varies.

Table 3.2........................................................................................................................................43
Geographic location, population code, latitude, longitude, number of samples (N) and population density for 20 populations of *Persoonia elliptica*.

Table 3.3........................................................................................................................................50
Genetic diversity parameters for six microsatellite loci used in this study for *Persoonia longifolia*. *Nₐ*: average number of alleles, *Hₒ*: observed heterozygosity, *Hₑ*: expected heterozygosity, *Fᵢₛ*: inbreeding in individuals relative to their population, *Fᵢₜ*: inbreeding in populations relative to the total species, *Fₛᵢ*: inbreeding in populations relative to total species

Table 3.4........................................................................................................................................50
Genetic diversity parameters for six microsatellite loci used in this study for *Persoonia elliptica*. *Nₐ*: average number of alleles, *Hₒ*: observed heterozygosity, *Hₑ*: expected heterozygosity, *Fᵢₛ*: inbreeding in individuals relative to their population, *Fᵢₜ*: inbreeding in populations relative to the total species, *Fₛᵢ*: inbreeding in populations relative to total species
Genetic diversity parameters from six polymorphic microsatellite markers for populations of *Persoonia longifolia*: \( N \): total number of individuals genotyped, \( N_A \): number of alleles; \( H_O \): observed heterozygosity, \( H_E \): expected heterozygosity, \( F \): fixation index, \( S \): private alleles, \( H_S \): gene diversity.

Genetic diversity parameters from six polymorphic microsatellite markers for populations of *Persoonia elliptica*. \( N \): total number of individuals genotyped, \( N_A \): number of alleles, \( H_O \): observed heterozygosity, \( H_E \): expected heterozygosity, \( F \): fixation index, \( S \): private alleles, \( H_S \): gene diversity.

Number of *Persoonia elliptica* seeds genotyped in 2010 and 2012 per maternal tree in Avon Valley National Park, Western Australia, including geographic location. * indicates the maternal tree died before sampling of seed in 2012.

Genetic diversity parameters for nine microsatellite loci used in this study. \( N_A \): number of alleles, \( H_O \): observed heterozygosity, \( H_E \): expected heterozygosity, \( F_{IS} \): inbreeding in individuals relative to their population, \( F_{IT} \): inbreeding in populations relative to the total species, \( F_{ST} \): inbreeding in populations relative to total species.

Genetic diversity parameters for the adult population, the 2010 and 2012 seed cohorts for *Persoonia elliptica* in Avon Valley National Park, Western Australia. \( N \): number of individuals, \( N_A \): average number of alleles per locus, \( H_O \): observed heterozygosity, \( H_E \): expected heterozygosity, \( F \): fixation index, \( S \): average number of private alleles.

Mating system parameters for two seed cohorts of *Persoonia elliptica* in Avon Valley National Park, Western Australia estimated with nine microsatellite loci, with standard errors. \( t_m \): multilocus outcrossing rate; \( t_s \): single locus outcrossing rate; \( t_m - t_s \): an estimate of biparental inbreeding; \( r_p \): multilocus correlation of paternity (fraction of outcrossed siblings that share the same sire).

Mating system parameters for each maternal *Persoonia elliptica* in Avon Valley National Park, Western Australia, over two years with standard errors. \( t_m \): multilocus outcrossing rate; \( t_s \): single locus outcrossing rate; \( t_m - t_s \): an estimate of biparental inbreeding; \( r_p \): multilocus correlation of paternity (fraction of outcrossed siblings that share the same sire).
Table 4.6 .............................................................................................................. 95
Mean number of seed tested, number of seed assigned paternity and number of unique pollen donors for two
seed populations of *Persoonia elliptica* in Avon Valley National Park, Western Australia estimated with nine
microsatellite loci.

Table 4.7 .............................................................................................................. 96
Location, number of seed tested, number of seed assigned paternity at 80% confidence interval and number of
unique pollen donors for each maternal per year of *Persoonia elliptica* in Avon Valley National Park, Western
Australia estimated with nine microsatellite loci.

Table 5.1 ............................................................................................................. 125
Genetic diversity parameters for the adult population, the 2010 and 2012 seed cohorts for *Persoonia elliptica*
in Avon Valley National Park, Western Australia. N: number of individuals, \( N_A \): average number of alleles,
\( A_R \): allelic richness (standardised number of alleles), \( H_O \): observed heterozygosity, \( H_E \): expected
heterozygosity, \( F \): fixation index, \( S \): average number of private alleles.
CHAPTER ONE

General Introduction

1.1 INTRODUCTION

Genetic variation is the raw material for evolutionary change within plant and animal populations. Genetic variation results from the combined action of migration, mutation, mating, selection and drift, which, in turn, operate within the historical and biological framework of each species (Loveless and Hamrick 1984; Slatkin 1987; Morjan and Rieseberg 2004). Genetic variation is spatially structured at different scales, such as population, subpopulation or among neighbouring individuals and this spatial genetic structure is a product of environmental influences (Loveless and Hamrick 1984; Heywood 1991; Hamrick and Godt 1996). The genetic divergence among conspecific populations from contrasting geographic regions has been a central focus of ecological genetics since its inception. Studying such divergence provides information on the role of gene movement in distributing genetic variation within and among populations and on the ways that populations and species respond to heterogeneous environments (Latta 2004; Bonin et al. 2007). Identifying the boundaries between distinct gene pools provides the scientific basis for defining units of evolutionary, management, conservation and/or taxonomic significance (Crandall et al. 2000; Holderegger et al. 2006). Understanding the roles of ecological determinants of genetic variation and its spatial structure within a species distribution in creating these units allows us to manage populations in a way that preserves the adaptive match of organisms to their local environments (Latta 2004). Ecological determinants include such factors as breeding systems, floral morphology mode of reproduction, pollination mechanisms, seed dispersal, seed dormancy, phenology, time of reproduction, geographic range, population size, population density and pollen dispersal (Loveless and Hamrick 19984). In addition, understanding genetic variation and its ecological determinants can inform us about how well a species may cope with current rapid climate change (Latta 2004). Ecological factors affecting reproduction and dispersal are likely to be particularly important in determining genetic variation and its structuring (Loveless and Hamrick 1984).
1.2 ECOLOGICAL DETERMINANTS OF GENETIC VARIATION

Ecological determinants contribute to the level of genetic variation and structure in populations and species (Loveless and Hamrick 1984; Ingvarsson 1997). Consequently, researching the roles and significance of key ecological determinants that shape the levels and spatial structure of genetic variation is a major goal of molecular ecology. An understanding of the key drivers of evolution within populations and species has consequences for determining the best strategies for conservation of genetic variation (Hamrick et al. 1979; Loveless and Hamrick 1984; Hamrick et al. 1989; Hamrick and Godt 1989; 1996). This thesis will focus on three key ecological determinants and how they affect genetic variation and its structuring - plant density, pollen dispersal and seed dispersal.

1.2.1 Plant Density

Density is the number of individuals per unit of area. In a biological sense, density can be described as the spacing between neighbouring individuals of the same species (Kunin 1997). Numerous reviews have identified density as an ecological determinate that affects genetic variation and how it is structured in plants (Hamrick et al. 1979; Loveless and Hamrick 1984; Hamrick et al. 1989; Hamrick and Godt 1989; 1996).

In both animal and wind pollinated plants, increased density usually results in decreased pollen dispersal distance and consequently decreased gene flow distances (Antonovics and Levin 1980). In the case of animal pollinated plants, density can affect a plants reproductive success, by affecting both the quantity and quality of pollination services received (Antonovics and Levin 1980; Kunin 1993; Lamont et al. 1993; Ingvarsson and Lundberg 1995; Groom 1998). Low density populations may be less apparent or attractive to pollinators, resulting in a lower number of pollinator visits to plants in these populations (Jennersten 1988).

Previous studies indicate that higher plant density is associated with higher visitation rates of insect pollinators (e.g. Kunin 1993) and this is likely to have greater impact for self-incompatible species which cannot compensate for lower pollinator abundance by selfing. Moreover, plant density probably affects the behaviour of pollinators which are more likely to move between individuals in dense populations, where flight distances are shorter, than
in sparse population where increased visits within a plant may favour within-plant pollen transfer (Antonovics and Levin 1980; Van Treuren 1992). In cases where pollinator visitation is not affected by population density, plants in low density populations may still suffer reduced seed set due to pollen quality (Kunin 1993; Lamont et al. 1993; Wolf and Harrison 2001). This may occur through the deposition of foreign rather than conspecific pollen, or through the transfer of incompatible pollen (Feinsinger et al. 1991; Kunin 1993; Aizen and Feinsinger 1994; Groom 1998; Ramsey and Vaughton 2000).

Low density may also affect populations in a deterministic way due to Allee effects, which are broadly defined as a decline in individuals’ fitness at low densities (Allee 1931; 1938). Allee effects may be due to a variety of genetic, demographic, and/or ecological factors, including skewed sex ratios, increased levels of inbreeding depression or genetic drift and reduced availability of mates (Allee 1938). Reproductive success may decline in low density populations because individuals in these populations are mate limited, particularly in species that depend on animal vectors for pollination (Groom 1998). Species that are obligate outcrossers that rely on animal vectors for pollination are most likely to be affected by Allee effects (Huenneke 1991; Forsyth 2003).

Plant density is also known to effect the structuring of genetic variation across a species range (Loveless and Hamrick 1984). Because of the reduced gene flow distances, the tendency for microdifferentation will be greater in high density populations than low density populations (Antonovics and Levin 1980; Loveless and Hamrick 1984). In addition, populations that occur at low densities have the potential for greater isolation-by-distance (IBD) due to higher local genetic drift leading to high variation within species (Heywood 1991; Vekemans and Hardy 2004). The effects of density on gene flow by seed dispersal are less well understood, however seed dispersal is generally not considered density-dependent (Antonovics and Levin 1980; Heywood 1991). Overall changes in density may have profound effects on genetic variation both within and among populations; however there is a need for more empirical studies into these effects.

1.2.2 Gene Dispersal
Gene dispersal is the movement of genes within and among populations and their subsequent establishment (Levin and Kerster 1974). Gene dispersal is a key issue in
Chapter One: General Introduction

population and ecological genetics because (i) it determines the extent to which individuals across the species range share a common gene pool, (ii) dispersal capabilities condition the demographic behaviour of populations and (iii) it determines the amount of local genetic subdivision (Wright 1951; Levin and Kerster 1974). For plant species, gene dispersal occurs primarily through pollen and/or seed dispersal (Levin and Kerster 1974; Ellstrand 1992).

1.2.2.1 Pollen Dispersal

Pollination is the sexual process for gene exchange between flowering plants and is an essential ecosystem process (Kremen et al. 2007; Lonsdorf et al. 2009; Menz et al. 2011). Although pollen dispersal lacks the colonization function of seeds, patterns of pollen dispersal play a central role in plant population genetics and influence effective population size and the degree of population subdivision resulting either from drift or differential selection (Levin and Kerster 1974).

Pollen dispersal is constrained by determinants such as plant density (Antonovics and Levin 1980; Loveless and Hamrick 1984; Kunin 1997), breeding system (Harris and Johnson 2004; Aguilar et al. 2006) and the presence, composition and behaviour of pollinators (Hobbs and Yates 2003; Harris and Johnson 2004). Plant density is known to affect patterns of pollen dispersal due to density-dependent changes in pollinator behaviour (Antonovics and Levin 1980; Friedman and Barrett 2008). Plant breeding systems determine fertilization success and indicate the degree to which plants species depend on pollinators (Bond 1994). Furthermore, outcrossing plant species are also influenced by the availability of genetically compatible pollen donors and are therefore even more dependent on wind or pollinator mediated gene flow (Aguilar et al. 2006). Typically, pollen is deposited on nearest neighbours, producing a leptokurtic pollen dispersal curve, with the probability of pollen deposition being highest for nearest neighbours and decreasing as distance increases (Levin and Kerster 1974). A leptokurtic pollen dispersal curve is characterised by a steep rate of decline near the origin with a long tail of relatively rare long-distance events (Levin and Kerster 1974). Foraging behaviour of pollinators also influences nearest neighbour mating, the number of potential mates and therefore the reproductive success of plants (Dick et al. 2003; Rymer et al. 2005).
Pollen dispersal in plants has now been examined in numerous species using a range of approaches based on parent-offspring genotype data (reviewed in Austerlitz et al. 2004; Smouse and Sork 2004; Burczyk and Koralewski 2005). While patterns of near neighbour pollen dispersal have been commonly examined, there is still limited research into long distance pollen dispersal events, particularly over large geographic scales. By identifying and genotyping all potential pollen sources within a large study area, direct paternity assignment can be used to categorically identify the sires, with little information lost for the estimation of the effective pollen dispersal distribution or other within-population reproductive parameters. Thus large geographically isolated populations can provide a unique opportunity to assess the tail end of the pollen dispersal.

1.2.2.2 Seed Dispersal

Seed dispersal also plays a vital role in ecological and evolutionary dynamics. It can influence the structure of plant populations through the spatial arrangement of individuals across the landscape, maintaining genetic diversity/connectivity and facilitating range expansions (Bowman et al. 2002; Trakhtenbrot et al. 2005). In plants, dispersal occurs through the movement of seeds away from the parent plant to a location where the seed is able to germinate and reach reproductive maturity. As plants are sessile, they have developed adaptations to assist in the movement of their seeds through the actions of specific dispersal vectors (Regale 1977; Stiles 1992; Herrera 1995). The foraging behaviour and consumption of fruits and seeds by an array of animal species means that they play a vital role as vectors for the dispersal of seeds in many species (Westcott et al. 2005). The effective dispersal of seeds by animals relies on the quantity of seeds dispersed, the quality of the dispersal site and the distance the seed is able to travel with the fauna species (Wotton and Kelly 2011). In most plant species, the majority of seeds are dispersed relatively short distances from the source, rarely exceeding a few dozen meters (Nathan 2006; 2008; Nathan et al. 2008). Long distance dispersal (LDD) (seed dispersal to a distance of at least 100 times greater than plant height (Nathan et al. 2003)) events are typically rare, yet have important consequences for species range, migration rates, metapopulation dynamics and gene flow (Calviño-Cancela et al. 2006). Despite the importance of seed dispersal, direct measurements of realised long distance dispersal are rare, largely due to the inherent difficulty of quantifying the tail of the dispersal distance curve.
1.2.2.3 Measuring Gene Dispersal

Neutral genetic markers have been widely used to infer patterns of gene dispersal (e.g. Mitchell et al. 2005; Hoebee et al. 2007; Nakanishi et al. 2012) and existing molecular approaches all attempt to reconstruct patterns of parentage in the population; however, they differ in their methodologies for achieving this goal (Jones and Arden 2003). Methods of parentage analysis can be placed into six categories; exclusion, categorical allocation, fractional allocation, full probability parentage analysis, parental reconstruction and sibship reconstruction. Several reviews (Jones and Arden 2003; Jones et al. 2010) have characterised these methods in detail and hence why will not be discussed here. Selecting the appropriate method for parentage analysis is largely dictated by the types of samples obtained from the study system. Factors include: (1) how many of the unknown parents are sampled, (2) are large groups of full or half-sibs sampled and (3) how many parents of each offspring are known a priori. In light of these parameters, categorical allocation is the appropriate method to use for this study and is the most commonly used method of parentage analysis (Jones et al. 2010).

Categorical allocation employs likelihood-based approaches to select the most likely parent from remaining non-excluded candidate parents. This method involves calculating a logarithm of the likelihood ratio (LOD score) by determining the likelihood of an individual (or pair of individuals) being the parent (or parents) of a given offspring divided by the likelihood of these individuals being unrelated (Jones and Arden 2003; Jones et al. 2010). After an exhaustive evaluation of all genetically possible parents, offspring are assigned to the parent (or parental pair) with the highest LOD score. When all parent–offspring relationships show zero likelihood, offspring are unassigned. Contrary to strict exclusion methods, likelihood-based allocation methods usually allow for some degree of transmission errors due to genotype misreading or mutation (Jones and Arden 2003; Jones et al. 2010).

Overall, the recent proliferation of molecular markers (such as microsatellites) has been accompanied by a surge of techniques in parentage analysis in both natural and experimental populations (Jones and Arden 2003). Consequently, the potential for empirical studies of parentage is at an all-time high, but more future studies are needed and
they should combine molecular parentage assignment with the study of ecological processes (Ashley 2010).
Chapter One: General Introduction

1.3 PERSOONIA

1.3.1 Persoonia in Australia

*Persoonia* (Proteaceae) is an endemic Australian genus of ca. 100 species (Weston 2003), with two main centres of radiation: southeastern and southwestern Australia (Weston 2003). *Persoonia* consist of both obligate-seeding (the above ground plants are killed by fire and population persistence relies on successful establishment from long-lived seed bank) and resprouting (plants survive fire and respond from lignotubers, epicormic shoots or rhizomes) life histories (Benson and McDougall 2000). Plant growth-form varies from prostrate shrubs to small trees. The leaves are highly variable in size and shape among species (Weston 2003). Flowers are generally yellow, symmetrical, with recurved tepals and stamens of similar length and a central carpel with a stigma terminating the style, above an ovary containing one or two ovules. The timing of peak flowering is species-dependant, but is generally between late spring and early autumn, although there may be sporadic flowering throughout the year in some species. Once fertilisation has occurred, the fruits mature on the plant until release in the following spring (Weston 2003). The fruit is a fleshy drupe containing a single hard woody stone with one or two seeds. An embryo may contain up to nine cotyledons, making newly emerged seedlings of the species readily identifiable (Weston 2003).

1.3.2 Previous Studies on Persoonia

To date, most studies of *Persoonia* have concentrated on ecological characteristics of members from southeast Australia. For example, Bauer et al. (2004) examined fruit processing, seed viability and dormancy mechanisms of *P. sericea* and *P. virgata* and found that dormancy in these species is primarily due to physical restriction of the embryo by the hard endocarp. McKenna (2007) studied the demographic and ecological indicators of rarity in a suite of obligate-seeding *Persoonia* shrubs in New South Wales, Australia. By making comparisons of common and rare species using a matrix modelling approach, McKenna (2007) was able to highlight differences in the life histories of the common and rare species and subsequently suggest management approaches. Rymer et al. (2005) found that the poorer reproductive success in rare *Persoonia* species was associated with lower pollinator effectiveness, which is exacerbated by frequent fires and introduced honeybees. Additionally, Field et al. (2005) tested the effects of local plant density and plant size on pollinator activity on the endangered plant *P. bargoensis* in two remnant populations. They
found that reliance on pollinators and a preference for outcrossed pollen implies that the observed pollinator behaviour would reduce the reproductive output of the species at low local densities.

Previous molecular investigations on *Persoonia* have been largely confined to members from southeast Australia (e.g. Krauss 1994a; Krauss and Peakall 1998; Krauss 2000a; b; Ayre et al. 2009). These studies were primarily focused on *P. mollis* and include genetic studies of evolutionary pattern and process, role of post-pollination sexual selection and the characterization of realised pollen dispersal. Using allozyme electrophoresis single- and multilocus estimates outcrossing rates for *P. mollis* were consistently equal to or greater than one (i.e. complete outcrossing) and 70% of all pollen received was on average the paternal plant's immediate neighbour (Krauss 1994a). Furthermore, a slight excess of heterozygotes, as revealed by a negative mean fixation index, was found in the seed cohort (Krauss 1994). Using amplified fragment length polymorphism markers, another study on the mating system of *P. mollis* supported high outcrossing rates (98.8%) and realised pollen flow distances showed a leptokurtic distribution (Krauss 2000b). In addition, mean genetic dissimilarity among all seeds was not significantly different from the mean genetic dissimilarity among all adults, which indicates an overall absence of inbreeding (Krauss 2000b). A population genetic study using microsatellite data examined pollen and seed dispersal after fire in *P. mollis* and found no evidence that successive fires produced either a decline in genetic diversity or the genetic differentiation of adult and successive seedling cohorts (pairwise F_{ST} = -0.01 to <0.01) and spatial genetic structure as measured by the S_{p} statistic was found to be low in seedlings ranging from S_{p} = 0.02 to 0.06 (Ayre et al. 2009). Furthermore, assessment of the breeding system of *P. mollis* also indicated that selfing is possible, but that there is preferential outcrossing with an emphasis on flexibility and post-zygotic choice following pre-zygotic "pseudo" self-incompatibility (Krauss 1994a).

There has been limited research into the southwest Australian (SWWA) *Persoonia* species, with the few studies focused on seed dormancy and recruitment failure. For example, germination trials of *P. longifolia* for ecological restoration found that fruits should be buried *in-situ* in forest soil for 18 months immediately following seed fall and treated with gibberellic acid, following exhumation (Norman and Koch 2008). There have also been several studies on recruitment failure and growth rates in *P. elliptica* (Abbott 1984a; 1984b;
Abbott and Van Heurck 1988; Monaco 2012). Fruit production has been found to be low in *P. elliptica* with only 9% of flowers becoming mature fruits (Monaco 2012). In addition, fresh seeds have a high level of viability (Monaco 2012), but germination could not be induced in the laboratory or forest even after physical and chemical treatments (Abbott 1984a; 1984b). In the forest, the passage of low intensity fire over leaf litter is known to cause abundant germination of dispersed seeds, but few seedlings survived due to browsing by vertebrates, assumed to be kangaroos and wallabies (Abbott and Van Heurck 1988). For example, a recent study (Monaco 2012) confirmed that macropods exhibit a strong browsing preference for *P. elliptica* when presented with fresh foliage, and as such are also likely to consume seedlings. This level of browsing pressure is thought to be a potential cause of recruitment failure (Monaco 2012). Furthermore, height growth of seedlings and lignotuberous seedlings and the diameter growth of trees has found to be slow where stem diameter at breadth height of 10 cm is attained. On average, recruitment of *P. elliptica* over much of the northern jarrah forest ceased around ca 1900 (Abbott and Van Huerck 1988). There has also been an investigation on leaf and androecium evolution of *P. elliptica* which indicate that it shows characteristics of an Australian rainforest relict plant (Melville 1975).

To date, there has been one study using molecular ecology methods in SWWA *Persoonia*. Stingemore and Krauss (2012) addressed how distance from source population of seed affected restoration success in *P. longifolia*. Using 66 polymorphic amplified fragment length polymorphism (AFLP) markers genetic variation within, and differentiation among 12 potential seed source populations was assessed. Results showed that 92% of the total genetic variation was within and 8% among populations, indicating relatively weak but statistically significant population genetic differentiation. Ordination of genetic data showed marked west/east and north/south gradients. They also found that pairwise population genetic dissimilarity was correlated with both geographic distance and environmental distance derived from five climate variables. However, partial Mantel tests showed that the relationship between genetic and geographic distance was not independent of environmental distance, suggesting a non-neutral signature in these markers. Bayesian outlier analysis identified two markers, and spatial analysis method tests identified highly significant associations between these two markers and three environmental variables. Frequency differences at these markers across populations suggested the possibility of climatically adapted provenances. Overall, there is limited empirical knowledge on the
Chapter One: General Introduction

ecological determinants of genetic variation and its structuring for the *Persoonia* genus, and for SWWA plant species in general.

SWWA is home to one of the global biodiversity hot spots - the Southwest Australian Floristic Region (SWAFR) (Myers et al. 2000). The SWAFR occupies 302, 627 km$^2$ on a temperate margin of the world’s most arid and insular populated continent. It contains almost 6,000 plant species of which 79% are endemic (Hopper and Goia 2004). The SWAFR is topographically unique among the world’s five regions of Mediterranean climate (Hopper 1979; Cowling et al. 1996; Dallman 1998), being a flat, stable, highly weathered, low plateau dominated by old landscapes with nutrient-deficient soils (Hopper et al. 1996a). Furthermore, it has been hypothesized that natural selection has resulted in mechanisms promoting local persistence rather than wide dispersal (Hopper et al. 1996; Hopper 1997; 2000). Given the unique characteristics of SWWA, research in this region is critical to provide important empirical information into ecological determinants of genetic variation and its structuring that underpin the conservation and management of its biota.
1.4 STUDY SPECIES

1.4.1 *Persoonia elliptica* R.Br.

*Persoonia elliptica* R.Br (spreading snottygobble) is an erect spreading shrub or tree (2 – 8 m), with a hard corky grey bark, which survives fire by resprouting (Bell et al. 1993; Florabase 2013). *Persoonia elliptica* occurs largely as well-separated trees in populations of very low densities. It occurs within 50 km of the coast, from Perth to Albany in SWWA jarrah forest, in sandy soil or laterite (Figure 1.1). Leaves are dark green, flat, hairless and are obovate to elliptic (30-110 mm long and 10-50 mm wide) (Figure 1.2). *Persoonia elliptica* flowers from October to February, with yellow green flowers that are hermaphroditic and generally comprise four symmetrical, recurved yellow tepals that are 8 – 12.5 mm long and glabrous to sparsely hairy on the outside (Florabase 2013) (Figure 1.2).

To date, the mating system is largely unknown for this species, but in general *Persoonia* species are preferentially outcrossing (Krauss 1994) and are pollinated by native bees (e.g. species of *Amegilla*, *Megachilidae* and *Leioproctus*; Bernhardt and Weston 1996). The introduced European honeybee (*Apis mellifera*) is also a frequent visitor but it is still unclear whether this species is an efficient pollinator. Once fertilisation has occurred, the fruits remain in the canopy until release in August to November (Weston 2003). Fruits are drupes, oval in shape and change from bright green to deep purple in colour when ripe. The fleshy fruits are clearly adapted for animal dispersal but it is not known whether mammals (i.e. macropods) or birds (i.e. emus) are the most important dispersers (Bernhardt and Weston 1996). Emu scat can often consist almost entirely of *Persoonia* seed during the period of fruit fall and these birds are thought to have an important role as dispersal vectors (Vigilante 1996). Trees can produce large quantities of fruit and viability of the freshly fallen fruit is also high (Abbott 1984; Monaco 2012). The fruit is edible by humans (Daw et al. 1997) and has been a food source for Aboriginal people (Mullins et al. 2002).

There is an absence of seedlings and/or saplings in *P. elliptica* populations and there is little evidence for recruitment since around 1900 (Abbott and Van Heurck 1988). *Persoonia elliptica*’s slow growth rate can leave seedlings and saplings within a susceptible zone of browsing pressure for an extended period of time and is speculated to lead to recruitment failure (Abbott and Van Heurck 1988; Monaco 2012).
Figure 1.1: Natural distribution of *Persoonia elliptica* in Western Australia. Maps sourced from Nature Map, Department of Environment and Conservation.
Chapter One: General Introduction

Figure 1.2: Photographs of a) *Persoonia elliptica* tree in its natural habitat, b) bark characteristics, c) leaf shape, d) c.a. thirty-year old *P. elliptica*, e) seedling, f) *P. elliptica* tree in its natural habitat, g) fruit fall and h) introduced honeybee (*Apis mellifera*) collecting pollen from flower. All photographs taken by author.
1.4.2 Persoonia longifolia R.Br.

Persoonia longifolia R.Br. (snottygobble) is an erect shrub or tree (1 – 5 m high) that survives fire by resprouting and has flaky reddish papery layered bark (Bell et al. 1993; Florabase 2013). Persoonia longifolia occurs in more-or-less continuous populations of moderate sizes and relatively high densities (up to ca. 300 plants/ha). It is most common in gravelly soils of upland jarrah forests in SWWA (Havel 1975) but is distributed as far south as Albany on the south coast of Western Australia (Marchant et al. 1987) (Figure 1.3). Persoonia longifolia has distinctive light green leaves, that are linear and somewhat curved (70-200 mm long and 2-10 mm wide), flat, without prominently raised marginal ribs, hairless and more or less pointed (Florabase 2013) (Figure 1.4). Flowers are hermaphroditic, yellow to orange and occur mostly in November to February; floral segments are free, all similar narrowly elliptic, 10-16 mm long, with appressed brownish hairs; anther cells are linear, attached just below middle of floral segments, often becoming recurved (Figure 1.4).

P. longifolia is reported to share pollen and seed dispersal vectors with P. elliptica, (Abbott 1984a), however fruits are round and fruit fall is from June to August. Persoonia longifolia also produces large quantities of fruit and viability of the freshly fallen fruits, which have been known to be eaten by humans (Daw et al. 1997), is also high (Abbott 1984a).

In contrast to P. elliptica, seedlings and saplings of P. longifolia can be found throughout its natural range (pers. obs) and it has a growth rate of ca. 5 cm per year (Chia unpublished). The size of the tree, the attractive drooping foliage and reddish flaky bark make the species important for the horticultural industry where it has potential as a garden plant. The foliage is currently harvested for the cut flower trade (Mullins et al. 2002).
Figure 1.3: Left to right, natural distribution of *Persoonia longifolia* in Western Australia. Maps sourced from Nature Map, Department of Environment and Conservation.
Figure 1.4: Photographs of a) *Persoonia longifolia* in natural habitat, b) unripe fruit, c) flowers, d) bark characteristics, e) leaf shape, f) *P. longifolia* resprouting after fire, g) seedling and h) *P. longifolia* occurring near a mine site. All photographs taken by author.
Chapter One: General Introduction

1.5 THESIS AIMS AND OUTLINE

This study will contribute new knowledge toward an understanding of key drivers of population genetic variation and its structuring by assessing the interactions between density, pollen dispersal and seed dispersal within two co-occurring species with markedly different population densities. The core objective of this thesis is to provide the disciplines of population genetics, conservation genetics, restoration ecology and evolutionary biology with a better understanding of the role and consequences of key processes affecting genetic variation and its structuring.

The objective in Chapter Two is the isolation and characterisation of microsatellite markers for *Persoonia elliptica* and *P. longifolia*. Microsatellite markers developed for the southeast Australian *P. mollis* are initially screened for amplification in individuals of both *P. elliptica* and *P. longifolia*. Following this, microsatellite markers developed for *P. elliptica* from microsatellite enriched libraries are tested for cross amplification in *P. longifolia*. In addition, microsatellite markers developed for *P. longifolia* by 454 pyrosequencing are tested for cross amplification in *P. elliptica*. The microsatellites identified in this study will enable the examination of population and spatial structuring of genetic diversity and pollen and seed dispersal characteristics in *P. elliptica* and *P. longifolia*.

Chapter Three utilises these microsatellite markers to quantify genetic variation and its structuring for 20 and 22 (respectively) populations across the natural ranges of *P. elliptica* and *P. longifolia*. Density for each population of *P. longifolia* is calculated using the point-quadrat method, whereas populations of *P. elliptica* are exhaustively sampled and absolute population density determined at each population. This information is used to test the hypotheses that plant density will affect levels of genetic diversity, inbreeding, as well as within and among population genetic structure.

Chapter Four utilises a large, geographically isolated population of the insect-pollinated *P. elliptica* to characterise pollen dispersal patterns. Every *P. elliptica* tree (n = 450) in a ca. 1,300 ha study plot, together with canopy seed collected from ten maternal trees over two flowering seasons, is mapped and genotyped. Paternity assignment methods are employed to test the hypotheses that low plant density promotes high multiple paternity and a
departure from nearest neighbour mating and also whether the spatial position of a plant within a population affects genetic diversity and inbreeding rates.

Chapter Five further addresses gene flow in the same study plot of *P. elliptica* and employs motion sensor cameras to test the hypotheses that *Dromaius novaehollandiae* (emus) are a major seed dispersal vector for *P. elliptica*. Once identified, scat belonging to key dispersal vectors is systematically searched for *P. elliptica* seed, which are then mapped and genotyped. Parentage assignment is then used to infer dispersal distance of that seed and from that generate a seed dispersal distribution that characterises the scale and nature of long distance seed dispersal. The hypothesis that LDD away from parent plants will reflect high mobility and movement patterns of dispersal vectors is also tested.

Chapter Six offers a general summary of the knowledge gained from this thesis and addresses potential new research questions. Future directions that have arisen from this work are also discussed.
2.1 ABSTRACT

Microsatellite markers were developed and cross-species transferability assessed for *Persoonia elliptica* and *P. longifolia*, two broadly distributed southwest Australian tree species. Microsatellite enriched libraries and 454 GS-FLX shotgun sequencing were used to identity nine microsatellite loci for *P. elliptica* (one 454; eight cloning) and six loci for *P. longifolia* (three 454; three cloning). These loci were screened for variation in individuals from two study populations in southwest Australia. In *P. elliptica*, between 3 and 14 alleles per locus were found and observed and expected heterozygosities ranged from 0.46 - 0.93 and 0.42 - 0.88, respectively. For *P. longifolia*, between 2 and 13 alleles per locus were found and observed and expected heterozygosities ranged from 0.04 - 0.88 and 0.04 - 0.84, respectively. The microsatellites identified in this study will enable the examination of population and spatial structuring of genetic diversity and pollen and seed dispersal characteristics in *P. elliptica* and *P. longifolia*. 
Chapter Two: Microsatellite Development

2.2 INTRODUCTION

Much research to date has been dedicated to describing and quantifying population genetic divergence within and among species, through the use of molecular markers such as microsatellites (also known as simple sequence repeats), AFLPs (amplified fragment length polymorphisms), allozymes and RAPDs (random amplified polymorphic DNAs) (van Tienderen et al. 2002; Holderegger et al. 2006; Meudth and Clarke 2007). Microsatellites are commonly used in studies of biodiversity and the investigation of within versus among population genetic diversity and have become the marker of choice for ecological and evolutionary studies from the individual to the population level (Sunnucks 2000). Traditionally, microsatellites are identified and isolated from size-selected or enriched genomic libraries of the study species, by screening several thousand clones through hybridization with microsatellite probes (Grover et al. 2011). However, such methods yield only a fractional representation of the genomic microsatellites and are biased towards particular motifs used for screening (Gardener et al. 2011). With the revolution in sequencing technologies, it has now become feasible to screen the entire genome(s) for the presence of microsatellites even in the case of non-model species (Davey et al. 2011). One such method that has found to be extremely successful in marker development is 454 GS-FLX shotgun sequencing.

The 454 shotgun sequencing method has several advantages over enrichment methods for microsatellite development. Firstly, there is no need to decide a priori what motif types (e.g. AC, AT, AG, CG) to enrich for (Gardener et al. 2011). Second, a potential problem inherent with enrichment methods is the use of restriction enzymes to cut the DNA into fragments of manageable size. If the particular enzyme cuts within transposable elements, there is the potential for over-representation of fragments that contain other classes of repetitive elements. The identified microsatellite loci may be associated with these repetitive sequences as mobile elements have been implicated in the generation of microsatellites, confounding the use of the recovered loci (Gardner et al. 2011). Third, there is no need to edit sequence chromatograms, when using the 454 pyrosequencing method. Finally, large numbers of sequences can be obtained at significantly reduced prices and in less time than with traditional approaches. For these reasons, 454 pyrosequencing is in the process of taking over from enrichment as the preferred method for developing microsatellites.
Chapter Two: Microsatellite Development

The genus *Persoonia* is an endemic Australian genus of ca. 100 species (Weston 2003), with two main centres of radiation: southeastern and southwestern Australia. Many of these species have high conservation significance and are important key components in ecological restoration activities. *Persoonia elliptica* R.Br. and *P. longifolia* R.Br. are widespread, small trees within the southwest Australian (SWWA) jarrah forest. These two sympatric congeners are key components of the jarrah forest and share pollinators and seed dispersers, but contrast markedly in their population densities. To date, no genetic markers have been developed for SWWA *Persoonia* species. While nuclear microsatellite markers have been developed for ecological studies on the southeast Australian species *P. mollis* (Ayre et al. 2009), evolutionary distance between the two centres of radiation indicates marker transfer is unlikely to be successful. This makes the identification of suitable molecular makers in SWWA *Persoonia* species important for both biologists and resource managers.

Given the increased use of 454 pyrosequencing to develop microsatellites for non-model organisms compared to microsatellite enriched libraries, the Biodiversity and Conservation Centre, Botanic Gardens and Parks Authority was interested in investigating a change of development approach. Thus this study reports on the isolation and characterisation of microsatellite markers for *P. elliptica* and *P. longifolia* using microsatellite enriched libraries and 454 GS-FLX shotgun sequencing, with the specific intent to:

- Test microsatellite primers developed for *P. mollis* for cross amplification in *P. elliptica* and *P. longifolia*.
- Develop microsatellites using microsatellite enriched libraries for *P. elliptica* and test for cross amplification in *P. longifolia*.
- Develop microsatellites using 454 pyrosequencing for *P. longifolia* and test for cross amplification in *P. elliptica*. 
2.3 METHODS AND MATERIALS

2.3.1 DNA Extraction

Fresh leaf material was collected from two populations of *P. elliptica* (Pe-AE -31.572E 116.181S and Pe-AW -31.613E 116.151S) and two populations of *P. longifolia* plants (Pl-MD -32.176E 116.245S and Pl-CE -33.356E 116.345S). The fresh leaf material was stored at -80°C until DNA extraction. Nuclear DNA was extracted using a procedure modified from Jobes et al. (1995). Approximately 1 - 2 g of leaves were frozen in liquid nitrogen and ground to fine powder using a mortar and pestle. The powder was mixed in a 10 mL tube with 5 mL pre-heated Jobes buffer and 4 µL of proteinase-K and incubated at 55°C for one hour. 900 µL of 20% sodium dodecyl sulfate and 100 µL of deionised water were then added and the samples were again incubated at 55°C for one hour. After centrifugation at 12,000 rpm for 10 minutes at room temperature, the top layer was transferred to a new 10 mL tube and purified by mixing with 2 mL of cold 5M potassium acetate. Following storage at -20°C for 20 minutes, then centrifugation at 12,000 rpm for 10 minutes at 4°C, the aqueous phase was collected. DNA was then precipitated using 4 mL of cold isopropanol, mixed gently for five minutes and then centrifuged at 12,000 rpm for 10 minutes at room temperature. The supernatant was poured off and the resultant pellet was purified in 1 mL of deionised water. 0.5 mL of 4M sodium chloride was added to the sample and mixed well. Then 3 mL of cold 95% ethanol was added, the sample gently mixed for five minutes and then centrifuged at 12,000 rpm for 10 minutes at room temperature to collect nucleic acids. The pellet was dissolved in 500 µL of deionised water and transferred to a 1.5 mL tube. RNA was then precipitated with one third volume of 8M lithium chloride and incubated at 4°C overnight. The RNA was recovered by centrifugation at 13,000 rpm for fifteen minutes and the supernatant was transferred to a new 1.5 mL tube. Contaminating proteins were removed by two sequential extractions with phenol chloroform, followed by one extraction with chloroform to remove trace amounts of phenol and proteins. DNA was precipitated by adding cold 95% ethanol, mixed gently for five minutes and then centrifuged at 13,000 rpm for fifteen minutes. The pellet was washed with 70% ethanol and centrifuged at 13,000 rpm for five minutes. The ethanol was then poured off and the pellet was left to dry. Once dry, the pellet was then dissolved in 200 µL of low TE buffer and stored at -80°C. Genomic DNA from silica dried *P. mollis* samples from The Royal Botanic Gardens, Sydney, was extracted using the same procedure.
2.3.2 Cross Amplification from P. mollis Microsatellites

Twelve microsatellite primers developed for P. mollis (Ayre et al. 2009) were screened for amplification in eight individuals of both P. elliptica and P. longifolia, plus seven P. mollis samples and a negative control. Amplification of the microsatellite loci was carried out in a total volume of 10 µL, containing approximately 10 ng genomic DNA template, 1x PCR Polymerization Buffer containing dNTPs (Fisher Biotech), 0.2 µM each of unlabelled forward and reverse primer (GeneWorks), 0.5 Units Taq DNA polymerase (Fisher Biotech, Australia) and 2 mM of MgCl₂ (Fisher Biotech). PCR was carried out with an initial activation step at 95°C for 15 minutes; followed by 35 cycles of: 95°C for 30 seconds; annealing at 59°C for 90 seconds; extension at 72°C for 90 seconds; followed by a final extension at 72°C for 15 minutes. PCR products were separated on a 2% agarose gel stained with SYBR safe (Invitrogen Corporation) and fragment sizes determined by comparison to a Low DNA Mass Ladder (Invitrogen).

2.3.3 Isolation and Characterisation of Microsatellite Markers

2.3.3.1 Microsatellite Enriched Libraries

Genetic Identification Services, California, USA (http://www.genetic-id-services.com/) was employed to develop microsatellite enriched libraries for P. elliptica for four different repeat motifs (CAₙ, GAₙ, ACCₙ and ATGₙ). Briefly, genomic DNA was restricted with seven blunt-end cutting enzymes (RsaI, HaeIII, BsrB1, PvuII, StuI, ScaI and EcoRV). Fragments in the size range of 300-750 bp were linker adapted with oligonucleoties that contained a HindIII site and then subjected to magnetic bead capture (CPG Inc.) Molecules were restricted with HindIII and ligated into the HindIII site of the pUC19 plasmid. Ligation products were introduced into E. coli strain DH5 alpha (ElectroMax, Invitrogen) by electroporation. Blue-white selection was used to identify recombinant clones for sequencing on an ABI 377 (Applied Biosystems) using Amersham’s DYEnamic Terminator Cycle Sequencing Kit (Amersham Biosciences).

One hundred and six clones were sequenced, including 22 from the CA library, 22 from the GA library, 20 from the AAC library and 22 from the ATG library. Eighty-two different microsatellite containing clones were identified from the four libraries and primers were designed for 72 sequences using DesignerPCR version 1.03 (Research Genetics Inc.) and synthesized for 24. These primer pairs were initially tested to verify amplification,
determine the optimum annealing temperature and to establish size ranges for later polymerase chain reaction (PCR) multiplexing, using DNA from eight individuals of *P. elliptica*. PCR was carried out in a total volume of 10 µL, containing approximately 10 ng genomic DNA template, 1x PCR Polymerization Buffer containing dNTPs (Fisher Biotech), 0.2 µM each of unlabelled forward and reverse primer (GeneWorks), 0.5 Units *Taq* DNA polymerase (Fisher Biotech, Australia) and 2 mM of MgCl$_2$ (Fisher Biotech). PCR was carried out in a Veriti 96 Well Thermal Cycler (Applied Biosystems) using the following reaction conditions: an initial activation step at 95ºC for 15 minutes, followed by 35 cycles of 95ºC for 30 seconds, annealing at 59ºC for 90 seconds; extension at 72ºC for 90 seconds, followed by a final extension at 72ºC for 15 minutes. PCR products were separated on a 2% agarose gel stained with SYBR safe (Invitrogen Corporation) and fragment sizes determined by comparison to a Low DNA Mass Ladder (Invitrogen).

The nine loci that amplified successfully were then screened on eight individuals of *P. elliptica* to test for polymorphism and trial multiplexing groups. Multiplexing was performed on four groups of primers (Table 2.1) using the QIAGEN Multiplex Kit (QIAGEN) in 12.5 µL reaction volumes containing 5-30 ng DNA template, 6.25 µL QIAGEN Multiplex PCR Master Mix, 1.25 µL Q solution, 0.1 µM of each forward primer (labelled; unique to primer), 0.1 µM of each reverse primer (unlabelled) and sterile H$_2$O to 12.5 µL. The multiplex PCR was conducted in a Veriti 96 Well Thermocycler (Applied Biosystems) with the same PCR cycle as listed above. Following PCR, samples were diluted 1:30 in sample loading solution (Beckman Coulter) with the addition of 0.4 µL fluorescently labelled 400 bp size standard per sample (Beckman Coulter) for capillary electrophoresis on a CEQ 8800 Genetic Analysis System (Beckman Coulter). Fragment peaks were visualized using CEQ Genetic Analysis System software (Beckman Coulter) and fragment (allele) sizes were scored manually. Eight individuals of *P. longifolia* were also genotyped using the above protocol, to test for cross species amplification for these nine loci.

### 2.3.3.2 454 GS-FLX Shotgun Sequencing

Genomic DNA of *P. longifolia* was sent to the Australian Genomic Research Facility (AGRF), Adelaide, Australia for shotgun sequencing on a Titanium GS-FLX (454 Life Sciences/Roche FLX), following Gardner et al. (2011). The sample occupied 6.25 % of a
plate and produced 108,806 individual sequences, with an average read length of 367 bp. The average GC content of these data was 37.82%. The program QDD v. 1 (Meglécz et al. 2010) was used to screen the raw sequences for ≥ eight di-, tri-, tetra- or penta-base repeats, remove redundant sequences and design primers using Primer3 (Rozen and Skaletsky 2000). Software running parameters were set to default values, except PCR product lengths, which were set to 80 - 480 base pairs. 14.2 % of all reads were identified containing microsatellite loci. Dinucleotide motifs were the most frequent (79,604) followed by tri-, tetra- and then penta- nucleotide motifs (14,550, 1,072 and 445 respectively) (Meglécz et al. 2012). Primer pairs were designed for 108 loci and from these, loci were excluded that contained imperfect repeats, had a > 2 °C difference between the forward and reverse primer annealing temperature, short repeat motifs within the flanking region or primer sequence or had poly A/T runs of more than seven base pairs as there is an association between a high degree of poly A/T and instability (Li et al. 2002).

From the remainder, 28 loci were arbitrarily selected and then guidelines from Gardner et al. (2011) were followed for further development and initially, the loci were trialled for amplification. Initial PCR amplification was carried using the same method as for cloned microsatellites, mentioned above.

The 12 loci that amplified successfully were then screened on eight individuals of *P. longifolia* to test for polymorphism and trial multiplexing groups. Multiplexing was performed on four groups of primers (Table 2.1) using the QIAGEN Multiplex Kit in 12.5 µL reaction volumes containing 5-30 ng DNA template, 6.25 µL QIAGEN Multiplex PCR Master Mix, 1.25 µL Q solution, 0.1µM of each forward primer (labelled; unique to primer), 0.1µM of each reverse primer (unlabelled) and sterile H₂O to 12.5 µL. The multiplex PCR was conducted in a Veriti 96 Well Thermocycler (Applied Biosystems) with an initial activation step at 95°C for five minutes, followed by nine cycles of: 95°C for 30 seconds, a 1°C touchdown starting at 65°C for 180 seconds and 72°C for 15 seconds, this was followed by 25 cycles at 95°C for 30 seconds, 56°C for 180 seconds and 72°C for 15 seconds and then a final extension at 60°C for 30 minutes. Capillary electrophoresis and fragment scoring was carried out using the same method as for cloned microsatellites, mentioned above. Eight individuals of *P. elliptica* were also genotyped using the above protocol, to test for cross species amplification for these 12 loci.
2.3.4 Genetic Diversity

Fifteen plus individuals from the two populations each of *P. elliptica* and *P. longifolia* were then genotyped using loci, which were both polymorphic and amplified reliably from the previous screening. Genetic diversity parameters and deviation from Hardy-Weinberg Equilibrium (HWE) were calculated using GenAlEx version 6.5 (Peakall and Smouse 2006; 2012). MICROCHECKER 2.2.3 (van Oosterhout et al. 2004) was used to check each locus for evidence of null alleles, scoring error due to stuttering and large allele drop out.
2.4 RESULTS

2.4.1 Cross Amplification from P. mollis Microsatellites

The cross amplification of primers developed for P. mollis primers was unsuccessful for both study species, with ten of the primers not amplifying and two not amplifying reliably.

2.4.2 Isolation and Characterisation of Microsatellite Markers

One of the nine loci isolated from microsatellite enriched libraries for P. elliptica did not amplify consistently in the target species (Table 2.1). Six out of 12 loci isolated from 454 pyrosequencing for P. longifolia were monomorphic and three did not amplify reliably (Table 2.1). Cross species amplification to P. longifolia from microsatellites developed via enriched libraries was successful for three loci (Table 2.2). Cross species amplification to P. elliptica from microsatellites developed via 454 pyrosequencing was successful at one locus (Table 2.1).

2.4.3 Genetic Diversity

Genetic diversity parameters and deviation from HWE were calculated using the nine (P. elliptica) and six (P. longifolia) loci. In P. elliptica, between 3 and 14 alleles per locus were found. Observed ($H_O$) and expected ($H_E$) heterozygosities ranged from 0.46 to 0.93 and 0.42 to 0.88, respectively (Table 2.2). For P. longifolia, between 2 and 13 alleles per locus were found. Observed ($H_O$) and expected ($H_E$) heterozygosities ranged from 0.04 to 0.88 and 0.04 to 0.84, respectively (Table 2.2). Significant departure from HWE was detected in four of the nine loci for P. elliptica and three of the six loci for P. longifolia (Table 2.2). No loci showed significant null allele frequencies, large allele drop out or evidence of scoring error due to stuttering.
Table 2.1: Microsatellite primers developed in *Persoonia elliptica* (listed first where relevant) and *P. longifolia*. Shown for each primer are locus name, development method, forward primer sequence, reverse primer sequence, repeat motif, size range, WellRED dye label (multiplex marker set) and GenBank Accession Number.

<table>
<thead>
<tr>
<th>Locus name</th>
<th>Method</th>
<th>Primer sequences (5’ to 3’)</th>
<th>Repeat motif</th>
<th>Size range (bp)</th>
<th>Dye label</th>
<th>Genbank Acc. No</th>
</tr>
</thead>
<tbody>
<tr>
<td>PeA102</td>
<td>GIS</td>
<td>F: ACCCACCAGCTAACTCTTTTG</td>
<td>(CA)$_{14}$</td>
<td>94-138</td>
<td>D3 (3)</td>
<td>JX987704</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: CCATCTGATCGTACCAACGTA</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PeA103</td>
<td>GIS</td>
<td>F: CCTAATAACCCCACCTACTAC</td>
<td>(GT)$_{15}$</td>
<td>243-299; 243-289</td>
<td>D3 (2)</td>
<td>JX987705</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: AGGTCCTTTCCACCTTTTGAT</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PeA106</td>
<td>GIS</td>
<td>F: TCCAAGTGATGCACCACCCTC</td>
<td>(GC)$<em>{3}$(AC)$</em>{15}$</td>
<td>179-187; 167-169</td>
<td>D2 (1)</td>
<td>JX987706</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: TCTTGGGATCGGATGCACCACG</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PeB103</td>
<td>GIS</td>
<td>F: AATGGGGGTTGTGCTGTCTC</td>
<td>(GA)$_{19}$</td>
<td>272-314; 270-282</td>
<td>D4 (2)</td>
<td>JX987707</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: TCCCATCTCTTTCCACACCC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PeB106</td>
<td>GIS</td>
<td>F: TGCTTTCTTGATGCAAGAAGAG</td>
<td>(GTT)$_{9}$</td>
<td>164-226</td>
<td>D2 (3)</td>
<td>JX987708</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: CTTCTAAGGGAGAGCTACAGAG</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PeB2</td>
<td>GIS</td>
<td>F: TTCGCTTTCCTTTTGAAAGAG</td>
<td>(CT)$_{19}$</td>
<td>285-313</td>
<td>D4 (1)</td>
<td>JX987709</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: AGGGAGGATCGACAGGACAGGAC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PeC104</td>
<td>GIS</td>
<td>F: CTTGAGCATGAGCTGAGATC</td>
<td>(GTT)$_{9}$</td>
<td>146-168</td>
<td>D4 (1)</td>
<td>JX987710</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: ATCAACCATGAGGAGAAGAGAGAG</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PeC2</td>
<td>GIS</td>
<td>F: GATCCCTAGGTTGGTTTCTTG</td>
<td>(GTT)$_{10}$</td>
<td>235-267</td>
<td>D3 (1)</td>
<td>JX987711</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: TGCCCTATCAAGCATAGTTATG</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pl13</td>
<td>454</td>
<td>F: GAGGCGATATAGGTTGGTGA</td>
<td>(AT)$_{8}$</td>
<td>na; 110-132</td>
<td>D2 (5)</td>
<td>JX987701</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: CACAGGATATGAGCTGCGCTC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pl15</td>
<td>454</td>
<td>F: ATAATGGGCCCTAATGATGCG</td>
<td>(GA)$_{9}$</td>
<td>145-163; 135-181</td>
<td>D3 (3)</td>
<td>JX987702</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: ACTCCCTAGGCTATGCTC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pl18</td>
<td>454</td>
<td>F: TGGACACTTCTAGGAGCTAACA</td>
<td>(GA)$_{15}$</td>
<td>na; 188-206</td>
<td>D2 (5)</td>
<td>JX987703</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: GGGAGGCTATCCACTCTTTG</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 2.2: Results of primer screening in two populations each of *Persoonia elliptica* and *P. longifolia*. For each primer pair the locus name, number of individuals sampled (n), number of alleles ($N_A$), observed ($H_O$) and expected heterozygosity ($H_E$) and deviation from Hardy Weinberg equilibrium (HWE) (*$P<0.05$; **$P<0.01$; ***$P<0.001$; ns. = not significant) are given. All values are based on samples from populations in southwest Australia (*P. elliptica*: Pe-AE -31.644E 116.297S, Pe-AW -31.676E 116.180S and *P. longifolia*: Pl-MD -32.176E 116.245S, Pl-CE -33.356E 116.345S).

<table>
<thead>
<tr>
<th>Locus</th>
<th>Pe-AW (n = 15)</th>
<th>Pe-AN (n =15)</th>
<th>Pl-MD (n = 30)</th>
<th>Pl-CE (n = 25)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$N_A$</td>
<td>$H_O$</td>
<td>$H_E$</td>
<td>HWE</td>
</tr>
<tr>
<td>PeA102</td>
<td>10</td>
<td>0.46</td>
<td>0.84</td>
<td>*</td>
</tr>
<tr>
<td>PeA103</td>
<td>7</td>
<td>0.80</td>
<td>0.78</td>
<td>*</td>
</tr>
<tr>
<td>PeA106</td>
<td>3</td>
<td>0.47</td>
<td>0.42</td>
<td>ns</td>
</tr>
<tr>
<td>PeB103</td>
<td>14</td>
<td>0.87</td>
<td>0.86</td>
<td>ns</td>
</tr>
<tr>
<td>PeB106</td>
<td>13</td>
<td>0.69</td>
<td>0.84</td>
<td>**</td>
</tr>
<tr>
<td>PeB2</td>
<td>8</td>
<td>0.93</td>
<td>0.84</td>
<td>ns</td>
</tr>
<tr>
<td>PeC104</td>
<td>4</td>
<td>0.73</td>
<td>0.66</td>
<td>ns</td>
</tr>
<tr>
<td>PeC2</td>
<td>6</td>
<td>0.93</td>
<td>0.78</td>
<td>*</td>
</tr>
<tr>
<td>Pl13</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Pl15</td>
<td>10</td>
<td>0.86</td>
<td>0.85</td>
<td>ns</td>
</tr>
<tr>
<td>Pl18</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
Chapter Two: Microsatellite Development

2.5 DISCUSSION

The microsatellite loci developed in this study will be useful for the examination of levels and spatial structuring of genetic diversity and mating systems in *P. elliptica* and *P. longifolia*. Furthermore, the cross-species transferability of simple sequence repeats supports the suggestion that based on morphological similarity and life history traits, they are closely related species.

The effort required in developing microsatellite markers has long been an important consideration, especially for low budget studies on non-model organisms (Squirrell et al. 2003). Next Generation Sequencing (NGS) techniques such as 454 pyrosequencing have now been widely used for the development of microsatellite markers more rapidly and cheaply than traditional cloning approaches (Gardner et al. 2011; Malausa et al. 2011).

These results have demonstrated that although there were efficiencies to be gained from employing NGS for microsatellite marker development, it is not a panacea and it can require a significant investment in time and effort in finding, screening and optimizing amplification of polymorphic microsatellite markers. Indeed, when post-454-sequencing demands on time are factored into an assessment of microsatellite marker development, the experience from this research was that efficiencies and success of 454 over traditional cloning approaches were minimal. While unable to identify the cause of difficulties, elsewhere it has not worked well due to poor read quality (Harismendy et al 2009; Nui et al 2010). The paucity of published failures, however, makes it difficult to understand the extent and causes of when difficulties have been encountered in the development of microsatellite markers from 454 pyrosequencing. A significant investment in time and effort in the screening, and optimization of polymorphic microsatellite markers is still required with this relatively new approach to marker development.
CHAPTER THREE

Plant density effects on genetic variation and its spatial structuring in two co-occurring *Persoonia* species

3.1 ABSTRACT

An understanding of ecological and life history traits and their effects on genetic patterns and processes, is a core objective of molecular ecology. Numerous reviews have identified that plant density can effect genetic variation both within and among populations. In this study, the effects of plant density on genetic variation and its structuring were evaluated using six microsatellite markers for two co-occurring *Persoonia* congers. *Persoonia longifolia* and *P. elliptica* occur in the jarrah forest of southwest Australia and have densities that are typically three orders of magnitude different, yet near identical life-history parameters. Despite the marked contrast in density, the two species were found to have similarly high genetic diversity. This study did find a significant relationship between density and both the number of alleles and fixation indices. These relationships were driven by very low density *P. elliptica* populations, indicating that density begins to have an effect on number of alleles and fixation indices below a threshold of ca. 0.01 plants per ha. Below this density threshold the number alleles decreased, however fixation indices showed a heterozygote excess. While both species displayed moderate genetic differentiation among geographically proximate populations, the high density species (*P. longifolia*) displayed significantly stronger within population genetic structure. On average, *P. longifolia* populations displayed significant positive genetic structure at inter plant distances of up to 25 m. Overall, it seems that the retention of historical genetic patterns and/or sufficient gene flow between populations are negating negative effects often associated with low population density.
Chapter Three: Density Effects

3.2 INTRODUCTION

Genetic variation is the raw material for evolutionary change within plant and animal populations. Genetic variation results from the combined action of mating, mutation, migration, selection and drift, which, in turn, operate within the historical and biological framework of each species (Loveless and Hamrick 1984). The amount of genetic variation, and its spatial structure, is especially affected by the biological characteristics of a species (Hamrick and Godt 1996). For plants, life history traits such as pollination mechanisms, seed dispersal and longevity, among other traits, including geographic range and plant density, have an impact on genetic variation.

A core objective of molecular ecology is to understand the role, impact and consequences of life history and ecological traits on the levels and structuring of population genetic variation. Consequently, there exists an extensive literature relating to the correlation between selected ecological attributes of species and the level and apportionment of genetic diversity within and between populations. Literature reviews (Hamrick et al. 1979; Loveless and Hamrick 1984; Hamrick et al. 1989; Hamrick and Godt 1989; 1996) have identified density as an ecological parameter that affects the levels and partitioning of genetic variation in plants. Density is the number of individuals per unit of area. In a biological sense, density can be described as the spacing between neighbouring individuals of the same species (Kunin 1997).

Plant density is known to affect mating systems because of the density-dependent changes in pollinator behaviour (Antonovics and Levin 1980; Friedman and Barrett 2008) for animal-pollinated plant species. For example, optimal foraging theory predicts that in sparse patches, pollinators prefer to visit more flowers in sequence on individual plants, which may enhance geitonogamous selfing in self-compatible species (Bosch and Waser 2001). Most previous studies have confirmed this prediction by indicating a positive correlation between plant density and outcrossing rate (e.g. Murawski and Hamrick 1991; van Treuren et al. 1993; Karron et al. 1995; Brunet and Sweet 2006). However, others did not find a density effect on outcrossing rate (e.g., Lu 2000; Brunet and Sweet 2006; Barnaud et al. 2008; Friedman and Barrett 2008).
Plant density is also known to affect genetic diversity and inbreeding (Antonovics and Levin 1980) in animal-pollinated plant species. Low density populations are predicted to have lower genetic diversity than high density populations, reducing their long term adaptive potential (Gitzendanner and Soltis 2000; Leimu et al. 2006; Bouzat 2010). This is a result of higher plant density having higher visitation rates of insect pollinators (Kunin 1993), which can result in greater pollen flow and higher genetic diversity. Inbreeding is expected to increase as plant density decreases due to reduced mate diversity (Keller and Waller 2002; Biebach and Keller 2010) and given that near neighbours may be closely related (Ellstrand et al. 1992). Several previous studies have confirmed these predictions by indicating a correlation between density and diversity and inbreeding (Tarayre and Thompson 1997; Coates and Sokolowski 1992), but see also Ferrer et al. (2004) and Gram and Sork (2001) for contradictory findings.

Genetic structuring of populations is also effected by plant density (Loveless and Hamrick 1984). In animal-pollinated species, higher plant densities generally lead to shorter pollinator flights (Levin and Kerster 1974). Because of the reduced gene flow distances, the tendency for microdifferentiation (within population spatial genetic structure) will be greater in high density populations than low density populations (Antonovics and Levin 1980; Loveless and Hamrick 1984). In addition, species that occur at low densities have the potential for greater isolation-by-distance (IBD) due to higher local genetic drift (Heywood 1991; Vekemans and Hardy 2004). As such it can be expected that less dense populations would show greater spatial genetic structure among populations than more dense populations. Previous reviews and studies have confirmed these predictions by indicating a correlation between plant density and relative genetic isolation (Murawski and Hamrick 1991; Shapcott 1996; Vekemans and Hardy 2004).

Low population densities are also expected to affect populations in a deterministic way. As plants become increasingly isolated reproductive processes, such as pollen dispersal, begin to function less efficiently leading to Allee effects whereby declining population density or abundance results in a disproportionate decline in reproductive output and population viability. The ‘Allee effect’ (Allee et al. 1949) is defined by a negative relationship between density and any components of fitness (Stephens et al. 1999) and sometimes referred to as ‘inverse density dependence’ (Courchamp et al. 1999). It can therefore be expected that as a
result of Allee effects, plants growing at low density can suffer from loss of heterozygosity, leading to decreased fitness (Stephens et al. 1999). Furthermore, a strong Allee effect can lead to a threshold density below which populations tend to decrease toward extinction (Ghazoul 2005). In models incorporating Allee effects, extinction rates increase dramatically as density declines and below some density threshold extinction is virtually certain (Dennis 1989; Kunin and Iwasa 1996). Threshold densities have become much studied, largely because of their potential role in extinctions of already endangered, rare or dramatically declining species (Fowler and Baker 1991; Stephens and Sutherland 1999; Barnett 2001). Theoretical studies have identified density thresholds below which extinction is almost inevitable (Kunin and Iwasa 1996; Veit and Lewis 1996). However, there is little information on threshold densities and it is difficult to verify these theoretical results with real population data, although extinction thresholds have been suggested in some studies (Lamont et al. 1993; Groom 1998).

The effects of density on gene flow by seed dispersal are less well understood, however seed dispersal is not considered density-dependent (Heywood 1991). Seed dispersal by large mobile animals is expected to influence the genetic structure of plants at various spatial scales. At the local spatial scale (i.e. within a population), long seed dispersal distances are expected to lead to high gene flow and little genetic structure within a given population. Similarly, at the regional spatial scale (i.e. among populations) long seed dispersal distances should lead to high gene flow and little genetic structure among populations (Nathan 2006; Hardy et al. 2006; Garcia et al. 2007).

Like plant density, population size is known to affect genetic variation and its structuring (Kunin 1997, Bosch and Waser 1999). The strong correlation between plant density and population size (Arden 1996) means that it is difficult to separate the influence of size from density (and vice versa). Consequently, where this study addresses density issues, I recognise that size may also influence the results. Thus for the purpose of this study only the influence of plant density genetic variation and its structuring will be discussed.

To date, studies on the effect of plant density on genetic variation and its structuring have largely been restricted to northern hemisphere species. There has been little research on the drivers of genetic diversity and structure on the Western Australian flora. The South West
Australian Floristic Region (SWAFR) is recognized as one of the biodiversity hotspots of the world, ranking alongside tropical rainforest for animal and plant diversity (Myers et al. 2000). The SWAFR contains almost 6,000 plant species on only about 35,000 km², of which 79% are endemic (Hopper and Goia 2004). Furthermore, the SWAFR is topographically unique in that it is a flat, stable, highly weathered, low plateau dominated by old landscapes with nutrient-deficient soils (Hopper et al. 1996). Given the unique characteristics of SWWA, research in this region can provide important empirical information on the effects of density on genetic variation and its structuring. Yet, despite these unique characteristics, knowledge of the genetic variation of these species and the impact of different life history traits on their genetic variation is limited. Apart from the floristic significance for the SWWA, the region provides an excellent system to study the impact of density on genetic variation using a comparative approach, since the region contains numerous congeners with similar life history traits and analysing closely related species improves the comparability of genetic data.

This study assesses the effect of plant density on genetic diversity and spatial genetic structure in two co-occurring SWAFR tree congeners, chosen because they have densities that are typically three orders of magnitude different, yet have nearly identical life-history traits. Persoonia longifolia occurs in the southwest jarrah forest of SWWA in relatively high densities (Mullins et al. 2002), while P. elliptica occurs largely at very low densities (Abbott 1984a). These congeners provide a powerful model system to study the impact of density on genetic variation using a comparative approach, because they are sympatric with similar habitats, flowers and fruits, shared pollinators and seed dispersers, but contrast markedly in their population densities.

Specifically, this research aims to characterise spatial patterns of genetic variation and structuring across the natural ranges of P. longifolia and P. elliptica, by testing whether:

- Populations that occur at low densities have lower genetic diversity than those that occur at relatively higher densities.
- Populations that occur at low densities have greater levels of inbreeding, than those that occur at relatively higher densities.
Chapter Three: Density Effects

- A species with high population densities shows stronger spatial genetic structure within populations than a species with less dense populations.
- A species with low densities across its range shows greater spatial genetic structure among populations than a species with higher density across its range.
3.3 METHODS AND MATERIALS

3.3.1 Study Species

Persoonia (Proteaceae) is an endemic Australian genus of woody perennial plants containing 100 species, 43 of which are found in Western Australia (Weston 1995). Persoonia longifolia R.Br. (commonly known as snottygobble) is an erect shrub or tree (1 – 5 m high) found in the jarrah forest in SWWA (Figure 3.1). Persoonia longifolia flowers from November to February with yellow flowers (Marchant et al. 1987). It is a very conspicuous species because of its distinctive, flaky layered bark and light green slender foliage. Persoonia longifolia occurs in more-or-less continuous populations at relatively high densities (up to ca. 300 plants/ha). It is most common in upland jarrah forests in SWWA (Havel 1975) but is distributed as far south as Albany on the south coast of Western Australia (Marchant et al. 1987). Persoonia elliptica R.Br. (spreading snottygobble) is an erect spreading shrub or tree (2 – 8 m high) and is also found in the jarrah forest in SWWA (Figure 3.2). Persoonia elliptica flowers from October to February, with yellow green flowers. Persoonia elliptica occurs largely as well-separated (ca. > 50 m) trees in populations of very low densities. It occurs within 50 km of the coast, from Perth to Albany SWWA (Weston 1995). Both species are pollinated by native bees (Bernhardt and Weston 1996) and the introduced honeybee (Apis mellifera) is also a frequent visitor but it is unclear whether this species is an efficient pollinator. The fleshy fruits, which fall from April to August, are clearly adapted for animal dispersal but it is not known whether mammals (i.e. macropods) or birds (e.g. emus, Dromaius novaehollandiae) are the most important dispersers (Bernhardt and Weston 1996). Both species survive fire by resprouting (Benson and McDougall 2000).

3.3.2 Sample Collection

Twenty-two and 20 sites were sampled across the natural ranges of P. longifolia and P. elliptica (respectively) within the jarrah forest of SWWA (Figure 3.1, 3.2; Table 3.1, 3.2). These sites were selected without prior assumptions about population boundaries. Initially it was planned to sample both species at all sites, however the species co-occurred at only seven sampled sites. For P. longifolia, 30 plants from each site were sampled.
Figure 3.1: Geographic location of 22 sampled populations of *Persoonia longifolia* in southwest Western Australia. Population codes correspond to those used in Table 3.1. The grey dots indicate herbarium collections and show the range of the species. Maps sourced from Nature Map, Department of Environment and Conservation.
Figure 3.2: Geographic location of 20 sampled populations of *Persoonia elliptica* in southwest Western Australia. Population codes correspond to those used in Table 3.2. The grey dots indicate herbarium collections and show the range of the species. Maps sourced from Nature Map, Department of Environment and Conservation.
Table 3.1: Geographic location, population code, latitude, longitude, number of samples (N) and population density for 22 populations of *Persoonia longifolia*. *At least 30 individuals were sampled for each population, however due to DNA extraction and genotyping difficulties the number of genotyped accessions varies.

<table>
<thead>
<tr>
<th>Location</th>
<th>Code</th>
<th>Latitude</th>
<th>Longitude</th>
<th>N</th>
<th>Density (plants/ha)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Albany Hwy South</td>
<td>AS</td>
<td>-32.52</td>
<td>116.38</td>
<td>28</td>
<td>37.86</td>
</tr>
<tr>
<td>Boddington</td>
<td>BD</td>
<td>-32.77</td>
<td>116.41</td>
<td>27</td>
<td>7.98</td>
</tr>
<tr>
<td>Bokerup</td>
<td>BK</td>
<td>-34.34</td>
<td>116.87</td>
<td>21</td>
<td>4.83</td>
</tr>
<tr>
<td>Collie East</td>
<td>CE</td>
<td>-33.42</td>
<td>116.41</td>
<td>30</td>
<td>10.39</td>
</tr>
<tr>
<td>Chester Pass</td>
<td>CP</td>
<td>-34.89</td>
<td>117.94</td>
<td>17</td>
<td>4.46</td>
</tr>
<tr>
<td>Collie West</td>
<td>CW</td>
<td>-33.33</td>
<td>116.05</td>
<td>28</td>
<td>14.90</td>
</tr>
<tr>
<td>Denmark</td>
<td>DN</td>
<td>-34.79</td>
<td>117.14</td>
<td>28</td>
<td>32.19</td>
</tr>
<tr>
<td>Del Park Rd</td>
<td>DP</td>
<td>-32.65</td>
<td>116.05</td>
<td>26</td>
<td>2.81</td>
</tr>
<tr>
<td>Grimwade</td>
<td>GW</td>
<td>-33.59</td>
<td>116.02</td>
<td>30</td>
<td>103.07</td>
</tr>
<tr>
<td>Jarrahwood</td>
<td>JW</td>
<td>-33.68</td>
<td>115.74</td>
<td>30</td>
<td>17.69</td>
</tr>
<tr>
<td>Keysbrook</td>
<td>KY</td>
<td>-32.48</td>
<td>116.07</td>
<td>27</td>
<td>16.96</td>
</tr>
<tr>
<td>Larego Form</td>
<td>LF</td>
<td>-32.96</td>
<td>116.05</td>
<td>29</td>
<td>4.86</td>
</tr>
<tr>
<td>Lower Hotham</td>
<td>LH</td>
<td>-32.86</td>
<td>116.35</td>
<td>29</td>
<td>5.42</td>
</tr>
<tr>
<td>Mt Dale</td>
<td>MD</td>
<td>-32.17</td>
<td>116.26</td>
<td>25</td>
<td>130.61</td>
</tr>
<tr>
<td>Mt Frankland</td>
<td>MF</td>
<td>-34.67</td>
<td>116.71</td>
<td>26</td>
<td>14.08</td>
</tr>
<tr>
<td>Muirs Hwy</td>
<td>MH</td>
<td>-34.66</td>
<td>117.52</td>
<td>19</td>
<td>1.74</td>
</tr>
<tr>
<td>Milyeannup</td>
<td>MY</td>
<td>-34.28</td>
<td>115.65</td>
<td>30</td>
<td>48.56</td>
</tr>
<tr>
<td>Nanga Brook</td>
<td>NB</td>
<td>-32.84</td>
<td>116.06</td>
<td>29</td>
<td>35.22</td>
</tr>
<tr>
<td>Phillips Brook</td>
<td>PB</td>
<td>-34.89</td>
<td>117.78</td>
<td>6*</td>
<td>182.62</td>
</tr>
<tr>
<td>Quinadaning</td>
<td>QD</td>
<td>-33.08</td>
<td>116.42</td>
<td>27</td>
<td>11.73</td>
</tr>
<tr>
<td>The Stinkwoods</td>
<td>ST</td>
<td>-33.26</td>
<td>116.39</td>
<td>29</td>
<td>38.46</td>
</tr>
<tr>
<td>Stirling Reservoir West</td>
<td>SW</td>
<td>-33.17</td>
<td>116.02</td>
<td>28</td>
<td>36.73</td>
</tr>
<tr>
<td>MEAN</td>
<td></td>
<td></td>
<td></td>
<td>25</td>
<td>34.69</td>
</tr>
</tbody>
</table>
Table 3.2: Geographic location, population code, latitude, longitude, number of samples (N) and population density for 20 populations of *Persoonia elliptica*.

<table>
<thead>
<tr>
<th>Location</th>
<th>Code</th>
<th>Latitude</th>
<th>Longitude</th>
<th>N</th>
<th>Density (plants/ha)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ashcroft</td>
<td>AC</td>
<td>-32.86</td>
<td>116.41</td>
<td>10</td>
<td>0.01</td>
</tr>
<tr>
<td>Albany Hwy North</td>
<td>AN</td>
<td>-32.51</td>
<td>116.37</td>
<td>12</td>
<td>0.04</td>
</tr>
<tr>
<td>Avon Valley</td>
<td>AV</td>
<td>-31.58</td>
<td>116.16</td>
<td>20</td>
<td>0.34</td>
</tr>
<tr>
<td>Boonanarring</td>
<td>BN</td>
<td>-31.17</td>
<td>115.85</td>
<td>3</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Collie East</td>
<td>CE</td>
<td>-33.42</td>
<td>116.41</td>
<td>12</td>
<td>0.02</td>
</tr>
<tr>
<td>Collie West</td>
<td>CW</td>
<td>-33.35</td>
<td>116.10</td>
<td>3</td>
<td>0.01</td>
</tr>
<tr>
<td>Dempster Rd</td>
<td>DM</td>
<td>-34.93</td>
<td>118.07</td>
<td>3</td>
<td>0.02</td>
</tr>
<tr>
<td>Denmark</td>
<td>DN</td>
<td>-34.78</td>
<td>117.14</td>
<td>15</td>
<td>0.04</td>
</tr>
<tr>
<td>Del Park Rd</td>
<td>DP</td>
<td>-32.64</td>
<td>116.03</td>
<td>8</td>
<td>0.05</td>
</tr>
<tr>
<td>Dwellingup</td>
<td>DW</td>
<td>-32.77</td>
<td>116.08</td>
<td>15</td>
<td>0.05</td>
</tr>
<tr>
<td>Forest Grove</td>
<td>FG</td>
<td>-34.09</td>
<td>115.22</td>
<td>4</td>
<td>0.01</td>
</tr>
<tr>
<td>Kent Rd</td>
<td>KR</td>
<td>-33.02</td>
<td>116.09</td>
<td>11</td>
<td>0.06</td>
</tr>
<tr>
<td>Keysbrook</td>
<td>KY</td>
<td>-32.47</td>
<td>116.06</td>
<td>21</td>
<td>0.05</td>
</tr>
<tr>
<td>Mt Dale</td>
<td>MD</td>
<td>-32.17</td>
<td>116.26</td>
<td>8</td>
<td>0.03</td>
</tr>
<tr>
<td>Mt Frankland</td>
<td>MF</td>
<td>-34.67</td>
<td>116.71</td>
<td>2</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Nanga</td>
<td>NG</td>
<td>-32.79</td>
<td>116.08</td>
<td>5</td>
<td>0.03</td>
</tr>
<tr>
<td>Porongurup</td>
<td>PO</td>
<td>-34.68</td>
<td>117.91</td>
<td>15</td>
<td>0.01</td>
</tr>
<tr>
<td>Stirling Reservoir East</td>
<td>SE</td>
<td>-33.16</td>
<td>116.11</td>
<td>3</td>
<td>0.01</td>
</tr>
<tr>
<td>Vasse Hwy</td>
<td>VH</td>
<td>-34.30</td>
<td>115.77</td>
<td>3</td>
<td>0.01</td>
</tr>
<tr>
<td>Zigzag Rd</td>
<td>ZZ</td>
<td>-32.92</td>
<td>116.05</td>
<td>9</td>
<td>0.02</td>
</tr>
<tr>
<td>MEAN</td>
<td></td>
<td></td>
<td></td>
<td>9</td>
<td>0.04</td>
</tr>
</tbody>
</table>
Six to ten intact leaves were collected from each plant and GPS coordinates were recorded. An area in the south west of *P. longifolia*’s natural range was not genotyped, due to deterioration in collected plant material from which DNA could not be extracted. Exhaustive (every tree) sampling was conducted for *P. elliptica* at all sites, with samples collected from 2 – 20 plants off each site. The leaf samples were returned to the laboratory and frozen at - 80ºC until DNA was extracted.

### 3.3.3 Population Density

For *P. longifolia*, the point-quadrat method (Evans and Love 1957) was used to calculate density. This method employs a transect through the study site and random points along this transect. The area near each random point was divided into four quadrants. Within each quadrat, the distance from the random point to centre of the nearest *P. longifolia* individual was measured. To calculate the density, the mean sum point-to-tree distance was calculated. The square of this number is equal to the mean area occupied per tree and density is equal to the inverse of the area per tree. The density was then converted to the number of plants per hectare. As populations of *P. elliptica* were exhaustively sampled absolute population density was determined at each site sampled. This was done using the number of plants encountered and calculating the total area sampled (i.e. nature reserve) or the area encompassing the sampled plants. The density was then converted to the number of plants per hectare.

### 3.3.4 DNA Extraction

DNA was extracted from leaf material using a procedure modified from Jobes et al. (1995), as described in Chapter Two.

### 3.3.5 Microsatellite Procedure

Multiplex PCR was performed using six microsatellite loci previously optimised for *P. elliptica* (*PeA103, PeA106, PeB103, PeB2, PeC104 and PeC2*) and *P. longifolia* (*PeA103, PeA106, PeB103, *Pl*13, *Pl*15 and *Pl*18) (Chapter Two). PCR amplification, fragment separation and allele size scoring were conducted as described previously for all individuals (see Chapter Two). Positive-control samples were replicated within and between plates to ensure consistency of allele size scoring.
3.3.6 Data Analysis

3.3.6.1 Genetic Diversity

Potential genotyping errors caused by stuttering, large allele dropout and the presence of null alleles in each population were estimated using MICROCHECKER version 2.2.3 (van Oosterhout et al. 2004), using the “Oosterhout” method to calculate the null allele frequency (r) for each locus. Null alleles are predicted when a significant excess of homozygotes is observed (according to HWE predictions), which was evenly distributed across all allele sizes (van Oosterhout et al. 2004).

Genetic diversity statistics per locus were calculated for each species in GenAlEx v 6.5 (Peakall and Smouse 2006; 2012) for the total number of alleles (N_A), observed and expected heterozygosities (H_O and H_E respectively), inbreeding in individuals relative to their population (F_IS), inbreeding in populations relative to the total sample (F_IT) and inbreeding in populations relative to total sample (F_ST).

Genetic diversity statistics were calculated for each population. The number of alleles (N_A), observed and expected heterozygosities (H_O and H_E respectively), fixation indices (F) and total number of private alleles (S), per locus for each population were measured using GenAlEx v 6.5. As populations of P. elliptica were exhaustively sampled, the number of alleles per population was a direct count per population, with no standard error. Gene diversity (H_S; equivalent to H_E) was estimated for each population of P. longifolia and P. elliptica using FSTAT version 2.9.3.2 (Goudet 1995; 2001). FSTAT utilizes a rarefaction method to account for uneven sample size (El Mousadik and Petit 1996). A two-sample t-test using SPSS (PASW Statistics for Windows, Version 18.0, Chicago) assessed the statistical significance of differences in genetic diversity statistics between species at the species and population level.

The association between population density (expressed as the log of number of plants per hectare) and indices of genetic diversity were investigated by regression analysis using SPSS.
Chapter Three: Density Effects

3.3.6.2 Genetic Structure

Spatial genetic structure (SGS) within populations of *P. longifolia* and *P. elliptica* was assessed using Spatial Autocorrelation Analysis (SAA) in GenAlEx v 6.5. Analysis was conducted using all loci and variable distance classes with 15 classes in total. Spatial structure graphs (correlograms) were used to display the correlation between genetic distances of trees and geographic distance. Correlograms were produced for both species with calculated correlation coefficient $r$ and with upper and lower confidence limits, as generated by 999 random permutations of the data and bound by the 95% confidence interval about the null hypothesis of no spatial structure (Peakall and Lindenmayer 2003; Peakall and Smouse 2006). The distance where $r$ intercepts the x-axis provides an estimate of the spatial extent of genetic structure, beyond which gene flow is no longer effective in connecting populations.

SGS for each species was also quantified by calculating the $S_p$ statistic using SPAGeDi 1.3 (Hardy and Vekemans 2002). The $S_p$ statistic was determined as $b / (1 - F_t)$, where $b$ is the mean regression slope with natural log of distance (ln) and $F_t$ is the mean pairwise kinship coefficient for the smallest distance class (Vekemans and Hardy 2004). The $S_p$ statistic accounts for differences in spatial genetic structure due to variation in sampling schemes by considering average kinship across individuals relative to the extent of the decrease in $F$ across distance intervals. A low $S_p$ value means that genetic structure is weak and can be used to compare the extent of spatial genetic structure across species and studies (Vekemans and Hardy 2004).

To investigate the effects of density on SGS of populations of *P. longifolia* and *P. elliptica*, several analyses were applied and compared for each species. First, an Analysis of Molecular Variance (AMOVA) was performed to apportion total genetic variation to within-population and among-population components. Second, the correlation between individual genetic distance and geographic distance was assessed by Mantel tests (Smouse et al. 1986; Smouse and Long 1992) with significance determined by 1000 (999 plus the observed value) permutations of the correlation coefficient $r$. Finally, differentiation among populations was determined by obtaining species level values of $G_{ST}$' (equivalent estimator of $F_{ST}$, independent of the number of samples), using FSTAT version 2.9.3.2. All of the above analyses were performed using GenAlEx v 6.5, unless stated otherwise. A two
sample t-test using SPSS assessed the statistical significance of differences in $G_{ST}$ values between populations.

To visualise genetic similarities among populations, a non-metric Multi Dimensional Scaling (nMDS) ordination was generated for *P. longifolia* and *P. elliptica* populations, using PRIMER V6 (Clarke and Gorley 2006) with Nei’s genetic distance calculated in GenAlEx v 6.5. nMDS ordinations were also generated for individuals of *P. longifolia* and *P. elliptica*. nMDS ordinations represent the samples as points in a 2-dimensional plane, such that the relative distances between all points are in the same rank order as the relative dissimilarities of the samples (Clarke and Gorley 2006). Thus the interpretation of an nMDS is that points that are relatively close together represent samples that are genetically similar and points that are relatively far apart represent samples that are more genetically dissimilar. The extent of mismatching is measured by a “stress” coefficient, which has a value of zero for a perfect match, up to a value of one. To aid interpretation in the ordination, populations were classified as either northern or southern depending on their location.

The Bayesian clustering program STRUCTURE v 2.3.2.1 (Pritchard et al. 2000) was used to identify the presence of population genetic structure across the species range in both *P. longifolia* and *P. elliptica*. The method assigns individuals to ‘K’ number of genetically homogeneous clusters, independent of sampling location and *a priori* population assignments. Initial test runs were performed using the admixture model assuming correlated allele frequencies, which suggested the presence of weak population structure (poor assignment values to any one cluster for several populations; data not shown). Therefore, in subsequent runs the LOCPRIOR model was used, which has the ability to use the sampling location information to assist clustering and is recommended by the authors when the signal of structuring in datasets is weak (Hubisz et al. 2009; Pritchard et al. 2010). The program was run with 100,000 burn-ins and 50,000 MCMC repetitions and K was modelled from K equal to 1 to 22 (*P. longifolia*) and K equals 1 to 20 (*P. elliptica*), with four iterations of each K. Structure Harvester version 0.6 (Earl and von Holdt 2011) was used to determine the optimal value of delta K clusters according to the Evanno et al. (2005) method. The optimal value of K was also considered by visual assessment of the change in slope of lnP (K) with each increasing K value (Pritchard et al. 2010).
3.4 RESULTS

3.4.1 Population Density

To test the accuracy of the point-quadrat method, estimates were compared against a georeferenced dataset from a population of all known individuals with known density (Avon Valley National Park - Chapter Four). The mean density obtained from the point-quadrant method estimates (0.42, 0.56, 0.09 and 0.07; mean = 0.29 plants per hectare) was not significantly different to the actual density (0.34 plants per hectare).

Mean population density was three orders of magnitude higher for *P. longifolia* compared to *P. elliptica* (34.69 and 0.04 plants per hectare, respectively) (Table 3.1, 3.2). For *P. longifolia*, the number of plants per hectare ranged from 1.74 to 182.63. For *P. elliptica*, the number of plants per hectare ranged from <0.01 to 0.34. The highest density *P. elliptica* population was only one fifth as dense as the lowest density *P. longifolia* population.

3.4.2 Data Analysis

3.4.2.1 Genetic Diversity

MICROCHECKER gave no support for stuttering or large allele dropout for either species. Null alleles were detected for *P. longifolia* in low frequencies for PeA103 in populations at MH, MY, ST and SW ($r = 0.07 – 0.22$), PeA106 in AS and MF populations ($r = 0.18 – 0.23$), Pl15 at AS, CP, DN, GW and MF populations ($r = 0.11 – 0.21$), PeB103 at MY only and Pl18 at JW only ($r = 0.12, r = 0.17$, respectively). Null alleles were detected for *P. elliptica* at high frequencies for PeA106 at DP and PeC104 at KY only ($r = 0.33, r = 0.31$ respectively). In addition, null alleles were predicted in moderate frequency for PeA103 at AH and PeB103 at DW only ($r = 0.21, r = 0.17$, respectively). Null alleles were not detected consistently across all populations, suggesting they are likely a sampling effect from small sample sizes.

In total, 568 individuals of *P. longifolia* and 182 individuals of *P. elliptica* were genotyped. Eighty-five alleles were detected across the six loci for *P. longifolia* and 104 alleles were detected across the six loci for *P. elliptica*. All six loci included in this study for *P. longifolia* were variable, with the number of alleles ranging from 6 to 20 per locus, with a mean of 14.17 ± 2.19 (Table 3.3). Gene diversities were high with $H_O$ and $H_E$ per locus averaging 0.58 ± 0.02 and 0.58 ± 0.02, respectively. $F_{IS}$ was negative for most loci, with the
exceptions of PeA103 and Pl15 and $F_{IT}$ was positive for all loci (Table 3.3). $F_{ST}$ was positive, with a mean of $0.18 \pm 0.02$ (Table 3.3). All six loci included in this study for $P.\ elliptica$ were variable, with the number of alleles ranging from 10 to 31 per locus, with a mean of $17.33 \pm 3.08$ (Table 3.4). Gene diversities were high with $H_O$ and $H_E$ per locus averaging $0.57 \pm 0.03$ and $0.55 \pm 0.02$, respectively. $F_{IS}$ was negative for most loci, with the exceptions of PeB103, and PeC104 and $F_{IT}$ was positive for all loci (Table 3.4). $F_{ST}$ was positive and close to zero for loci, with a mean of $0.30 \pm 0.01$ (Table 3.4).

Heterozygosity was moderate for $P.\ longifolia$ populations with $H_E$ ranging from $0.42 \pm 0.13$ to $0.72 \pm 0.06$ and $H_O$ ranging from $0.45 \pm 0.14$ to $0.74 \pm 0.07$. Heterozygosity was also moderate for populations of $P.\ elliptica$, with $H_E$ ranging from $0.17 \pm 0.11$ to $0.81 \pm 0.03$ and $H_O$ ranging from $0.22 \pm 0.17$ to $0.79 \pm 0.06$. There was no significant difference between mean expected heterozygosity ($0.58 \pm 0.02$, $0.55 \pm 0.02$; $p = 0.39$) and mean observed heterozygosity ($0.58 \pm 0.02$, $0.57 \pm 0.03$; $p = 0.93$) for populations of $P.\ longifolia$ and $P.\ elliptica$, respectively (Table 3.5; 3.6).

Gene diversity in populations of $P.\ longifolia$ ranged from $0.43 \pm 0.11$ to $0.73 \pm 0.06$, with a mean of $0.59 \pm 0.10$, and from $0.19 \pm 0.12$ to $0.83 \pm 0.03$ (mean $0.60 \pm 0.09$) in populations of $P.\ elliptica$. There was no significant difference between the mean values of gene diversity number of private alleles in populations of $P.\ longifolia$ and $P.\ elliptica$ ($p = 0.88$).

Fixation indices were low or negative in populations of $P.\ longifolia$, ranging from -$0.21 \pm 0.13$ to $0.19 \pm 0.13$, with a mean close to zero ($0.00 \pm 0.02$). Fixation indices were also low or negative in populations of $P.\ elliptica$, ranging from -$0.40 \pm 0.14$ to $0.27 \pm 0.14$, with a mean of -$0.07 \pm 0.04$. There was no significant difference between mean fixation indices for $P.\ longifolia$ and $P.\ elliptica$, ($p = 0.11$) (Table 3.5; 3.6).

The mean number of private alleles in populations of $P.\ longifolia$ ranged from $0.00$ to $0.67 \pm 0.49$ and from $0.00$ to $1.67 \pm 0.67$ in populations of $P.\ elliptica$. There was no significant difference between the mean number of private alleles in populations of $P.\ longifolia$ and $P.\ elliptica$ ($0.13 \pm 0.11$ and $0.28 \pm 0.16$, respectively; $p = 0.21$).
Table 3.3: Genetic diversity parameters for six microsatellite loci used in this study for *Persoonia longifolia*. 

- $N_A$: average number of alleles, $H_O$: observed heterozygosity, $H_E$: expected heterozygosity, $F_{IS}$: inbreeding in individuals relative to their population, $F_{IT}$: inbreeding in populations relative to the total species, $F_{ST}$: inbreeding in populations relative to total species.

<table>
<thead>
<tr>
<th>Locus</th>
<th>$N_A$</th>
<th>$H_O$</th>
<th>$H_E$</th>
<th>$F_{IS}$</th>
<th>$F_{IT}$</th>
<th>$F_{ST}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>PeA103</td>
<td>20</td>
<td>0.67</td>
<td>0.72</td>
<td>0.06</td>
<td>0.21</td>
<td>0.16</td>
</tr>
<tr>
<td>PeA106</td>
<td>6</td>
<td>0.26</td>
<td>0.23</td>
<td>-0.14</td>
<td>0.17</td>
<td>0.27</td>
</tr>
<tr>
<td>PeB103</td>
<td>12</td>
<td>0.54</td>
<td>0.53</td>
<td>-0.02</td>
<td>0.15</td>
<td>0.17</td>
</tr>
<tr>
<td>Pl13</td>
<td>12</td>
<td>0.53</td>
<td>0.50</td>
<td>-0.06</td>
<td>0.14</td>
<td>0.18</td>
</tr>
<tr>
<td>Pl15</td>
<td>20</td>
<td>0.74</td>
<td>0.82</td>
<td>0.09</td>
<td>0.19</td>
<td>0.11</td>
</tr>
<tr>
<td>Pl18</td>
<td>15</td>
<td>0.71</td>
<td>0.68</td>
<td>-0.04</td>
<td>0.16</td>
<td>0.20</td>
</tr>
<tr>
<td>Mean</td>
<td>14.17</td>
<td>0.58</td>
<td>0.58</td>
<td>-0.02</td>
<td>0.17</td>
<td>0.18</td>
</tr>
<tr>
<td>SE</td>
<td>2.19</td>
<td>0.02</td>
<td>0.02</td>
<td>0.03</td>
<td>0.01</td>
<td>0.02</td>
</tr>
</tbody>
</table>

Table 3.4: Genetic diversity parameters for six microsatellite loci used in this study for *Persoonia elliptica*. 

- $N_A$: average number of alleles, $H_O$: observed heterozygosity, $H_E$: expected heterozygosity, $F_{IS}$: inbreeding in individuals relative to their population, $F_{IT}$: inbreeding in populations relative to the total species, $F_{ST}$: inbreeding in populations relative to total species.

<table>
<thead>
<tr>
<th>Locus</th>
<th>$N_A$</th>
<th>$H_O$</th>
<th>$H_E$</th>
<th>$F_{IS}$</th>
<th>$F_{IT}$</th>
<th>$F_{ST}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>PeA103</td>
<td>31</td>
<td>0.59</td>
<td>0.56</td>
<td>-0.06</td>
<td>0.25</td>
<td>0.30</td>
</tr>
<tr>
<td>PeA106</td>
<td>10</td>
<td>0.54</td>
<td>0.53</td>
<td>-0.02</td>
<td>0.29</td>
<td>0.30</td>
</tr>
<tr>
<td>PeB103</td>
<td>19</td>
<td>0.53</td>
<td>0.53</td>
<td>0.00</td>
<td>0.33</td>
<td>0.33</td>
</tr>
<tr>
<td>PeB2</td>
<td>17</td>
<td>0.62</td>
<td>0.61</td>
<td>-0.01</td>
<td>0.26</td>
<td>0.27</td>
</tr>
<tr>
<td>PeC104</td>
<td>11</td>
<td>0.40</td>
<td>0.41</td>
<td>0.01</td>
<td>0.33</td>
<td>0.32</td>
</tr>
<tr>
<td>PeC2</td>
<td>16</td>
<td>0.75</td>
<td>0.64</td>
<td>-0.18</td>
<td>0.13</td>
<td>0.27</td>
</tr>
<tr>
<td>Mean</td>
<td>17.33</td>
<td>0.57</td>
<td>0.55</td>
<td>-0.05</td>
<td>0.27</td>
<td>0.30</td>
</tr>
<tr>
<td>SE</td>
<td>3.08</td>
<td>0.03</td>
<td>0.02</td>
<td>0.03</td>
<td>0.03</td>
<td>0.01</td>
</tr>
</tbody>
</table>
Table 3.5: Genetic diversity parameters from six polymorphic microsatellite markers for populations of *Persoonia longifolia*: N: total number of individuals genotyped, \(N_A\): number of alleles; \(H_O\): observed heterozygosity, \(H_E\): expected heterozygosity, \(F\): fixation index, \(S\): private alleles, \(H_S\): gene diversity. * At least 30 individuals were sampled for each population, however due to DNA extraction and genotyping difficulties number of genotyped accessions vary.

<table>
<thead>
<tr>
<th>POP</th>
<th>N</th>
<th>Density (plants/ha)</th>
<th>(N_A)</th>
<th>(H_O)</th>
<th>(H_E)</th>
<th>(F)</th>
<th>(S)</th>
<th>(H_S)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AS</td>
<td>28</td>
<td>37.86</td>
<td>37 ± 1.85</td>
<td>0.57 ± 0.13</td>
<td>0.58 ± 0.11</td>
<td>0.16 ± 0.19</td>
<td>0.00 ± 0.00</td>
<td>0.59 ± 0.11</td>
</tr>
<tr>
<td>BD</td>
<td>27</td>
<td>7.98</td>
<td>32 ± 1.57</td>
<td>0.55 ± 0.06</td>
<td>0.54 ± 0.08</td>
<td>-0.05 ± 0.05</td>
<td>0.17 ± 0.17</td>
<td>0.55 ± 0.08</td>
</tr>
<tr>
<td>BK</td>
<td>21</td>
<td>4.83</td>
<td>25 ± 1.46</td>
<td>0.51 ± 0.11</td>
<td>0.48 ± 0.10</td>
<td>-0.08 ± 0.06</td>
<td>0.00 ± 0.00</td>
<td>0.49 ± 0.11</td>
</tr>
<tr>
<td>CE</td>
<td>30</td>
<td>10.39</td>
<td>37 ± 1.91</td>
<td>0.46 ± 0.10</td>
<td>0.51 ± 0.12</td>
<td>0.07 ± 0.04</td>
<td>0.17 ± 0.17</td>
<td>0.52 ± 0.12</td>
</tr>
<tr>
<td>CP</td>
<td>17*</td>
<td>4.46</td>
<td>24 ± 1.45</td>
<td>0.56 ± 0.06</td>
<td>0.49 ± 0.07</td>
<td>-0.21 ± 0.13</td>
<td>0.17 ± 0.17</td>
<td>0.50 ± 0.07</td>
</tr>
<tr>
<td>CW</td>
<td>28</td>
<td>14.90</td>
<td>46 ± 2.03</td>
<td>0.55 ± 0.13</td>
<td>0.56 ± 0.13</td>
<td>0.02 ± 0.06</td>
<td>0.67 ± 0.49</td>
<td>0.57 ± 0.13</td>
</tr>
<tr>
<td>DN</td>
<td>28</td>
<td>32.19</td>
<td>35 ± 1.69</td>
<td>0.64 ± 0.06</td>
<td>0.63 ± 0.03</td>
<td>-0.03 ± 0.12</td>
<td>0.00 ± 0.00</td>
<td>0.64 ± 0.03</td>
</tr>
<tr>
<td>DP</td>
<td>26</td>
<td>2.81</td>
<td>37 ± 2.02</td>
<td>0.59 ± 0.12</td>
<td>0.60 ± 0.12</td>
<td>0.02 ± 0.02</td>
<td>0.00 ± 0.00</td>
<td>0.62 ± 0.12</td>
</tr>
<tr>
<td>GW</td>
<td>30</td>
<td>103.07</td>
<td>36 ± 1.99</td>
<td>0.56 ± 0.07</td>
<td>0.56 ± 0.08</td>
<td>-0.04 ± 0.08</td>
<td>0.00 ± 0.00</td>
<td>0.57 ± 0.08</td>
</tr>
<tr>
<td>JW</td>
<td>30</td>
<td>17.69</td>
<td>45 ± 2.06</td>
<td>0.54 ± 0.11</td>
<td>0.63 ± 0.10</td>
<td>0.16 ± 0.07</td>
<td>0.33 ± 0.21</td>
<td>0.64 ± 0.10</td>
</tr>
<tr>
<td>KY</td>
<td>27</td>
<td>16.96</td>
<td>40 ± 1.72</td>
<td>0.69 ± 0.10</td>
<td>0.66 ± 0.10</td>
<td>-0.06 ± 0.03</td>
<td>0.17 ± 0.17</td>
<td>0.67 ± 0.10</td>
</tr>
<tr>
<td>LF</td>
<td>29</td>
<td>4.86</td>
<td>40 ± 1.98</td>
<td>0.64 ± 0.13</td>
<td>0.64 ± 0.13</td>
<td>-0.02 ± 0.04</td>
<td>0.00 ± 0.00</td>
<td>0.65 ± 0.14</td>
</tr>
<tr>
<td>LH</td>
<td>29</td>
<td>5.42</td>
<td>43 ± 1.97</td>
<td>0.62 ± 0.09</td>
<td>0.61 ± 0.08</td>
<td>-0.03 ± 0.04</td>
<td>0.17 ± 0.17</td>
<td>0.62 ± 0.09</td>
</tr>
<tr>
<td>MD</td>
<td>25</td>
<td>130.61</td>
<td>34 ± 1.61</td>
<td>0.60 ± 0.12</td>
<td>0.59 ± 0.11</td>
<td>-0.02 ± 0.07</td>
<td>0.17 ± 0.17</td>
<td>0.60 ± 0.11</td>
</tr>
<tr>
<td>MF</td>
<td>26</td>
<td>14.08</td>
<td>39 ± 1.77</td>
<td>0.54 ± 0.12</td>
<td>0.61 ± 0.10</td>
<td>0.19 ± 0.13</td>
<td>0.00 ± 0.00</td>
<td>0.62 ± 0.10</td>
</tr>
<tr>
<td>MH</td>
<td>19</td>
<td>1.74</td>
<td>28 ± 1.56</td>
<td>0.59 ± 0.09</td>
<td>0.58 ± 0.07</td>
<td>-0.06 ± 0.16</td>
<td>0.00 ± 0.00</td>
<td>0.60 ± 0.07</td>
</tr>
<tr>
<td>MY</td>
<td>30</td>
<td>48.56</td>
<td>42 ± 1.81</td>
<td>0.65 ± 0.06</td>
<td>0.72 ± 0.06</td>
<td>0.08 ± 0.08</td>
<td>0.33 ± 0.33</td>
<td>0.73 ± 0.06</td>
</tr>
<tr>
<td>NB</td>
<td>29</td>
<td>35.22</td>
<td>42 ± 2.17</td>
<td>0.74 ± 0.07</td>
<td>0.71 ± 0.08</td>
<td>-0.06 ± 0.07</td>
<td>0.00 ± 0.00</td>
<td>0.72 ± 0.08</td>
</tr>
<tr>
<td>PB</td>
<td>6*</td>
<td>182.62</td>
<td>16 ± 1.1</td>
<td>0.47 ± 0.13</td>
<td>0.43 ± 0.10</td>
<td>-0.14 ± 0.21</td>
<td>0.00 ± 0.00</td>
<td>0.47 ± 0.11</td>
</tr>
<tr>
<td>QD</td>
<td>27</td>
<td>11.73</td>
<td>27 ± 1.53</td>
<td>0.45 ± 0.14</td>
<td>0.42 ± 0.13</td>
<td>-0.07 ± 0.03</td>
<td>0.00 ± 0.00</td>
<td>0.43 ± 0.14</td>
</tr>
<tr>
<td>ST</td>
<td>29</td>
<td>38.46</td>
<td>40 ± 1.82</td>
<td>0.55 ± 0.11</td>
<td>0.57 ± 0.12</td>
<td>0.01 ± 0.06</td>
<td>0.33 ± 0.21</td>
<td>0.58 ± 0.12</td>
</tr>
<tr>
<td>SW</td>
<td>28</td>
<td>36.73</td>
<td>47 ± 2.12</td>
<td>0.58 ± 0.13</td>
<td>0.62 ± 0.13</td>
<td>0.19 ± 0.18</td>
<td>0.17 ± 0.17</td>
<td>0.63 ± 0.13</td>
</tr>
<tr>
<td>MEAN</td>
<td>25</td>
<td>34.69</td>
<td>36 ± 1.78</td>
<td>0.58 ± 0.02</td>
<td>0.58 ± 0.02</td>
<td>0.00 ± 0.02</td>
<td>0.13 ± 0.11</td>
<td>0.59 ± 0.10</td>
</tr>
</tbody>
</table>
Table 3.6: Genetic diversity parameters from six polymorphic microsatellite markers for populations of *Persoonia elliptica*. N: total number of individuals genotyped, $N_A$: number of alleles, $H_O$: observed heterozygosity, $H_E$: expected heterozygosity, $F$: fixation index, $S$: private alleles, $H_S$: gene diversity.

<table>
<thead>
<tr>
<th>POP</th>
<th>Density (plants/ha)</th>
<th>$N_A$</th>
<th>$H_O$</th>
<th>$H_E$</th>
<th>$F$</th>
<th>$S$</th>
<th>$H_S$</th>
</tr>
</thead>
<tbody>
<tr>
<td>AC</td>
<td>10</td>
<td>0.01</td>
<td>37</td>
<td>0.68 ± 0.08</td>
<td>0.71 ± 0.06</td>
<td>0.04 ± 0.06</td>
<td>0.33 ± 0.33</td>
</tr>
<tr>
<td>AN</td>
<td>12</td>
<td>0.04</td>
<td>35</td>
<td>0.68 ± 0.09</td>
<td>0.70 ± 0.04</td>
<td>0.02 ± 0.11</td>
<td>0.33 ± 0.21</td>
</tr>
<tr>
<td>AV</td>
<td>20</td>
<td>0.34</td>
<td>44</td>
<td>0.76 ± 0.11</td>
<td>0.72 ± 0.09</td>
<td>-0.04 ± 0.07</td>
<td>1.50 ± 0.67</td>
</tr>
<tr>
<td>BN</td>
<td>3</td>
<td>&lt;0.01</td>
<td>16</td>
<td>0.72 ± 0.10</td>
<td>0.52 ± 0.07</td>
<td>-0.40 ± 0.14</td>
<td>0.17 ± 0.17</td>
</tr>
<tr>
<td>CE</td>
<td>12</td>
<td>0.02</td>
<td>19</td>
<td>0.60 ± 0.10</td>
<td>0.49 ± 0.07</td>
<td>-0.19 ± 0.08</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>CW</td>
<td>3</td>
<td>0.01</td>
<td>18</td>
<td>0.44 ± 0.17</td>
<td>0.52 ± 0.11</td>
<td>0.19 ± 0.24</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>DM</td>
<td>3</td>
<td>0.02</td>
<td>8</td>
<td>0.22 ± 0.17</td>
<td>0.17 ± 0.11</td>
<td>-0.33 ± 0.39</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>DN</td>
<td>15</td>
<td>0.04</td>
<td>23</td>
<td>0.51 ± 0.12</td>
<td>0.43 ± 0.09</td>
<td>-0.18 ± 0.11</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>DP</td>
<td>8</td>
<td>0.05</td>
<td>38</td>
<td>0.52 ± 0.11</td>
<td>0.70 ± 0.06</td>
<td>0.27 ± 0.14</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>DW</td>
<td>15</td>
<td>0.05</td>
<td>36</td>
<td>0.50 ± 0.09</td>
<td>0.61 ± 0.08</td>
<td>0.19 ± 0.07</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>FG</td>
<td>4</td>
<td>0.01</td>
<td>20</td>
<td>0.69 ± 0.15</td>
<td>0.60 ± 0.07</td>
<td>-0.14 ± 0.24</td>
<td>0.17 ± 0.17</td>
</tr>
<tr>
<td>KR</td>
<td>11</td>
<td>0.06</td>
<td>23</td>
<td>0.53 ± 0.11</td>
<td>0.52 ± 0.09</td>
<td>-0.03 ± 0.11</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>KY</td>
<td>21</td>
<td>0.05</td>
<td>53</td>
<td>0.72 ± 0.11</td>
<td>0.81 ± 0.03</td>
<td>0.12 ± 0.12</td>
<td>1.00 ± 0.68</td>
</tr>
<tr>
<td>MD</td>
<td>8</td>
<td>0.03</td>
<td>29</td>
<td>0.54 ± 0.06</td>
<td>0.64 ± 0.04</td>
<td>0.15 ± 0.10</td>
<td>0.33 ± 0.33</td>
</tr>
<tr>
<td>MF</td>
<td>2</td>
<td>&lt;0.01</td>
<td>11</td>
<td>0.33 ± 0.21</td>
<td>0.29 ± 0.14</td>
<td>-0.11 ± 0.42</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>NG</td>
<td>5</td>
<td>0.03</td>
<td>20</td>
<td>0.67 ± 0.08</td>
<td>0.54 ± 0.07</td>
<td>-0.23 ± 0.04</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>PO</td>
<td>15</td>
<td>0.01</td>
<td>31</td>
<td>0.79 ± 0.06</td>
<td>0.57 ± 0.04</td>
<td>-0.04 ± 0.01</td>
<td>1.67 ± 0.67</td>
</tr>
<tr>
<td>SE</td>
<td>3</td>
<td>0.01</td>
<td>15</td>
<td>0.56 ± 0.11</td>
<td>0.46 ± 0.08</td>
<td>-0.24 ± 0.19</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>VH</td>
<td>3</td>
<td>0.01</td>
<td>12</td>
<td>0.39 ± 0.18</td>
<td>0.30 ± 0.14</td>
<td>-0.33 ± 0.09</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>ZZ</td>
<td>9</td>
<td>0.02</td>
<td>27</td>
<td>0.59 ± 0.10</td>
<td>0.61 ± 0.07</td>
<td>0.02 ± 0.12</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>MEAN</td>
<td>9</td>
<td>0.04</td>
<td>25.75</td>
<td>0.57 ± 0.03</td>
<td>0.55 ± 0.02</td>
<td>-0.07 ± 0.04</td>
<td>0.28 ± 0.16</td>
</tr>
</tbody>
</table>
3.4.2.2 Genetic Diversity and Population Density

The association between population density (expressed as the log of number of plants per hectare) and indices of genetic diversity were investigated by regression analysis. A significantly positive correlation was observed between density and number of alleles \( (r^2 = 0.31, p < 0.01) \) (Figure 3.3a). There was also a significant positive relationship between density and fixation index \( (r^2 = 0.10, p = 0.02) \) (Figure 3.3b). When the regression analysis was done separately for each species, \( P. longifolia \) showed no significant correlation \( (r^2 = -0.05, p = 0.87) \) between density and number of alleles, whereas \( P. elliptica \) showed a significant correlation \( (r^2 = 0.47, p < 0.01) \). Furthermore, a regression analysis of log density and fixation index showed no significant correlation \( (r^2 = -0.04, p = 0.64) \) for \( P. longifolia \), whereas \( P. elliptica \) showed a significant correlation \( (r^2 = 0.19, p = 0.03) \). These results suggest that the relationship between population density and number of alleles and fixation index is largely driven by \( P. elliptica \) and that there is a threshold below which density effects become evident. For both number of alleles and fixation index this threshold appears to be \(-\log_{10}(10)\) or 0.01 plants per ha. Furthermore the regression analysis was on a log scale, so the extent of decline below this threshold is great. There was no correlation between density and observed heterozygosity, expected heterozygosity, number of private alleles and gene diversity \( (r^2 = 0.02, p = 0.63; r^2 = 0.05, p = 0.10; r^2 = -0.02, p = 0.70; \text{ and } r^2 = -0.02, p = 0.72, \text{ respectively}) \).

3.4.2.3 Genetic Structure

Spatial Autocorrelation Analysis in GenAlEx v 6.5 identified significant SGS within \( P. longifolia \) populations. Significant positive genetic structure was found at inter plant distances of 0 – 25 m, with \( r \) falling outside the 95% confidence levels (Figure 3.4a). The \( r \) value at 25 m \( (r = 0.06; p = 0.01) \) indicates moderate genetic structure at these distances. Beyond 25 m there was no significant genetic structure. SAA did not identify any significant genetic structure within \( P. elliptica \) populations (Figure 3.4b).

SGS was further quantified for each species by calculating the \( S_p \) statistic. For \( P. longifolia \) the regression slope \( (b) \) was negative (-0.012) and the kinship coefficient estimate for adjacent individuals in the first distance interval was 0.023. For \( P. elliptica \) the regression slope \( (b) \) was also negative (-0.013) and the kinship coefficient estimate for adjacent individuals in the first distance interval was 0.030. From these data, SGS as measured by
the $S_p$ statistic, was similarly weak in both *P. longifolia* ($S_p = 0.013$) and *P. elliptica* ($S_p = 0.013$).
Figure 3.3: Relationship between (a) number of alleles ($r^2 = 0.39$) and (b) fixation index with log density ($r^2 = 0.21$) for populations of *Persoonia longifolia* (pink) and *P. elliptica* (blue). Solid line represents logarithmic line of best fit.
Figure 3.4: Spatial autocorrelation analysis correlograms for populations of (a) *Persoonia longifolia* and (b) *P. elliptica* showing the genetic correlation coefficient ($r$) for increasing distance class sizes, with 95% confidence intervals about $r$ as determined by bootstrapping. Distance class option on x-axis is start point.
An AMOVA of hierarchical genetic diversity partitioned 83% of the total genetic variation within populations and 17% among populations for *P. longifolia*. For *P. elliptica*, AMOVA partitioned 78% of the total genetic variation within populations and 22% among populations. Mantel tests identified a significant relationship between genetic and geographic distances for *P. longifolia* ($R_{xy} = 0.28$, $p = 0.01$) and for *P. elliptica* ($R_{xy} = 0.19$, $p = 0.01$). Both *P. longifolia* and *P. elliptica* demonstrated a significant degree of genetic differentiation among populations ($G_{ST} = 0.16$ and 0.23, respectively), and the value for *P. longifolia* was significantly lower than that for *P. elliptica* ($p = 0.04$). These values are comparable to the mean $F_{ST}$ values per locus, calculated for *P. longifolia* and *P. elliptica* ($F_{ST} = 0.18$ and 0.30, respectively (Table 3.3; 3.4).

nMDS scaling was performed to visually summarize the genetic differentiation among the populations of *P. longifolia* and *P. elliptica*. To aid interpretation in the ordination, populations were classified into northern or southern regions. There was a distinct division between the northern and southern region populations for *P. longifolia* (Figure 3.5), while the nMDS ordination for *P. elliptica* did not cluster populations by region (Figure 3.6). The low stress coefficients of 0.07 and 0.06 (*P. longifolia* and *P. elliptica*, respectively) indicated a good fit between the arrangement of populations in the ordination space and their relative genetic distances.

nMDS ordinations for individuals of *P. longifolia*, showed individuals from the same population typically falling close to each other but not to the exclusion of substantial overlap with individuals from other populations (Figure 3.7). Individuals from the same region (northern or southern) typically fell close to each other (Figure 3.7). nMDS ordinations of *P. elliptica*, showed individuals from the same populations typically falling close to each, but not distinct regional clustering (Figure 3.8). The low stress coefficients of 0.13 and 0.14 (*P. longifolia* and *P. elliptica*, respectively) indicated a good fit between the arrangement of populations in the ordination space and their relative genetic distances.

STRUCTURE analysis revealed that for *P. longifolia*, delta K was maximum at $K = 2$, but a smaller peak was also observed at $K = 5$ (Figure 3.9). The plot of lnP(K) did not show an obvious point at which the values “levelled off” (Figure 3.9). The two genetic clusters corresponded to predominantly northern (red) versus southern (green) populations, with a
Chapter Three: Density Effects

‘transition zone’ between northern and southern populations in the geographic centre of the species range (Figure 3.9).

For *P. elliptica*, STRUCTURE analysis revealed delta K was maximum at \( K = 2 \), but a smaller peak was also observed at \( K = 6 \) (Figure 3.10). Again, the plot of \( \ln P(K) \) did not show an obvious point at which the values “levelled off” (Figure 3.10). The two genetic clusters corresponded to predominantly northern (red) versus southern (green) populations, with a ‘transition zone’ between northern and southern populations in the geographic centre of the species range (Figure 3.10). The assignment of populations to northern or southern clusters was stronger for *P. longifolia*, than *P. elliptica.*
Figure 3.5: nMDS ordination of Nei’s genetic distance for 22 populations of *Persoonia longifolia*. Population symbols correspond to geographic region (northern: open triangle, southern: closed triangle).

Figure 3.6: nMDS ordination of Nei’s genetic distance for 20 populations of *Persoonia elliptica*. Population symbols correspond to geographic region (northern: open triangle, southern: closed triangle).
Chapter Three: Density Effects

Figure 3.7: nMDS ordination of genetic distance for 568 individuals of *Persoonia longifolia*. Individuals are labelled according to population.

Figure 3.8: nMDS ordination of genetic distance for 182 individuals of *Persoonia elliptica*. Individuals are labelled according to population.
Figure 3.9: STRUCTURE results for *Persoonia longifolia*, a) delta K peaks at $K = 2$, b) plot of $\ln P(K)$ and c) bar chart of relative proportional membership of each individual (single bars) within each sampling location/population (solid lines) to one of two genetic clusters (red, green).
Figure 3.10: STRUCTURE results for *Persoonia elliptica*, a) delta K peaks at $K = 2$, b) plot of lnP (K) and c) bar chart of relative proportional membership of each individual (single bars) within each sampling location/population (solid lines) to one of two genetic clusters (red, green).
3.5 DISCUSSION

Mean population density for *P. longifolia* is three orders of magnitude higher than that of its sympatric congener *P. elliptica*. Despite this marked contrast in density, the two species were found to have similarly high levels of genetic diversity. While both species were found to display moderate genetic differentiation among geographically proximate populations, both $G_{ST}$ and $F_{ST}$ were greater for *P. elliptica*. *Persoonia longifolia* displayed significantly stronger within population genetic structure, than *P. elliptica*.

3.5.1 Effects Of Density On Genetic Diversity

Species that occur at low densities are predicted to have lower genetic diversity and higher levels of inbreeding than species that occur at relatively higher densities (Ellstrand and Elam 1993; Leimu et al. 2006). In spite of these expectations, most measures of genetic diversity were similar for *P. elliptica* and its relatively high density congener, *P. longifolia*. In addition, the low density species did not show signs of elevated inbreeding, as revealed by fixation indices. This suggests that despite low density, density dependent effects are not limiting genetic diversity parameters in populations of *P. elliptica* and therefore not affecting plant fitness (Fischer and Matthies 1998; Stephens and Sutherland 1999).

Despite theoretical and empirical predictions for density dependent effects, this study joins others that did not to detect decreased genetic diversity with decreased density. For example, high levels of genetic diversity were found in the low density *Baillonella toxisperma* Pierre, in Central Africa (Ndiade-Bourobou et al. 2010). In addition, a study of two low density fig species (*Ficus hispida* and *F. exasperata*) in the Indian Western Ghats found high levels of microsatellite diversity in both species (Dev et al. 2010). Furthermore, a review of low density rainforest species by Dick et al. (2008) found that low density species often maintain high levels of genetic diversity. These studies conclude that these low density species maintain high levels of genetic diversity because they are predominantly outcrossed and have extensive gene flow.

Several explanations might account for population density having no significant effect on most measures of genetic diversity ($H_O$, $H_E$, $S$ and $H_S$) and levels of inbreeding in this data. Firstly, gene flow within long-lived woody plant populations (such as *Persoonia*) is sufficient to maintain high levels of genetic diversity (Hamrick and Godt 1989). Secondly,
high genetic diversity in low density plant species can be an indication of historically larger populations (Shapcott et al. 2007). It is not known whether *P. elliptica* had historically larger populations, but the species does display characteristics attributed to having undergone a recent bottleneck. Bottlenecks have been known to increase demographic stochasticity (Luikart et al. 1998), which is evident by the predominance of the adult stage in all populations and low recruitment rate (Cabrera-Toledo et al. 2008), as is the case for *P. elliptica*, where there has been little evidence of recruitment since around 1900 (Abbott and Van Heurck 1988). Bottleneck analyses were not undertaken as part of this study, as most analyses (e.g. Cornuet and Luikart 1997) require minimum numbers of individuals higher than available here. However, this predominance of the adult stage in all populations and low recruitment rate could simply reflect a long-term effect, probably caused by herbivory of seedlings/saplings. Another explanation for density having no significant effect on genetic diversity is the combination of a self-incompatibility system and long distance pollen flow (Murawski and Hamrick 1991; Ferrer et al. 2004). This hypothesis is addressed further by characterizing realised pollen dispersal in a large isolated population of *P. elliptica* (Chapter Four).

Whilst density had no effect on most measure of genetic diversity there was a significant positive relationship between density and number of alleles. This relationship is driven by very low density *P. elliptica* populations, with only the lowest density populations having fewer alleles per population. These results indicate that density begins to have a negative effect on number of alleles below a density threshold of ca. 0.01 plants per ha. The reduced number of alleles at very low density populations indicates a loss of genetic variation below the density threshold. In general, the potential consequences of this loss of genetic variation are the evolutionary potential of species to adapt to changing environments is decreased and inbreeding depression (Ellstrand and Elam 1993).

The data also suggests a significant relationship between very low density *P. elliptica* populations and strongly negative fixation indices. Again, this indicates that density begins to have an effect at a threshold of 0.01 plants per ha, however in this case the density threshold is not having a negative impact. Negative values indicate an excess of heterozygosity compared to Hardy-Weinberg expectations, due to negative assortative mating (avoidance of mating between individuals that share a particular phenotype).
Chapter Three: Density Effects

(Wright 1951) or heterotic selection. Stoeckel et al. (2006) suggest that heterozygote excess revealed by negative fixation index has several potential causes, including self-incompatibility systems, wide outcrossing and low effective population size. Studies of small or subdivided populations have found that it is common for few reproductive individuals to be involved in producing the next generation and that there is often differential natural selection towards heterozygous individuals to avoid the effects of genetic drift (Lesica and Allendorf 1992; Luijten et al. 2000). This effect will probably reflect heterozygote excess in future generations and, if so, this should be detectable in the seedling stage (Stoeckel et al. 2006). This hypothesis is assessed further by analysing measures of genetic diversity in seeds of *P. elliptica* in Chapters Four and Five.

It should be noted that the different spatial scales of sampling for the two species can influence the statistics. In particular, as all trees were sampled in populations of *P. elliptica* all the genetic diversity was sampled. On the other hand, all trees were not sampled in populations of *P. longifolia* and thus not all alleles were sampled, meaning genetic diversity could be greater than reported.

### 3.5.2 Genetic Structure Within Populations

High density populations are expected to show stronger SGS within populations than less dense populations due to reduced gene flow via pollen movement (Antonovics and Levin 1980; Loveless and Hamrick 1984). In agreement with these theoretical expectations, SAA correlograms revealed that on average, *P. longifolia* populations displayed significant positive genetic structure at inter plant distances of up to 25 m, whereas *P. elliptica* populations displayed no significant genetic structure at any spatial scale. These results presumably reflect the effects of plant density on pollinator behaviour, where for *P. longifolia* the relatively higher density of neighbouring trees enables pollinators to travel shorter distances among more related trees, acting to produce seeds that have higher kinship than those of plants in less dense populations. For the less dense *P. elliptica*, pollinators would have to be able to fly considerable distances to find neighbouring sexually reproductive individuals or pollinators do not find the few existing plants at all, resulting in more within-plant movements (de Jong et al. 1993). However, strong selection against the products of self-pollination results in preferential outcrossing in *Persoonia* (Krauss 1994b; Chapter Four). Furthermore, this difference between the high and low density populations
could be a consequence of the cumulative structuring effects of nearest neighbour pollen transfer by insects among plants of *P. longifolia* that are already structured by local seed dispersal.

In contrast to the above findings, SGS as calculated by the $S_p$ statistic was similarly weak (0.013) in both species, regardless of density. These findings are comparable to a meta-analysis of species with similar reproductive ecology and life form to those reported by Vekemans and Hardy (2004), which summarises mean values for outcrossed ($S_p= 0.012$), animal-dispersed pollen ($S_p= 0.017$) and animal-dispersed seed ($S_p= 0.009$) tree species. The review of $S_p$ statistics by Vekemans and Hardy (2004) did report that the $S_p$ statistic is consistently higher in low density, as compared to high density populations. However, this comparison was only conducted on six pairwise comparisons and the difference was only marginally significant ($p= 0.07$ for a two-tailed test). As both high and low density populations showed a similar $S_p$ statistic, the overall effects of density on SGS appear to be weak and may reflect the excess heterozygosity found in the lowest density populations.

### 3.5.3 Genetic Structure Among Populations

Species that occur at low densities can be expected to show greater spatial genetic structure among populations than more dense populations due to higher local genetic drift (Heywood 1991; Vekemans and Hardy 2004). In agreement with this prediction, while both species displayed isolation-by-distance (IBD) (as revealed by Mantel tests), the low density populations of *P. elliptica* showed greater among population variation, than the dense populations of *P. longifolia*, as revealed by AMOVA results. In addition, *P. elliptica* had significantly higher $G_{ST}^\prime$ and $F_{ST}$ values demonstrating a higher degree of genetic differentiation among populations than *P. longifolia*.

These results support the findings by Hamrick et al. (1992) and Hamrick and Godt (1996) who used allozymes to summarise the effects of life history traits on genetic diversity in plant species. For long-lived, outcrossing woody species Hamrick and Godt (1996) used an equivalent estimator of $F_{ST}$ and found that 9 – 22% of genetic variation is partitioned among populations. These values are consistent with the $G_{ST}^\prime$ (0.16 and 0.23) and $F_{ST}$ (0.18 and 0.30) values found in this study found for *P. longifolia* and *P. elliptica* (respectively). The effects of plant density on genetic variation among populations from this present study
also reflect findings of other studies. For instance, Shapcott (1996) found that *Pinanga* species with higher densities generally also had lower $F_{ST}$ values (equivalent to $G_{ST}'$ values) than the lower density species, suggesting a correlation between plant density and relative genetic isolation. Murawski and Hamrick (1991) also found that the mean $F_{ST}$ was negatively correlated with tree density, for nine species of mass-flowering rain forest trees. However, Ferrer et al. (2004) found there was no genetic differentiation between sites of *Flourensia cernua* as measured by $F_{ST}$, regardless of population density, suggesting that high gene flow among populations may counter within population density effects.

Ordinations of the genetic data for both species showed individuals from the same population clustering together. This result is in agreement with previous results in that both high and low density species displayed IBD. Interestingly, whilst *P. elliptica* showed greater spatial genetic structure among populations (as indicated by AMOVA), *P. longifolia* showed evidence for regional genetic differentiation (as shown from nMDS). Specifically, there was strong evidence for genetic divergence between *P. longifolia* populations from widely geographically separated regions, whereas *P. elliptica* revealed no significant differentiation between the widely isolated geographic regions. This result is reflected by the stronger assignment of *P. longifolia* populations to northern versus southern clusters, in the STRUCTURE analysis, than populations of *P. elliptica*. Given that dispersal of seed by large mobile animals can act to homogenise populations across a species range and seed dispersal generally is not density-dependent (Heywood 1991), this may play a role in the results found for *P. elliptica*.

This genetic delineation of northern and southern populations for *P. longifolia* is most likely a reflection of sampling focus, as the genetic delineation between the two regions corresponds to the gap in the sample distributions. Given that there is a natural disjunction in the distribution of *P. elliptica*, it would be expected to see a genetic disjunction of northern and southern populations for *P. elliptica*. However, this is not the case and a possible explanation for the differences in population structure for the two species could be the species’ life histories. Whilst these two sympatric congeners share similar habitats, fruits, pollinators and seed dispersers, there are some key differences. For example, there is an absence of seedlings and/or saplings in *P. elliptica* populations and there is little evidence for recruitment since around 1900 (Abbott and Van Heurck 1988). Furthermore,
Chapter Three: Density Effects

*P. elliptica* has a slow growth rate and reaches reproductive maturity above a diameter-at-breast-height of 11cm (Abbott and Van Heurck 1988). This slow growth rate can leave *P. elliptica* seedlings and saplings within a susceptible zone of browsing pressure for an extended period of time, leading to recruitment failure (Abbott and Van Heurck 1988; Monaco 2012). On the other hand, seedlings and saplings of *P. longifolia* can be found throughout its natural range (pers. obs) and it has a growth rate of ca. 5 cm per year (Chia unpublished). For both species, seed viability is high in new season seeds (Abbott 1984; Monaco 2012).

While both species produce viable seeds, *P. elliptica*’s slower growth rate means it is more susceptible to browsing pressure and more likely to experience low recruitment rates. This recruitment limitation delays the establishment of reproductive adults (Epperson and Alvarez-Buyll 1997; Mariette et al. 1997), with the net result that, despite extensive seed dispersal by efficient frugivores (Chapter Five), the genetic make-up of the reproductive population represents historical genetic patterns. Moreover, for outcrossed, insect pollinated and animal dispersed species, high within-population genetic diversity would be maintained chiefly by a transient, diffuse genetic structuring with substantial build-up over repeated generations of the life cycle (Epperson and Alvarez-Buyll 1997). The pattern documented for *P. elliptica* can therefore be interpreted as a combination of efficient dispersal of seeds by frugivores contributing to high within-population genetic diversity and a process of IBD related to highly fragmented populations with frequent recruitment limitation.

3.5.4 Conclusions

While theory suggests that low densities populations show lower genetic diversity and greater levels of inbreeding, this study found that low density populations maintain high levels of genetic diversity and no evidence of inbreeding. In fact, this study found heterozygote excess in very low density populations. Theory also suggests that low densities populations can show weaker SGS within population and greater SGS among populations. In this study, both high and low density species displayed evidence of IBD. However, whilst high densities populations displayed high SGS within populations, the effects of SGS among populations were unclear, likely due to present and past recruitment failure. Given the results, it seems most likely that the retention of historical genetic patterns and/or sufficient gene flow between populations are counteracting negative effects
often associated with low population density. Overall, these findings highlight that pollen and seed dispersal play an important role in maintaining the amount of genetic variation and how it is spatially structured.
CHAPTER FOUR

Paternity assignment reveals long distance dispersal of pollen in *Persoonia elliptica*

4.1 ABSTRACT

Dispersal of genes in plants occurs through the movement of pollen or seed. In flowering plants, pollination is the sexual process for gene exchange between plants and thus characterizing the distance and direction of pollen dispersal is of critical importance for a good understanding of the processes that determine the local genetic structure. Paternity analysis based on nine microsatellite loci was used to investigate realised pollen dispersal patterns of the bee-pollinated tree, *Persoonia elliptica*. In a geographically isolated study population covering an area of ca. 1,300 ha, every adult tree ($n = 450$) was mapped and genotyped, together with 343 seeds from ten maternal plants over two flowering seasons. Effective pollen dispersal in *P. elliptica* showed a departure from predominantly nearest neighbour mating, with long-distance pollen dispersal (up to 5.01 km, with a mean of 1.59 km over the two flowering years) and high multiplicity of detected paternity (in 2010 84% and in 2012 86% of pollination events had a unique sire). Spatial Autocorrelation Analysis detected population genetic structure up to 500 m and trended towards a pattern of isolation by distance. These high dispersal distances indicate that trees within a low density population are connected by extensive gene flow, due to the ability and tendency of pollinator bee species to travel long distances. In addition, regardless of a maternal tree’s spatial position within the study population there was no significant difference in any mating parameters measured for the canopy seed. This reinforces that mating and pollen dispersal patterns are robust to position within the population, consistent with highly mobile insect pollinators.
4.2 INTRODUCTION

Plant mating patterns, as mediated by pollen flow, play a central role in plant population genetics, as they affect the level of genetic variation within and among populations, and how this variation is distributed (Levin and Kerster 1974; Hamrick 1989). Plant mating patterns also influence effective population size, the degree of population subdivision (resulting either from drift or differential selection) and levels of inbreeding (Ghazoul 2005). Therefore, detailed knowledge and understanding of the mating patterns in natural plant populations are fundamental pre-requisites for understanding the genetic architecture and evolutionary dynamics of plants.

Plant mating patterns may be largely driven by ecological factors that determine the availability and foraging behaviour of the pollinators involved (Richards et al. 1999; Ghazoul 2005). These factors may include the direct effects of life history traits or taxon-specific foraging behaviour of the pollinators (Schmitt 1980). However, effects of environmental variables that indirectly affect the foraging behaviour of pollinators, and in turn, the distance of pollen dispersal, can also be important (Ghazoul 2005).

Plant density is one environmental factor that can potentially influence mating patterns in plant populations. Pollen is most commonly deposited on nearest neighbours, especially when insect and wind pollination is dominant, producing a leptokurtic pollen dispersal curve (Ellstrand 1992; Ndiade-Bouroborou et al. 2010; Van Rossum et al. 2011; Ottewell 2012). A leptokurtic pollen dispersal curve is numerically dominated by short distance pollination events involving near neighbours and is characterised by a very steep rate of decline near the origin with a long tail of relatively rare long distance events (Levin and Kerster 1974). Consequently, the probability of siring is generally assumed to decrease rapidly with distance between mates, generating varying degrees of isolation by distance within and among populations (Levin and Kerster 1974; Ellstrand 1992). Pollen dispersal between nearest neighbours that are more related than on average across a population promotes biparental inbreeding and reinforces local spatial genetic structure and genetic neighbourhoods (Turner et al. 1982). However, low density populations have been found to shape pollen dispersal patterns differently by promoting long pollen dispersal distances and high multiple paternity (Klinkhamer 1990; Murawski and Hamrick 1991; Ghazoul 2005; Ward et al. 2005; Wagenius 2006). Therefore, given that mating patterns and pollen flow
within populations play an important function in plant population genetics, understanding how plant density affects these parameters is important.

The spatial position of a plant in a population represents another environmental factor potentially influencing mating patterns in plant populations. The abundant centre distribution model is expected when plant abundance is typically greatest at the centre of its geographical range and uniformly low toward the edge (Andrewartha and Birch 1954; Whittaker 1975; Brown 1984). This model predicts that plants on the periphery of a species range will display lower genetic diversity and higher inbreeding rates compared to the centre (Lesica and Allendorf 1995), due to a decrease in density towards the periphery of the species range (Mimura and Aitken 2007). Furthermore, in many cases the ecological conditions in the periphery of the population will be different from those in the centre (Lesica and Allendorf 1995). Low habitat quality at the population margin may affect plant performance and reduce reproduction and dispersal (Pigott and Huntley 1981; Dorken and Eckert 2001). Thus, it might be expected that plants on the periphery of a population produce seed that show lower genetic diversity and higher inbreeding rates, than plants in the core. To date, the effects of a plants’ spatial position in a population on mating patterns and genetic variation have rarely been tested.

There have been numerous attempts to track the physical movement of pollen using dyes (Van Rossum et al. 2011; Mayer et al. 2012) or traps (Yazdani et al. 1989; Caron and Leblanc 1992), whereas in other studies, the actual movements of pollinators were recorded (Levin and Kerster 1974). Whilst these indirect methods have their value (e.g. cost effectiveness) they can prove to be problematic. For instance, pollen traps will collect whatever pollen the trap intercepts and in nearly all cases, this pollen will be from multiple individuals. Hence, the direct estimation of dispersal distances is not possible using this approach. Additionally, these approaches only assess pollen movement and deposition, rather than actual patterns of fertilization and gene flow. Nuclear genetic markers provide an alternative to these indirect methods and enable the assessment of actual patterns of fertilization and gene flow. Methods based on genetic markers allow a more detailed description of the pattern of gene flow in natural populations. In particular, the development of highly polymorphic genetic markers, such as microsatellites, allows accurate paternity analyses (Jones et al. 2010) and
Chapter Four: Pollen Dispersal

thus direct estimates of effective pollen dispersal. Paternity analysis relies on sampling mother plants and offspring, as well as an enumeration and genetic characterization of the surrounding males. Paternity analysis methods attempt to detect whether paternity of the seed or offspring can be attributed to one of the sampled males present (Jones and Arden 2003; Jones et al 2010). Then, for the subset of offspring for which a credible on-site father has been found, the position of the mothers and the fathers can be used to identify realised pollen dispersal and from this construct realised pollen dispersal distributions (Oddou-Muratorio et al. 2005).

Pollen flow studies based on paternity analysis however have two major limitations. First, despite increasingly high-resolution genetic markers providing statistical power, total exclusion is not always possible due to limitation of the markers available or a very large population size (Chakraborty et al. 1988; Jones and Arden 2003; Jones et al. 2010). To overcome this likelihood-based procedures have been developed, providing unbiased estimates of male reproductive success (Meagher and Thompson 1986; Devlin and Ellstrand. 1992; Smouse and Meagher 1994). Secondly, paternity assignment requires the exhaustive sampling of males, which often limits the spatial scale of analysis, to the point that a significant proportion of offspring are found to have been sired by unidentified males from outside the study area. Consequently, even if assignment has been achieved for a portion of the sample, a gap will exist between the spatial scale of effective pollen dispersal in the population and the scale over which it is actually measured. Thus, inferences about pollen dispersal distribution will often be problematic (Smouse and Sork 2004). Given these limitations, geographically isolated populations of low density provide a powerful opportunity for pollen dispersal studies, as they enable exhaustive sampling of all potential sires at a biologically significant scale. In addition, if there is an absence of substantial pollen immigration due to spatial isolation, most of the observations can be converted into mating distances between plants/populations (Robledo-Armuncio and Gil 2004) and little information will be lost for the estimation of the effective pollen dispersal distribution or other within - population reproductive parameters.

To date, most studies assessing how pollen dispersal distances are affected by plant density and the spatial position of a plant in a population have focussed on northern hemisphere populations that consist of few individuals (e.g. Karron et al. 1995; Ndiade-Bourobo et al. 1995; Capelle et al. 1999; Wright et al. 1999; Jones et al. 2000; Traveset and Halfar 2000; Ndiade-Bourobo et al. 2001; Ndiade-Bourobo and Traveset 2001; Borregaard et al. 2002; Jones et al. 2003; Jones et al. 2010). These studies demonstrate that pollen dispersal distances can be highly variable, and that they are influenced by a number of factors, including plant density, the spatial position of a plant in a population, and the presence or absence of spatial isolation. For example, studies have shown that pollen dispersal distances tend to be shorter in populations with low plant density, and that they are longer in populations with high plant density. In addition, studies have shown that pollen dispersal distances tend to be longer when plants are located near the edges of a population, and that they are shorter when plants are located near the centers of a population. Finally, studies have shown that pollen dispersal distances tend to be longer in populations that are spatially isolated, and that they are shorter in populations that are not spatially isolated.
2010; Meeus et al. 2012; Moeller et al. 2012; Nakanishi et al. 2012). Furthermore, there is limited information on the long distance pollen movements in the southwest of Western Australia (SWWA), in particular of insect-mediated pollen dispersal other than observations of vagrant butterflies well outside of their normal distribution (Braby 2004). In this study, pollen dispersal distances and the role of pollinators are determined within each of two flowering years in a large population of the insect-pollinated tree species, *Persoonia elliptica*. Specifically, this research aims to characterise effective pollen dispersal by paternity assignment in *Persoonia elliptica*, with the intent to demonstrate whether:

- Low plant density is associated with a departure from nearest neighbour mating, leading to long distance pollen dispersal and a high multiple paternity.
- A plant's position within a population (core versus periphery) influences patterns of mating and pollen dispersal.
4.3 METHODS AND MATERIALS

4.3.1 Study Species

*Persoonia elliptica* R.Br (spreading snottygobble) is an erect spreading shrub or tree (2 – 8 m) found in the jarrah forest in SWWA (see Figure 1.1). *Persoonia elliptica* produces yellow green flowers from October to February, which are hermaphroditic and generally comprise four symmetrical, recurved yellow tepals that are 8 – 12.5 mm long and glabrous to sparsely hairy on the outside (Weston 1995). *Persoonia* species are preferentially outcrossing (Krauss 1994b) and are pollinated by native bees (i.e. species of *Amegilla*, *Megachilidae* and *Leioproctus*; Bernhardt and Weston 1996). Native bees have been found to visit only a single flower per plant and then fly some distance, often away from site (Anderson and Symon 1988; Rymer 2006). The introduced European honeybee (*Apis mellifera*) is also a frequent visitor but it is still unclear whether this species is an effective pollinator. This is because honey bees are generalist pollinators that harvest nectar from numerous flowering plants, in many cases without pollinating them (Paton 1997). Once fertilisation has occurred, the fruits mature on the plant until release in August to November (Weston 2003). The fruit is a fleshy drupe with a single hard woody stone containing one seed. An embryo may contain up to nine cotyledons, making a newly emerged seedling of this species readily identifiable (Weston 2003).

4.3.2 Study Site and Sample Collection

The study was conducted within Avon Valley National Park (AVNP) (-31.57E 116.16S) a 4,800 ha reserve located in SWWA (Figure 4.1). The study plot was located towards the northern edge of AVNP and encompasses ca. 1,300 ha of jarrah (*Eucalyptus marginata*) forest, where *P. elliptica* occurs almost continuously throughout, at a density of 0.34 plants per ha (Chapter Three). The eastern side of the study plot is bounded by wandoo (*Eucalyptus wandoo*) forest, the northern side by semi-cleared land, the southern side by granite outcrops; and the western side by jarrah forest, with no *P. elliptica* present. No *P. elliptica* were evident outside the study plot and these boundaries may act as a barrier to dispersal of long distance pollen movement into the study area. The nearest known population of *P. elliptica* is 25 km away to the northeast at Julimar National Park; however, there is the possibility of isolated *P. elliptica* trees being present within the remainder of AVNP. The relative isolation of this particular study population, the large distances between neighbouring individuals and the distinctive morphology and habit of these small
trees makes it possible to find and genotype every individual tree at a scale of up to 5 km, providing a unique opportunity to assess realised pollen dispersal across a large and biologically significant spatial scale.

All 450 *P. elliptica* trees in the study plot were sampled in 2010 (Figure 4.1). These trees are referred to as the adult population hereafter. The unique habit and leaf colouring of *P. elliptica* makes it easily identifiable at a distance up to ca. 50 m. To ensure sampling was exhaustive, north/south transects ca. 75 m apart were traversed throughout the study plot. Leaf material from each tree was collected and their GPS coordinates recorded. Adult trees were marked with flagging tape to avoid re-sampling individuals and to aid in future identification. The leaf samples were kept cool and returned to the laboratory where they were frozen at -80°C until DNA was extracted. During population sampling, only one seedling was encountered within the study plot, however, when re-visited a week later it appeared that death had occurred through herbivory.

Ten adult *P. elliptica* trees were chosen as maternal trees based on their spatial position within the study population, either on the periphery (five plants) or within the core of the population (also five plants). These trees are referred to as ‘maternal trees’ hereafter. Plants were classified as core if there was another conspecific in at least 75% of their perimeter, whereas edge plants were defined as such when there was not another conspecific within 75% of their perimeter (Figure 4.1). Twelve to twenty mature fruits were collected from each of the ten maternal trees in November 2010. In June 2012, 10 - 22 mature fruits (seed set was lower in 2012 than 2010, pers. obs) were collected from nine maternal trees, due to the death of one individual (Figure 4.1). Fruits from maternal trees were stored in separate bags. Seeds were treated by removing the outer fleshy covering and then stored at room temperature until DNA extraction. These seed cohorts are henceforth, classified as either 2010 combined (all seed collected in 2010), 2010 edge (all seed collected in 2010 from maternal trees on the periphery of the population), 2010 core (all seed collected in 2010 from maternal trees in the core of the population), 2012 combined (all seed collected in 2012), 2012 edge (all seed collected in 2012 from maternal trees on the periphery of the population) and 2012 core (all seed collected in 2012 from maternal trees in the core of the population). Site wide reproductive failure (possibly due to a dry winter in 2010, pers. obs) meant that no fruits were collected in 2011.
Figure 4.1: Geographic location of all 450 *Persoonia elliptica* trees in the study area within Avon Valley National Park, Western Australia. The pink dots indicate core maternal trees and the blue dots edge maternal trees from which fruit were sampled in 2010 and 2012. Numbers correspond to Table 4.1. Maternal tree 8 was only sampled in 2010, due to tree death.
4.3.3 DNA Extraction

Nuclear DNA was extracted from adult leaf material and canopy seeds using a procedure modified from Jobes et al. (1995), as described in Chapter Two.

4.3.4 Microsatellite Genotyping

Multiplex PCR was performed on adult and seed populations using nine microsatellite loci previously optimised for *P. elliptica* (*PeA102, PeA103, PeA106, PeB103, PeB106, PeB2, PeC104, PeC2 and Pl15*) (see Table 2.2). PCR amplification, fragment separation and allele size scoring were conducted as described previously in Chapter Two. Positive-control samples were replicated within and between plates to ensure consistency of allele size scoring.

4.3.5 Data Analysis

4.3.5.1 Genetic Diversity

Potential genotyping errors caused by stuttering and large allele dropout were estimated using MICROCHECKER version 2.2.3 (van Oosterhout et al. 2004).

Genetic diversity statistics for each locus were calculated in GenAlEx v 6.5 (Peakall and Smouse 2006; 2012). These included the total number of alleles (*N*<sub>A</sub>), observed and expected heterozygosities (*H*<sub>O</sub> and *H*<sub>E</sub> respectively), inbreeding in individuals relative to their population (*F*<sub>IS</sub>), inbreeding in populations relative to the total sample (*F*<sub>IT</sub>) and inbreeding in populations relative to total sample (*F*<sub>ST</sub>).

Population genetic diversity statistics were calculated for the adult population, 2010 combined, 2010 core, 2010 edge, 2012 combined, 2012 core and 2012 edge cohorts. The mean number of alleles per locus (*N*<sub>A</sub>), observed and expected heterozygosities (*H*<sub>O</sub> and *H*<sub>E</sub> respectively), fixation indices (*F*) and number of private alleles (*S*), for the adult and seed cohort populations were measured using GenAlEx v 6.5. A one-way Analysis of Variance (ANOVA) using SPSS (PASW Statistics for Windows, Version 18.0, Chicago) assessed the statistical significance of differences in genetic diversity statistics between i) adult and seed cohorts and ii) core and edge cohorts. Inheritance of all microsatellite marker loci was confirmed by verifying the presence of at least one of the maternal tree alleles in all progeny.
**4.3.5.2 Adult Population Genetic Structure**

Spatial genetic structure (SGS) within the population of *P. elliptica* was assessed using Spatial Autocorrelation Analysis (SAA) in GenAlEx v 6.5. The analysis was conducted using all loci and variable distance classes with 12 classes in total. Spatial structure graphs (correlograms) were used to display the correlation between genetic distances of trees by geographic distance. A correlogram was produced with calculated correlation coefficient \( r \) and with upper and lower confidence limits, as generated by 999 random permutations of the data and bound by the 95% confidence interval about the null hypothesis of no spatial structure (Peakall and Smouse 2006). The distance where \( r \) intercepts the x-axis provides an estimate of the spatial extent of genetic structure, beyond which gene flow is no longer effective in connecting populations (Peakall and Smouse 2006).

SGS was further quantified by calculating the \( S_p \) statistic using SPAGeDi 1.3 (Hardy and Vekemans 2002). The \( S_p \) statistic was determined as \( b / (1 - F_1) \), where \( b \) is the mean regression slope with natural log of distance (ln) and \( F_1 \) is the mean pairwise kinship coefficient for the smallest distance class (Vekemans and Hardy 2004). The \( S_p \) statistic accounts for differences in spatial genetic structure due to variation in sampling schemes by considering average kinship across individuals relative to the extent of the decrease in \( F \) across distance intervals. A low \( S_p \) value means that genetic structure is weak and it can be used to compare the extent of spatial genetic structure across species (Vekemans and Hardy 2004).

**4.3.5.3 Mating System Analysis**

The program MLTR 3.4 (Ritland 2002) was used to estimate maximum-likelihood mating system parameters for the 2010 combined, 2010 core, 2010 edge, 2012 combined, 2012 core and 2012 edge seed cohorts. Maximum-likelihood mating system parameters were also estimated for each maternal tree, for both years separately. Biparental inbreeding was estimated as the difference between the multilocus and single locus outcrossing rate \( (t_m - t_s) \) (Ritland 2002). Multilocus correlated paternity \( (r_p) \) was estimated as the probability that two siblings share the same pollen donor and are therefore outcrossed full-sibs (Ritland 2002). To obtain standard errors for mating system parameter estimates, 500 bootstraps of the data were performed. Maximum-likelihood mating system parameters are frequently
reported in the literature and therefore can readily be used to compare mating systems across species.

### 4.3.5.4 Paternity Analysis

Pollen flow was determined by assigning paternity to seeds collected from each year. Paternity was assigned using the software CERVUS v 3.0.3 (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 as the delta score. The simulation parameters for CERVUS to assign paternity to the most-likely individual with a known level of statistical confidence were as follows: 10,000 cycles of simulation, 450 candidate parents and 0.01 as the proportion of mistyped loci (as recommended by CERVUS) and confidence levels of 95% (strict) and 80% (relaxed). In addition, the estimated proportion of known potential sires was set at 95% as the population was exhaustively sampled for all potential pollen donors. Pollen dispersal distances were calculated for seeds based on the GPS position of the maternal tree and putative pollen parent within the plot.

Using the 80% confidence level, a pollen-dispersal curve for the 2010 and 2012 seed populations 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 0.50 km. This was then compared to the paired distance frequency of all plants within the population to the maternal tree with a Kolmogorov-Smirnov two-sample test (Siegel 1956) in SPSS to test for random mating. Mating distance curves for each maternal tree were also constructed using the same parameters as above.

The number of seeds genotyped for each maternal tree, number of seeds with assigned paternity and number of unique pollen sires was calculated for the seed classifications, as listed previously. Two-sample t-tests assessed the statistical significance of differences between the means of these measures.
Chapter Four: Pollen Dispersal

4.4 RESULTS
4.4.1 Genetic Diversity

Of the 453 adult trees from which DNA was extracted, 450 were genotyped using nine microsatellite markers. The three adults not in the study were eliminated as they failed to consistently amplify at least four markers. DNA was extracted from 343 seeds with 12-21 seeds per maternal tree (Table 4.1) and seeds were genotyped using the same nine microsatellite markers. There was no evidence for large allele dropout in the adult population or the seed cohorts from MICROCHECKER. There were no indications of stuttering in the microsatellite markers, for the adult, 2010 seed or 2012 seed cohorts.

All nine loci included in this study were highly variable, with the number of alleles ranging from 9 to 33 per locus, with a mean of 19.78 ± 3.24 (Table 4.2). Gene diversities were high with $H_O$ and $H_E$ per locus averaging 0.74 ± 0.02 and 0.77 ± 0.02, respectively. $F_{IS}$ and $F_{IT}$ were positive for most loci, with the exceptions of $PeB2$, $PeC104$ and $PeC2$ which were negative (Table 4.2). $F_{ST}$ was positive and close to zero for all loci (Table 4.2).

In total, 178 alleles were detected across nine loci for the adult and seed cohorts of *P. elliptica* (Table 4.1). The adult population had a significantly higher number of mean alleles per locus (19.22 ± 2.75) than both the 2010 combined (12.00 ± 1.58) and 2012 combined (12.44 ± 1.08) seed cohorts ($p = 0.02$). The adult, 2010 and 2012 combined seed cohorts displayed comparable levels of observed heterozygosities ($p = 0.48$), expected heterozygosities ($p = 0.58$) and fixation indices ($p = 0.36$) (Table 4.3).

To determine if the spatial position of a plant within the population affected genetic diversity, genetic parameters for edge and core seed cohorts were also measured. The edge and core seed cohorts displayed comparable levels of genetic diversity (Table 4.3) with mean number of alleles ($p = 0.63$), levels of observed heterozygosities ($p = 0.21$), expected heterozygosities ($p = 0.90$) and fixation indices ($p = 0.21$).
Table 4.1: Number of *Persoonia elliptica* seeds genotyped in 2010 and 2012 per maternal tree in Avon Valley National Park, Western Australia, including geographic location. * indicates the maternal tree died before sampling of seed in 2012.

<table>
<thead>
<tr>
<th>Maternal Location</th>
<th>2010 Seed</th>
<th>2012 Seed</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Edge</td>
<td>12</td>
<td>20</td>
</tr>
<tr>
<td>2 Edge</td>
<td>12</td>
<td>20</td>
</tr>
<tr>
<td>3 Core</td>
<td>20</td>
<td>21</td>
</tr>
<tr>
<td>4 Core</td>
<td>19</td>
<td>22</td>
</tr>
<tr>
<td>5 Edge</td>
<td>15</td>
<td>20</td>
</tr>
<tr>
<td>6 Core</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>7 Core</td>
<td>20</td>
<td>11</td>
</tr>
<tr>
<td>8 Edge</td>
<td>20</td>
<td>0*</td>
</tr>
<tr>
<td>9 Core</td>
<td>20</td>
<td>21</td>
</tr>
<tr>
<td>10 Edge</td>
<td>20</td>
<td>10</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>178</strong></td>
<td><strong>165</strong></td>
</tr>
</tbody>
</table>
Table 4.2: Genetic diversity parameters for nine microsatellite loci used in this study. \(N_A\): number of alleles, \(H_O\): observed heterozygosity, \(H_E\): expected heterozygosity, \(F_{IS}\): inbreeding in individuals relative to their population, \(F_{IT}\): inbreeding in populations relative to the total species, \(F_{ST}\): inbreeding in populations relative to total species.

<table>
<thead>
<tr>
<th>Locus</th>
<th>(N_A)</th>
<th>(H_O)</th>
<th>(H_E)</th>
<th>(F_{IS})</th>
<th>(F_{IT})</th>
<th>(F_{ST})</th>
</tr>
</thead>
<tbody>
<tr>
<td>PeA102</td>
<td>20</td>
<td>0.63</td>
<td>0.78</td>
<td>0.19</td>
<td>0.20</td>
<td>0.01</td>
</tr>
<tr>
<td>PeA103</td>
<td>26</td>
<td>0.81</td>
<td>0.80</td>
<td>-0.02</td>
<td>-0.01</td>
<td>0.01</td>
</tr>
<tr>
<td>PeA106</td>
<td>9</td>
<td>0.54</td>
<td>0.58</td>
<td>0.07</td>
<td>0.08</td>
<td>0.02</td>
</tr>
<tr>
<td>PeB103</td>
<td>26</td>
<td>0.80</td>
<td>0.81</td>
<td>0.01</td>
<td>0.02</td>
<td>0.01</td>
</tr>
<tr>
<td>PeB106</td>
<td>33</td>
<td>0.75</td>
<td>0.76</td>
<td>0.01</td>
<td>0.03</td>
<td>0.02</td>
</tr>
<tr>
<td>PeB2</td>
<td>21</td>
<td>0.81</td>
<td>0.86</td>
<td>0.06</td>
<td>0.06</td>
<td>0.01</td>
</tr>
<tr>
<td>PeC104</td>
<td>9</td>
<td>0.76</td>
<td>0.70</td>
<td>-0.09</td>
<td>-0.08</td>
<td>0.01</td>
</tr>
<tr>
<td>PeC2</td>
<td>16</td>
<td>0.86</td>
<td>0.80</td>
<td>-0.08</td>
<td>-0.07</td>
<td>0.00</td>
</tr>
<tr>
<td>Pl15</td>
<td>18</td>
<td>0.73</td>
<td>0.82</td>
<td>0.11</td>
<td>0.12</td>
<td>0.01</td>
</tr>
<tr>
<td>Mean</td>
<td>19.78</td>
<td>0.74</td>
<td>0.77</td>
<td>0.03</td>
<td>0.04</td>
<td>0.01</td>
</tr>
<tr>
<td>SE</td>
<td>3.24</td>
<td>0.02</td>
<td>0.02</td>
<td>0.03</td>
<td>0.03</td>
<td>0.00</td>
</tr>
</tbody>
</table>
Table 4.3: Genetic diversity parameters for the adult population, the 2010 and 2012 seed cohorts for *Persoonia elliptica* in Avon Valley National Park, Western Australia. N: number of individuals, $N_A$: average number of alleles per locus, $H_O$: observed heterozygosity, $H_E$: expected heterozygosity, $F$: fixation index, $S$: average number of private alleles.

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>$N_A$</th>
<th>$H_O$</th>
<th>$H_E$</th>
<th>$F$</th>
<th>$S$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adult</td>
<td>450</td>
<td>19.22 ± 2.75</td>
<td>0.75 ± 0.04</td>
<td>0.79 ± 0.04</td>
<td>0.06 ± 0.04</td>
<td>5.22 ± 1.14</td>
</tr>
<tr>
<td>2010 Seed Cohort</td>
<td>178</td>
<td>12.00 ± 1.58</td>
<td>0.78 ± 0.05</td>
<td>0.76 ± 0.02</td>
<td>0.02 ± 0.04</td>
<td>0.33 ± 0.17</td>
</tr>
<tr>
<td>2010 Edge Cohort</td>
<td>79</td>
<td>9.00 ± 1.05</td>
<td>0.77 ± 0.05</td>
<td>0.73 ± 0.03</td>
<td>-0.04 ± 0.06</td>
<td>0.22 ± 0.22</td>
</tr>
<tr>
<td>2010 Core Cohort</td>
<td>99</td>
<td>10.33 ± 1.40</td>
<td>0.78 ± 0.04</td>
<td>0.75 ± 0.02</td>
<td>-0.04 ± 0.04</td>
<td>0.11 ± 0.35</td>
</tr>
<tr>
<td>2012 Seed Cohort</td>
<td>165</td>
<td>12.44 ± 1.08</td>
<td>0.70 ± 0.03</td>
<td>0.75 ± 0.02</td>
<td>0.05 ± 0.04</td>
<td>0.11 ± 0.11</td>
</tr>
<tr>
<td>2012 Edge Cohort</td>
<td>70</td>
<td>9.56 ± 0.82</td>
<td>0.76 ± 0.03</td>
<td>0.75 ± 0.02</td>
<td>-0.02 ± 0.04</td>
<td>0.78 ± 0.32</td>
</tr>
<tr>
<td>2012 Core Cohort</td>
<td>95</td>
<td>10.89 ± 0.98</td>
<td>0.67 ± 0.03</td>
<td>0.73 ± 0.03</td>
<td>0.07 ± 0.05</td>
<td>1.11 ± 0.35</td>
</tr>
</tbody>
</table>
The number of non maternal tree alleles scored in the 2010 combined and 2012 combined seed cohorts suggests that some selfing may be present in both year cohorts (Figure 4.2), with 5% of the 2010 combined seeds and 4% of the 2012 combined seeds contained only maternal tree alleles (i.e. zero non-maternal tree alleles).

**4.4.2 Adult Population Genetic Structure**

The distance between all pairs of adult trees ranged from 0.01 – 5.41 km, with a mean of 1.79 km. The distance between all nearest neighbours ranged from 0.01 – 0.84 km, with a mean of 0.06 km. SAA identified significant genetic structure at inter-plant distances of up to 0.50 km, with $r$ falling outside the upper and lower confidence levels (95%) (Figure 4.3). The $r$ value at 0.50 km ($r = 0.05; p = 0.01$) indicates moderate genetic structure at these distances. Additionally, the correlogram shows a clear trend toward increasingly negative autocorrelation values with increasing distance.

Additional evidence of weak spatial genetic structure was supported by the estimate of $S_p$ from SPAGeDi 1.3. The regression slope ($b$) was negative (-0.006) and the kinship coefficient estimate for adjacent individuals in the first distance interval was 0.006. Consistent with this, was the limited genetic structure among adults overall as measured by the $S_p$ statistic ($S_p = 0.006$).
Figure 4.2: Number of non maternal tree alleles scored in the seed cohorts of *Persoonia elliptica* in Avon Valley National Park, Western Australia from 2010 (blue bars) and 2012 (green bars) from the nine microsatellite markers.
Figure 4.3: Spatial autocorrelation analysis correlogram for the study population of *Persoonia elliptica* in Avon Valley National Park, Western Australia showing the genetic correlation coefficient (r) for increasing distance class sizes, with 95% confidence intervals about r as determined by bootstrapping. Distance class option on x-axis is start point.
4.4.3 Realised Mating Patterns

Estimates of multi-locus outcrossing rates ($t_m$) were not significantly different from one for the 2010 combined, 2010 core, 2010 edge, 2012 combined, 2012 core and 2012 edge seed cohorts (Table 4.4), indicating that $P$. elliptica is predominantly outcrossing. The single-locus outcrossing rate ($t_s$) varied slightly among the 2010 combined, 2010 core, 2010 edge, 2012 combined, 2012 core and 2012 edge seed cohorts (0.90 ± 0.05, 0.99 ± 0.05, 0.84 ± 0.06, 0.85 ± 0.03, 0.83 ± 0.05 and 0.92 ± 0.05, respectively). Biparental inbreeding rates ($t_m - t_s$) were low and different to zero (Table 4.4), indicating that outcrossing events may occur between relatives. There was no evidence for correlated paternity ($r_p$) within progeny arrays with values ranging from 0.03 ± 0.17 to 0.33 ± 0.07 (Table 4.4), indicating that the probability of full-sibship within seed cohorts was low to moderate. Seed cohorts from 2010 combined and 2012 combined did not differ for any of the measured parameters (Table 4.4). Likewise, edge and core seed cohorts did not vary for any of the measured parameters (Table 4.4).

Estimates of multi-locus outcrossing rates ($t_m$) were not significantly different from one for each maternal tree (Table 4.5), again indicating that $P$. elliptica is predominantly outcrossing. The single-locus outcrossing rate ($t_s$) for each maternal tree was also not significantly different to one, with values ranging from 1.04 ± 0.00 to 1.20 ± 0.00 (1.20 is considered complete outcrossing). Biparental inbreeding rates ($t_m - t_s$) were low, and different to zero, indicating that mating among related plants occasionally takes place. There was a low degree of correlated paternity ($r_p$) within progeny cohorts (Table 4.5), indicating the contribution of several fathers to the pollen pool. Seed cohorts from each maternal tree did not differ for any of the measured parameters (Table 4.5).
Table 4.4: Mating system parameters for two seed cohorts of *Persoonia elliptica* in Avon Valley National Park, Western Australia estimated with nine microsatellite loci, with standard errors. $t_m$: multilocus outcrossing rate; $t_s$: single locus outcrossing rate; $t_m - t_s$: an estimate of biparental inbreeding; $r_p$: multilocus correlation of paternity (fraction of outcrossed siblings that share the same sire).

<table>
<thead>
<tr>
<th>Year</th>
<th>Multilocus outcrossing rate ($t_m$)</th>
<th>Single locus outcrossing rate ($t_s$)</th>
<th>Biparental inbreeding rate ($t_m - t_s$)</th>
<th>Correlation of paternity ($r_p$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2010</td>
<td>0.99 ± 0.07</td>
<td>0.90 ± 0.05</td>
<td>0.09 ± 0.07</td>
<td>0.33 ± 0.07</td>
</tr>
<tr>
<td>2010 Core</td>
<td>1.20 ± 0.00</td>
<td>0.99 ± 0.05</td>
<td>0.21 ± 0.05</td>
<td>0.22 ± 0.07</td>
</tr>
<tr>
<td>2010 Edge</td>
<td>0.97 ± 0.07</td>
<td>0.84 ± 0.06</td>
<td>0.13 ± 0.08</td>
<td>0.23 ± 0.06</td>
</tr>
<tr>
<td>2012</td>
<td>0.99 ± 0.06</td>
<td>0.85 ± 0.03</td>
<td>0.14 ± 0.06</td>
<td>0.14 ± 0.04</td>
</tr>
<tr>
<td>2012 Core</td>
<td>0.98 ± 0.07</td>
<td>0.83 ± 0.05</td>
<td>0.15 ± 0.07</td>
<td>0.11 ± 0.12</td>
</tr>
<tr>
<td>2012 Edge</td>
<td>1.20 ± 0.00</td>
<td>0.92 ± 0.05</td>
<td>0.28 ± 0.05</td>
<td>0.03 ± 0.17</td>
</tr>
</tbody>
</table>
Table 4.5: Mating system parameters for each maternal *Persoonia elliptica* tree in Avon Valley National Park, Western Australia, over two years with standard errors. $t_m$: multilocus outcrossing rate; $t_s$: single locus outcrossing rate; $t_m - t_s$: an estimate of biparental inbreeding; $r_p$: multilocus correlation of paternity (fraction of outcrossed siblings that share the same sire).

<table>
<thead>
<tr>
<th>Maternal, Year</th>
<th>Multilocus outcrossing rate ($t_m$)</th>
<th>Single locus outcrossing rate ($t_s$)</th>
<th>Biparental inbreeding rate ($t_m - t_s$)</th>
<th>Correlation of paternity ($r_p$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1, 2010</td>
<td>1.20 ± &lt;0.01</td>
<td>1.15 ± &lt;0.01</td>
<td>0.05 ± &lt;0.01</td>
<td>-0.99 ± &lt;0.01</td>
</tr>
<tr>
<td>1, 2012</td>
<td>1.20 ± &lt;0.01</td>
<td>1.19 ± &lt;0.01</td>
<td>0.01 ± &lt;0.01</td>
<td>-0.19 ± &lt;0.01</td>
</tr>
<tr>
<td>2, 2010</td>
<td>1.20 ± &lt;0.01</td>
<td>1.12 ± &lt;0.01</td>
<td>0.08 ± 0.04</td>
<td>-0.99 ± &lt;0.01</td>
</tr>
<tr>
<td>2, 2012</td>
<td>1.20 ± 0.51</td>
<td>1.08 ± 0.46</td>
<td>0.12 ± 0.05</td>
<td>-0.06 ± 0.41</td>
</tr>
<tr>
<td>3, 2010</td>
<td>1.20 ± &lt;0.01</td>
<td>1.20 ± &lt;0.01</td>
<td>0.00 ± &lt;0.01</td>
<td>-0.99 ± &lt;0.01</td>
</tr>
<tr>
<td>3, 2012</td>
<td>1.18 ± &lt;0.01</td>
<td>1.18 ± &lt;0.01</td>
<td>0.00 ± &lt;0.01</td>
<td>-0.99 ± &lt;0.01</td>
</tr>
<tr>
<td>4, 2010</td>
<td>1.20 ± &lt;0.01</td>
<td>1.10 ± &lt;0.01</td>
<td>0.10 ± &lt;0.01</td>
<td>-0.99 ± &lt;0.01</td>
</tr>
<tr>
<td>4, 2012</td>
<td>1.20 ± &lt;0.01</td>
<td>1.06 ± &lt;0.01</td>
<td>0.14 ± &lt;0.01</td>
<td>-0.68 ± &lt;0.01</td>
</tr>
<tr>
<td>5, 2010</td>
<td>1.20 ± &lt;0.01</td>
<td>1.20 ± &lt;0.01</td>
<td>0.00 ± &lt;0.01</td>
<td>-0.99 ± &lt;0.01</td>
</tr>
<tr>
<td>5, 2012</td>
<td>1.20 ± &lt;0.01</td>
<td>1.20 ± &lt;0.01</td>
<td>0.00 ± &lt;0.01</td>
<td>-0.40 ± &lt;0.01</td>
</tr>
<tr>
<td>6, 2010</td>
<td>1.20 ± &lt;0.01</td>
<td>1.15 ± &lt;0.01</td>
<td>0.05 ± &lt;0.01</td>
<td>-0.08 ± &lt;0.01</td>
</tr>
<tr>
<td>6, 2012</td>
<td>1.20 ± &lt;0.01</td>
<td>1.20 ± &lt;0.01</td>
<td>0.00 ± &lt;0.01</td>
<td>-0.03 ± &lt;0.01</td>
</tr>
<tr>
<td>7, 2010</td>
<td>1.20 ± &lt;0.01</td>
<td>1.06 ± &lt;0.01</td>
<td>0.14 ± &lt;0.01</td>
<td>-0.16 ± &lt;0.01</td>
</tr>
<tr>
<td>7, 2012</td>
<td>1.20 ± &lt;0.01</td>
<td>1.20 ± &lt;0.01</td>
<td>0.00 ± &lt;0.01</td>
<td>-0.99 ± &lt;0.01</td>
</tr>
<tr>
<td>8, 2010</td>
<td>1.18 ± &lt;0.01</td>
<td>1.14 ± &lt;0.01</td>
<td>0.05 ± &lt;0.01</td>
<td>-0.99 ± &lt;0.01</td>
</tr>
<tr>
<td>8, 2012</td>
<td>na</td>
<td>na</td>
<td>na</td>
<td>na</td>
</tr>
<tr>
<td>9, 2010</td>
<td>1.20 ± &lt;0.01</td>
<td>1.12 ± &lt;0.01</td>
<td>0.08 ± &lt;0.01</td>
<td>-0.99 ± &lt;0.01</td>
</tr>
<tr>
<td>9, 2012</td>
<td>1.20 ± &lt;0.01</td>
<td>1.12 ± &lt;0.01</td>
<td>0.08 ± &lt;0.01</td>
<td>-0.99 ± &lt;0.01</td>
</tr>
<tr>
<td>10, 2010</td>
<td>0.91 ± &lt;0.01</td>
<td>1.04 ± &lt;0.01</td>
<td>-0.13 ± &lt;0.01</td>
<td>0.05 ± &lt;0.01</td>
</tr>
<tr>
<td>10, 2012</td>
<td>1.20 ± &lt;0.01</td>
<td>1.17 ± &lt;0.01</td>
<td>0.03 ± &lt;0.01</td>
<td>-0.99 ± &lt;0.01</td>
</tr>
</tbody>
</table>
4.4.4 Paternity Analysis

CERVUS suggested loci with low null allele frequencies (p > 0.05) at loci PeC104 and Pl15. As alleles with low null allele frequencies should not cause any problems (providing the error rate used in likelihood calculations is greater than zero) (Marshall et al. 1998; Kalinowski et al. 2007), loci PeC104 and Pl15 were included in the paternity analysis and potential null alleles treated as typing errors.

For the 2010 seed cohort, paternity was assigned to 31 seeds (17%) with 95% confidence and 147 seeds (83%) with 80% confidence. For the 2012 seed cohort, paternity was assigned to 20 seeds (12%) with 95% confidence and 139 seeds (84%) with 80% confidence. Failure to confidently assign paternity to six seeds (4%) from the 2012 population was due to poor amplification, resulting from insufficient power in the microsatellite markers. From the 2010 assignments, with 95% confidence, the pollen dispersal distance ranged from 0.00 – 4.74 km with a mean of 1.26 km. From the 2010 assignments, with 80% confidence, the pollen dispersal distance ranged from 0.00 – 5.01 km with a mean of 1.51 km. From the 2012 assignments with 95% confidence, the pollen dispersal distance ranged from 0.00 – 4.16 km, with a mean of 1.30 km. From the 2012 assignments with 80% confidence, the pollen dispersal distance ranged from 0.00 – 5.01 km, with a mean of 1.66 km. For comparison, the mean distance between all individuals in the study plot to the maternal trees was 1.91 km, with a range of 0.01 – 5.41 km (Figure 4.4).

Overall, the pollen-dispersal curve showed a reversed J-shaped distribution (Figure 4.4). Pollen dispersal distributions were not significantly different from the plant pair distributions in the both the 2010 and 2012 seed cohorts (Kolmogorov-Smirnov two sample test, p = 0.81 and 0.99, respectively), indicating random mating. However, in the 0.50 km distance class, the pollen dispersal frequency was more than double that expected under random mating, suggested a degree of near neighbour mating (Figure 4.4). There was no significant difference in pollen distributions between the 2010 and 2012 seed populations (Kolmogorov-Smirnov two sample test, p = 0.99). Furthermore, there was no significant difference in pollen distributions between the 2010 core and edge seed cohorts and the 2012 core and edge cohorts (Kolmogorov-Smirnov two sample test, p = 0.64 and 0.85, respectively). Pollen dispersal curves for each individual maternal tree in the core of the
Chapter Four: Pollen Dispersal

population are shown in Figure 4.5. Four of the five maternal trees displayed pollen dispersal distances less than 3.5 km, which reflects the distribution of all plants within the population to the maternal trees. Pollen dispersal curves for each individual maternal tree on the periphery of the population are shown in Figure 4.6. All maternal trees displayed pollen dispersal distances more than 3.5 km, again this reflects the distribution of all plants within the population to the maternal trees.

Pollen dispersal distributions were not significantly different from the plant pair distributions in both the 2010 and 2012 seed populations (Kolmogorov-Smirnov two sample test, p = 0.81 and 0.99, respectively), indicating random mating. There was no significant difference in pollen distributions between the 2010 and 2012 seed populations (Kolmogorov-Smirnov two sample test, p = 0.99).

In 2010, 111 trees contributed paternity to 178 offspring with 80% or more confidence for the ten maternal trees tested. The number of seed genotyped per maternal tree ranged from 12 – 20, with an average of 17.8. Paternity assignment was 100% for the 2010 seed cohort. The number of unique sires per maternal tree ranged from 11 – 19, with an average of 14.9 (Table 4.6). In 2012, 109 trees contributed paternity to 159 offspring with 80% or more confidence for the nine maternal trees tested. The number of seed genotyped per maternal tree ranged from 11 – 22, with an average of 18.3. Paternity assignment was 97% for the 2012 seed cohort. The number of unique sires per maternal tree ranged from 6 – 20, with an average of 15.8 (Table 4.6). There was no significant difference between mean number of seed tested per maternal tree, number of seeds assigned paternity and number of unique sires between the 2010 and 2012 seed cohorts, as measured by two sample t-test (p = 0.77, 0.95 and 0.64, respectively).

For the 2010 core and edge cohorts, the number of seed tested per maternal tree ranged from 19 – 20 and 12 - 20, with an average of 19.8 and 15.8, respectively. Paternity assignment was 100% for the 2010 seed cohort. The number of unique pollen sires ranged from 13 – 19 and 11 - 18, with an average of 16.4 and 13.4, respectively (Table 4.6). For the 2012 edge and core cohort, number of seed tested per maternal tree ranged from 11 – 22 and 10 - 20, with an average of 19.0 and 17.5, respectively. Paternity assignment was 97% for the core seed cohort and 96% for the edge cohort. The number of unique pollen sires...
ranged from 8 – 20 and 6 - 19, with an average of 16.4 and 15.0 respectively (Table 4.6). There was no significant difference between mean number of seed tested per maternal tree, number of seeds assigned paternity and number of unique pollen sires between the 2010 core and edge cohorts, as measured by two sample t-test (p = 0.06, 0.06 and 0.11, respectively). Similarly, there was no significant difference between mean number of seed tested per maternal tree, number of seeds assigned paternity and number of unique pollen sires between the 2012 core and edge cohorts, as measured by two sample t-test (p = 0.65, 0.66 and 0.72, respectively) (Table 4.7).

In 2010, only four of 111 and in 2012 only three of 109 pollen donors sired more than five offspring of the 343 genotyped, with most pollen donors siring only one of the genotyped offspring. In 2010, nine seeds were selfed as determined by CERVUS (maternal trees 1, 6, 8 and 10) of which two seeds come from core maternal trees and seven from edge maternal trees. In 2012 seven seeds were selfed (maternal trees 6, 7, 9 and 10) of which five seeds come from core maternal trees and two edge maternal trees. Maps of sampled plants from the study site showing each maternal tree, 2010 and 2012 sires and all genotype individuals are shown in Figure 4.7.
Table 4.6: Mean number per maternal tree of seed tested, seed assigned paternity and unique pollen donors for two seed populations of *Persoonia elliptica* in Avon Valley National Park, Western Australia estimated with nine microsatellite loci.

<table>
<thead>
<tr>
<th>Year</th>
<th>Location</th>
<th>Mean No. of seed tested</th>
<th>Mean No. of seed assigned paternity</th>
<th>Mean No. of unique pollen donors</th>
</tr>
</thead>
<tbody>
<tr>
<td>2010</td>
<td>Combined</td>
<td>17.8</td>
<td>17.8 (100%)</td>
<td>14.9</td>
</tr>
<tr>
<td>2010</td>
<td>Core</td>
<td>19.8</td>
<td>19.8 (100%)</td>
<td>16.4</td>
</tr>
<tr>
<td>2010</td>
<td>Edge</td>
<td>15.8</td>
<td>15.8 (100%)</td>
<td>13.4</td>
</tr>
<tr>
<td>2012</td>
<td>Combined</td>
<td>18.3</td>
<td>17.8 (97%)</td>
<td>15.8</td>
</tr>
<tr>
<td>2012</td>
<td>Core</td>
<td>19.0</td>
<td>18.4 (97%)</td>
<td>16.4</td>
</tr>
<tr>
<td>2012</td>
<td>Edge</td>
<td>17.5</td>
<td>16.7 (97%)</td>
<td>15.0</td>
</tr>
</tbody>
</table>
Table 4.7: Location, number of seed tested, number of seed assigned paternity at 80% confidence interval and number of unique pollen donors for each maternal tree per year of *Persoonia elliptica* in Avon Valley National Park, Western Australia estimated with nine microsatellite loci.

<table>
<thead>
<tr>
<th>Maternal, Year</th>
<th>Location</th>
<th>No. of seed tested</th>
<th>No. of seed assigned paternity</th>
<th>No. of unique pollen donors</th>
</tr>
</thead>
<tbody>
<tr>
<td>1, 2010</td>
<td>Edge</td>
<td>12</td>
<td>12 (100%)</td>
<td>11</td>
</tr>
<tr>
<td>1, 2012</td>
<td>Edge</td>
<td>20</td>
<td>20 (100%)</td>
<td>19</td>
</tr>
<tr>
<td>2, 2010</td>
<td>Edge</td>
<td>12</td>
<td>12 (100%)</td>
<td>12</td>
</tr>
<tr>
<td>2, 2012</td>
<td>Edge</td>
<td>20</td>
<td>20 (100%)</td>
<td>17</td>
</tr>
<tr>
<td>3, 2010</td>
<td>Core</td>
<td>20</td>
<td>20 (100%)</td>
<td>13</td>
</tr>
<tr>
<td>3, 2012</td>
<td>Core</td>
<td>21</td>
<td>20 (95%)</td>
<td>20</td>
</tr>
<tr>
<td>4, 2010</td>
<td>Core</td>
<td>19</td>
<td>19 (100%)</td>
<td>18</td>
</tr>
<tr>
<td>4, 2012</td>
<td>Core</td>
<td>22</td>
<td>22 (100%)</td>
<td>18</td>
</tr>
<tr>
<td>5, 2010</td>
<td>Edge</td>
<td>15</td>
<td>15 (100%)</td>
<td>14</td>
</tr>
<tr>
<td>5, 2012</td>
<td>Edge</td>
<td>20</td>
<td>19 (95%)</td>
<td>18</td>
</tr>
<tr>
<td>6, 2010</td>
<td>Core</td>
<td>20</td>
<td>20 (100%)</td>
<td>15</td>
</tr>
<tr>
<td>6, 2012</td>
<td>Core</td>
<td>20</td>
<td>20 (100%)</td>
<td>17</td>
</tr>
<tr>
<td>7, 2010</td>
<td>Core</td>
<td>20</td>
<td>20 (100%)</td>
<td>17</td>
</tr>
<tr>
<td>7, 2012</td>
<td>Core</td>
<td>11</td>
<td>11 (100%)</td>
<td>8</td>
</tr>
<tr>
<td>8, 2010</td>
<td>Edge</td>
<td>20</td>
<td>20 (100%)</td>
<td>12</td>
</tr>
<tr>
<td>8, 2012</td>
<td>Edge</td>
<td>0</td>
<td>0 (na)</td>
<td>0</td>
</tr>
<tr>
<td>9, 2010</td>
<td>Core</td>
<td>20</td>
<td>20 (100%)</td>
<td>19</td>
</tr>
<tr>
<td>9, 2012</td>
<td>Core</td>
<td>21</td>
<td>19 (90%)</td>
<td>19</td>
</tr>
<tr>
<td>10, 2010</td>
<td>Edge</td>
<td>20</td>
<td>20 (100%)</td>
<td>18</td>
</tr>
<tr>
<td>10, 2012</td>
<td>Edge</td>
<td>10</td>
<td>8 (80%)</td>
<td>6</td>
</tr>
</tbody>
</table>
Figure 4.4: Frequency distribution of observed pollination distances for all *Persoonia elliptica* seeds screened in Avon Valley National Park, Western Australia sampled in 2010 (blue bars) and 2012 (green bars); and frequency distribution of all plants within the population to the maternal trees (pink bars).
Figure 4.5: Frequency distribution of observed pollination distances for maternal trees in the core of the study plot in Avon Valley National Park, Western Australia, for seeds sampled in 2010 (blue bars) and 2012 (green bars); and frequency distribution of all plants within the population to the maternal trees (pink bars). Maternal tree number is displayed on the top right of each graph. Combined refers to the average frequency distribution for all maternal trees on the edge of the population.
Figure 4.6: Frequency distribution of observed pollination distances for maternal trees on the edge of the study plot in Avon Valley National Park, Western Australia, for seeds sampled in 2010 (blue bars) and 2012 (green bars); and frequency distribution of all plants within the population to the maternal trees (pink bars). Maternal tree number is displayed on the top right of each graph. Combined refers to the average frequency distribution for all maternal trees on the edge of the population.
Figure 4.7: Map of sampled plants from the study site in Avon Valley National Park, Western Australia showing each maternal tree (pink dot), 2010 sires (blue dots), 2012 sires (green dots), 2010 and 2012 sires (orange dots) and other genotyped individuals (black dots) for each maternal tree. Dot size corresponds to number of seeds sired. Maternal tree number is displayed on the top right of each figure.
Figure 4.7 cont: Map of sampled plants from the study site in Avon Valley National Park, Western Australia showing each maternal tree (pink dot), 2010 sires (blue dots), 2012 sires (green dots), 2010 and 2012 sires (orange dots) and other genotyped individuals (black dots) for each maternal tree. Dot size corresponds to number of seeds sired. Maternal tree number is displayed on the top right of each figure.
Figure 4.7 cont: Map of sampled plants from the study site in Avon Valley National Park, Western Australia showing each maternal tree (pink dot), 2010 sires (blue dots), 2012 sires (green dots), 2010 and 2012 sires (orange dots) and other genotyped individuals (black dots) for each maternal tree. Dot size corresponds to number of sires. Maternal tree number is displayed on the top right of each figure.
4.5 DISCUSSION

Effective long distance pollen dispersal in *P. elliptica* showed a departure from the predominantly nearest neighbour mating that is typical of plants pollinated by insect vectors (Ellstrand 1992; Ndiade-Bourobou et al. 2010; Van Rossum et al. 2011; Ottewell 2012). Furthermore, due to the scale and density of plants genotyped this study has detected realised pollen movements up to 5 km and near random mating across ca. 1, 300 ha, revealing a scale and frequency of long distance pollen dispersal by insects rarely recorded up till now.

4.5.1 Realised Mating Patterns

As the multi-locus outcrossing rate was not significantly different from one, the species can be described as preferentially outcrossing. In a predominantly outcrossed, diploid species only one allele is expected to be truly maternal, however the lack of non-maternal alleles in both the 2010/2012 combined seed cohorts suggested that some selfing may have occurred in both years. On the basis of these points alone, selfing could be deemed as a less than parsimonious explanation, and that the seed with seemingly two maternal alleles may simply have inherited an allele via its paternal contributor that is also present within the maternal genotype. Yet, when combined with the results from paternity analysis, there is good evidence for a low number of self-pollinated seed.

Previous assessment of the breeding system of a related *Persoonia* species (*P. mollis*) indicated that selfing is possible, but that there is preferential outcrossing with an emphasis on flexibility and post-zygotic choice following pre-zygotic "pseudo" self-incompatibility (Krauss 1994b). Thus, it is worth noting that because of this, the observed level of selfing may depend on the developmental stage at which the progenies are analysed. Thus, the level of self-fertilization may have been underestimated because inbreeding levels were measured in the mature seeds (i.e., after early acting inbreeding depression may have selected out some inbred progeny). The results nevertheless reinforce a predominance of outcrossing in *P. elliptica*. This finding is consistent with the mating systems for other members of the Proteaceae, which indicate that most preferentially cross fertilise. For example available molecular marker data, pollen exclusion studies and experimental hand pollinations from studies of other Proteaceae have generally indicated either pre- or post-zygotic selection against self pollen with a preference for outcrossing (e.g. *P. mollis,*

Despite the observed high outcrossing rate, some biparental inbreeding was evident in the seed cohorts. This suggests that although outcrossing is high some cross-fertilization events occur between close relatives. Correlations of outcrossed paternity were also significantly greater than zero in each of the seed cohorts, indicating a significant percentage of the seeds are full-sibs (up to 33 % for one maternal tree). Several other studies have also found a correlation between low plant densities, levels of biparental inbreeding and correlated paternity (e.g. Coates and Hamley 1999; Eduardo et al. 2008; Feres et al. 2012; Kamm et al. 2011). These studies suggest that reduced plant density may increase intra-plant pollinator movement and increase the likelihood of a pair of flowers on the same plant receiving pollen from the same donor. Eduardo et al. (2008) also suggest that mating among relatives is indicative of spatial genetic structure and vice-versa. SAA found fine scale SGS in the present population of *P. elliptica* (up to 0.50 km), which indicates that near neighbours are more likely to be relatives. In combination with the evidence for near neighbour mating these results from SAA can explain the observed mating among relatives and the levels of biparental inbreeding in seed cohorts.

### 4.5.2 Realised Pollen Dispersal

Pollen flow distances have generally been observed to have a leptokurtic distribution (Fenster 1991) with most dispersal occurring at short distances and a long tail of low level pollen dispersal over larger distances (Levin and Kerster 1974). However, low plant density has been found to influence the extent of near-neighbor foraging activity of pollinators promoting long pollen dispersal distances and high multiple paternity (Ward et al. 2005; Ndiade-Bourobou et al. 2010; Ismail et al. 2012). In this study, the high level of pollen dispersal across large distances (up to 5 km) from a wide range of donors confirms this theory and extends to our understanding of how density facilitates long pollen dispersal distances and high multiple paternity.

The maximum detected pollination distance of 5 km was larger than those that have been reported for most other trees and shrubs of the Australian Proteaceae (Forrest et al. 2011; Llorens et al. 2012; Ritchie and Krauss 2012). In these studies, pollen dispersal displayed
leptokurtic distributions with most dispersal occurring at short distances with a long tail of low level pollen dispersal over larger distances. However, the prevalence of intermediate distance pollination events and long distance pollen dispersal observed for *P. elliptica* is similar to that documented in other recent studies. For example, Eduardo et al. (2008) found that the average pollen flow distance for a low density population of bat pollinated *Hymanea courbaril* was $827 \pm 429$ m. Jha and Dick (2010) found extensive gene flow by bees for the neotropical tree *Miconia affinis*, with pollination events spanning more than 1.80 km. In addition, Fuchs and Hamrick (2011) found pollen flow over 4 km in the tropical tree *Guaiacum sanctum*. Extensive pollen flow distances mediated by wind-borne wasps have also been reported in populations of *Ficus* species, typically 6 – 14 km (Nason et al. 1998). Moreover, a review by Ward et al. (2005) found that pollen dispersal is widespread among low density tropical trees, ranging from a mean of 200 m to over 19 km for species pollinated by small insects or bats. These studies concluded that the observed long pollen dispersal distance was probably a combination of the low density of reproductive trees in the site and highly mobile pollinators.

This theory is supported by comparing patterns of pollen dispersal between this low density population of *P. elliptica* and its southeast Australian relative *P. mollis*, which occurs in high density populations (mean of 42.5 plant per ha). In contrast to the long distance dispersal and departure from predominantly near neighbour mating found for *P. elliptica*, realised pollen dispersal distances for *P. mollis* showed that 99% of the pollen received by given females was donated by males on average within 33 m (with a maximum of 72 m) and that 70% of all pollen dispersal was on average to the paternal plant's immediate neighbour (Krauss 1994a). Given that these two species have similar life history characteristics it seems probable that the low density spatial distribution of *P. elliptica* trees is affecting pollinator behaviour leading to reduction in near neighbour mating. Given the high mean realised dispersal distances found in this study for *P. elliptica*, intriguing questions about the foraging behaviour of its pollinators arise.

### 4.5.3 Long Distance Pollen Dispersal By Bees

In this study, feral honeybees (*Apis mellifera*) were observed collecting nectar from *P. elliptica* flowers and are therefore perceived as possible pollinators. Globally, introduced honeybees visit roughly one-third of the plant species in local floras (Huryn 1997). In
Australia, honeybees have been recorded visiting a wide range of native flowering plants (Paton 1993). In fact, *A. mellifera* is the most abundant insect pollinator in SWWA (Phillips et al. 2010). However, honeybees have been reported as “pollen robbers” regularly grooming pollen from their body, storing it in the corbiculae making it inaccessible for transfer onto the stigmatic surface (Paton 1993; Bernhardt and Weston 1996). Furthermore, honeybee movements are expected to be mostly among inflorescences on the same plant rather than between different plants (Paton 1993; Gross 2001), raising the possibility that honeybees promote selfing, biparental inbreeding and reduce pollen dispersal. Given this combination of traits, introduced honey bees may be inefficient pollinators for *P. elliptica*, a predominantly outcrossing species.

In addition to feral honeybees, native bees (species of *Amegilla* and *Megachilidae*) were also observed collecting and contacting stigmas of *P. elliptica* flowers and are potential pollinators. Native bees have been found to visit only a single flower per plant and then fly some distance, often away from the site (Anderson and Symon 1988; Rymer 2006). Such behaviour supports the argument that native bee species are outcrossing agents transmitting pollen over long distances.

The behaviour of honeybee and natives bees has been observed in other *Persoonia* species from eastern Australia (*P. mollis* ssp. *nectans*, *P. mollis* ssp. *maxima*, *P. lanceolata* and *P. glaucescens*). A study on the pollination biology of the above *Persoonia* species (Rymer 2006) found that honeybees visited an average of eight flowers on a plant before moving to another plant, which was predominantly nearest neighbour (0.5 – 2.0 m away). On the other hand, native bees visited fewer flowers within plants (ca. two) and moved greater distances between *Persoonia* plants (usually out of sight, > 5.0 m away). Therefore, given the behaviour and movement of native bee species versus feral honey bees, it could be suggested that at AVNP, native bees contribute more to the effective long distance dispersal of pollen for *P. elliptica*, than honeybees despite their greater abundance (pers. obs).

It should be noted that plant size and number of flowers per plant could affect foraging behaviour of pollinators. However, these parameters were not measured in this present study. Nonetheless, this study does show that near random mating and long distance pollen
dispersal in *P. elliptica* and that near-neighbour mating occurs less frequently than expected for an outcrossing insect pollinated species.

### 4.5.4 Genetic Diversity

The adult population of *P. elliptica* displayed high measured levels of genetic diversity. Similarly, genetic diversity measures for both the 2010 and 2012 combined seed cohorts were high. Consistent with the mating system and pollen dispersal results, there was little evidence for inbreeding (as evident from close to zero fixation indices) in the adult population at AVNP. Consistent with the realised pollen dispersal results there was evidence of selfing in both the 2010 and 2012 combined seed cohorts, as evident by the lack of non maternal tree alleles. However, this result is in contrast to the multi- and single locus outcrossing rates which suggest that *P. elliptica* is predominately outcrossed. A likely explanation for this is that program MLTR 3.4 (Ritland 2002) only provides estimates of maximum-likelihood mating system parameters, whereas calculation of non maternal tree alleles does not.

This result of high levels of genetic diversity and little inbreeding supports the findings of other studies on Proteaceae species (e.g. He et al. 2004; Heliyanto et al. 2005; Ritchie and Krauss 2012). These studies conclude that little inbreeding and high levels of genetic diversity were maintained because the study species is predominantly outcrossed and has extensive gene flow, either from pollen and / or seed dispersal.

The results from the present study are also in agreement with results from Chapter Three, which indicate that population density has no significant negative effect on most measures of genetic diversity and inbreeding, across the natural range of *P. elliptica*. The similarly high levels of genetic diversity found in seed cohorts and adult population in this current study indicate that one explanation for this is that pollinators are resilient to low plant densities and their behaviour is not dramatically impacted by large interplant distances. The similarly high levels of genetic diversity found in seed cohorts and adult population, support this explanation.

Chapter Three reports evidence of heterozygote excess (negative fixation indices) in populations of *P. elliptica* and suggested that this excess could be reflected in future
generations and, if so, should be detectable in the seeds. Chapter Three hypothesised that heterozygote excess could be caused by self-incompatibility systems, outcross breeding and low effective population size. However, this present study found no evidence of heterozygote excess (fixation indices were close to zero in the canopy seed) and that *P. elliptica* is predominately outcrossed. In addition previous assessment of the breeding system of a related *Persoonia* species indicated that there is pre-zygotic "pseudo" self-incompatibility (Krauss 1994b). Thus the heterozygote excess found in Chapter Three is most likely a reflection of very low density, reinforcing the need for a greater understanding of threshold densities.

### 4.5.5 Adult Population Genetic Structure

SGS as measured by the $S_p$ statistic, was an order of magnitude lower for *P. elliptica* ($S_p = 0.006$) than the average ($S_p = 0.030$) reported for 47 plant species (Vekemans and Hardy 2004). In fact, estimates of SGS with the $S_p$ statistic are among the lowest recorded and matched by natural populations of other southwest Australian plant species *Banksia hookeriana* and *B. attenuata* (Krauss et al. 2009; Ritchie and Krauss 2012). Although $S_p$ indicated overall weak genetic structure in the *P. elliptica* population, there was SGS to a distance of 0.50 km and a long-distance cline that decreases continuously with increasing geographic distance. An explanation for this SGS could be contrasting impacts of pollen and seed dispersal and the random mating that is facilitated primarily by the behaviour of highly mobile insects as pollinators. This explanation is further examined in Chapter Five, where patterns of frugivore-mediated seed dispersal are characterised. The presence of the long distance cline is indicative of isolation by distance (Diniz-Filho and Telles 2002; Peakall et al. 2003).

Evidence for weak within population SGS for the study population of *P. elliptica* at AVNP, reflects findings from Chapter Three, in that low density populations show weak SGS within populations (Antonovics and Levin 1980; Loveless and Hamrick 1984). This result emphasizes the effect of wide pollen dispersal by bees on SGS in a population of *P. elliptica*.
4.5.6 Core Versus Edge Effects

The abundant centre distribution model (Andrewartha and Birch 1954; Whittaker 1975; Brown 1984) predicts that plants on the periphery of a population will display lower genetic diversity and higher inbreeding rates (Lesica and Allendorf 1995) compared to plants in the centre of the population. This is because plant density is expected to decrease towards the periphery of the population, thus affecting pollinator availability and behaviour leading to fewer pollinator visitors. This in turn results in lower pollen flow and lower genetic diversity. Inbreeding is expected to increase as plant density decreases due to reduced mate diversity (Keller and Waller 2002; Biebach and Keller 2010). Contrary to this prediction, maternal trees on the edge of the study population produced seed that displayed comparable levels of genetic diversity and inbreeding rates to those on the periphery.

Despite theoretical and empirical predictions for periphery versus core effects this study joins others that did not observe lower genetic diversity and higher inbreeding rates on the periphery of a population or a species range. For example, while some authors have concluded that genetic diversity declines toward range peripheries (e.g. Guries and Ledig 1982; Rajora et al. 2002), there is approximately equal empirical evidence against this prediction from studies where no differences in genetic diversity have been found among central and peripheral populations (e.g. Mouna et al. 1990; Gamache et al. 2003; Muir et al. 2004). Estimated multilocus outcrossing rate was slightly but not significantly higher in central compared to a peripheral population for both yellow star thistle (Centaurea solstitialis, Sun and Ritland 1998) and eastern white pine (Pinus strobus, Rajora et al. 2002). These studies conclude that pollen mediated gene flow is sufficient to maintain high levels of genetic diversity and outcrossing rates on the periphery of the species range. This is also a likely explanation for this population of P. elliptica as this study found evidence of long distance dispersal up to 5 km and a departure from the predominantly nearest neighbour mating. Furthermore, this study population exists within a national park of intact jarrah forest, where the abundance of co-flowering species provides an incentive for pollinators to travel to the core of the population well as the periphery.

It is important to note that these species have different mating systems to P. elliptica (i.e. eastern white pine is wind pollinated) and that these studies compare populations on the periphery to those in the centre of the species range. Therefore, these results should be
treated with caution. However, overall when comparing seed from each maternal tree in turn, there is no significant difference in any parameters measured. This reinforces that mating and pollen dispersal patterns are robust to position within the population, consistent with highly mobile insect pollinators.

4.5.7 Conclusions
Utilising a large, geographically isolated population to characterise pollen dispersal patterns of an insect-pollinated tree, this study has demonstrated that near random mating and long distance pollen dispersal is a characteristic of a very low density *P. elliptica* population and near-neighbour mating occurs less frequently than expected for an outcrossing insect pollinated species. Furthermore, this study found that the spatial position of a tree in a population does not affect patterns of paternity and pollen dispersal. This study is one of the first that delves into the patterns of pollen dispersal by nectarivorous insects in SWWA and has shown that for low plant density species *P. elliptica*, the low density is apparently no hindrance to extensive long-distance pollen flow.
CHAPTER FIVE

Long distance dispersal of *Persoonia elliptica* seed by macropods, inferred from parentage analysis

5.1 ABSTRACT

Seed dispersal plays a vital role in the ecological and evolutionary dynamics of a plant species, yet given the inherent difficulty in detecting long distance dispersal, there are limited empirical studies that quantify the tail of the dispersal curve. Nine microsatellite markers were used to measure long distance seed dispersal by assigning parentage to dispersed seed recovered from fauna scat in a natural population of the fleshy-fruited southwest Australian tree, *Persoonia elliptica*. Using motion sensor cameras, two macropod species (*Macropus fuliginous* and *M. irma*) were identified feeding on *Persoonia* fruit and therefore as potential seed dispersal vectors. One hundred and twenty-seven endocarps containing seed were collected from macropod scat and mapped and genotyped, together with every adult tree (n = 450) in a ca. 1,300 ha study site. Seed dispersal distances estimated from parentage assignments suggest that macropods moved seeds away from the parent tree and over long distances (mean 1.57 km and up to 4.40 km). This study emphasises the ecological and evolutionary significance of macropods as vectors for LDD of seed for *P. elliptica* and as such impacts on these macropods will have potential consequences for *P. elliptica*. 
5.2 INTRODUCTION

Gene dispersal is the movement and subsequent establishment of genes (Levin and Kerster 1974) and is a critical process in a healthy ecosystem. Dispersal influences the physical and genetic structure of populations and is a key determinant of the range of a species, migration rates and the capacity for colonisation of new habitat patches (Hamrick and Godt 1989; Schupp and Fuentes 1995; Hamrick and Godt 1996; Bowman et al. 2002; Trakhtenbrot et al. 2005). In seed plants, gene dispersal occurs during two independent life history stages: pollen and seed. Pollen is haploid (i.e. only male gametes are dispersed), whereas seeds are generally diploid and typically both male and female gametes are dispersed (Ghazoul 2005). Therefore, dispersed seeds that become established represent a gene flow event that is twice as effective as a pollen flow event (Hamrick and Trapnell 2011).

The frequency distribution of the dispersal distances reached by seeds constitutes the dispersal curve, and is typically characterised by a high frequency of short distance movements and a long tail of low frequency long distance dispersal (LDD) events. Long distance dispersal has been defined as a seed dispersal distance of at least 100 times greater than plant height (Nathan and Muller-Landau 2000; Nathan et al. 2003). Short distance dispersal events help to determine resource use, recruitment patterns species co-existence (Nathan and Muller-Landau 2000). LDD events are comparatively rare and have impacts at larger (regional/global) scales, directly affecting spatial spread and colonization rates (Nathan 2006; Nathan 2008). Thus, LDD affects both ecology (resource use, species co-existence and large-scale metapopulation dynamics) and evolutionary (gene flow, genetic structure and species diversity) trajectories (Ouborg et al. 1999; Cain et al. 2000; Webster et al. 2002; Nathan 2006). In spite of its importance to plant species, the, mechanisms of LDD are poorly understood, owing to a scarcity of empirical studies. In particular, there is a lack of knowledge on the role that animal vectors play in shaping patterns of seed dispersal.

As plants are sessile many have evolved traits to assist in the LDD of their seeds through the actions of specific dispersal vectors. There are numerous ways that LDD of seed can occur; these include animal dispersal (by adhesion or ingestion) (e.g. Ndiade-Bourobou et al. 2010; Sebben et al. 2010), wind dispersal (in updrafts and storms) (e.g. He et al. 2004; Freeland et al. 2012) and water dispersal (e.g. Soomers et al. 2012). Seed dispersal by
animals is a complex mutualistic interaction involving a great diversity of plant and animal species, and has significant ecological and evolutionary consequences (Howe and Smallwood 1982; Herrera 2002). Animals are a main seed disperser for many plant species, carrying seeds either internally (endozoochory, seeds consumed and passed through the gut) or externally (epizoochory, barbed or sticky seeds attached to their fur/feathers) (Regal 1977; Herrera 1995; Corlett 2002; Stiles 2002).

Fleshy-fruited species dominate the flora in many temperate and tropical habitats (Jordano 2000) and considerable information has accumulated in recent years on the ecology of endozoochory and seed dispersal in these habitats (Clark et al. 2004; Silvius and Fragoso 2003; Sezen et al. 2007; Haugaasen et al. 2010). However, fleshy-fruited species represent a remarkably small proportion of species in Australian ecosystems (Dennis et al. 2007) and information on the importance of endozoochory is limited. This is especially true in southwest Western Australia (SWWA) (Calvín-Cancela et al. 2006), where few animal species act as seed dispersers for fleshy-fruited species.

The emu (*Dromaius novaehollandiae*) is a large frugivorous bird that feeds on a variety of plant material and, as a consequence of long gut retention times and high mobility, provides opportunities for LDD of seed (Calvín-Cancela et al. 2006). Mammal species found within SWWA, such as the common brushtail possum (*Trichosurus vulpecular*), western grey kangaroo (*Macropus fuliginosus*), western brush wallaby (*Macropus irma*) and the bush rat (*Rattus fuscipes*), also provide an opportunity for LDD of seed as they are all known to consume fruits and act as seed dispersers for various SWWA species (Clifford and Drake 1985; Snow and Walter 2007; Calviño-Cancela 2011). Emus and macropods are the most abundant of these native vertebrate frugivores in SWWA (Calvín-Cancela et al. 2008). However, to date there has been limited research into their potential role in LDD (He et al. 2009) and these studies have primarily focused on the diversity of seed being dispersed (Calvín-Cancela et al. 2006; Calvín-Cancela et al. 2008), rather than actual seed dispersal distances. Despite the fundamental role of LDD events in dispersal ecology (Anderson 1991; Ouborg et al. 1999), there is a paucity of studies that have attempted to quantify the tail of the dispersal curve, especially for species in SWWA.

Neutral genetic markers are a powerful, and in many cases the only, tool to directly estimate contemporary seed dispersal patterns by genetically identifying the source of
dispersed fruits, seeds or established seedlings (Jones and Arden 2003). Likelihood-based parentage analysis is the most commonly used method for estimating seed dispersal distance for each mating event and the current rates of seed immigration into sampled populations (Smouse and Sork 2004; Hardy et al. 2006). Parentage analyses rely on biparentally inherited markers to identify the genetically most likely pair of adults for each seed or seedling (Jones and Arden 2003). This analysis requires identification of all potential parents within a population and knowledge of their multilocus genotypes as well as the multilocus genotypes of each seed or seedling being studied (Jones and Arden 2003). Furthermore, if the species is hermaphroditic or neither parent is known, it is often not possible to determine which member of the parent pair is the maternal/paternal individual (Godoy and Jordano 2001). Given the inherent difficulty in detecting LDD events, the majority of studies on seed dispersal have focused on short distance dispersal (Herrera et al. 1994; Calvinô-Cancela 2002; Gódinez-Alvarez et al. 2002), with relatively little empirical data on LDD (Nathan 2012).

Within SWWA, it has been hypothesized that natural selection has resulted in mechanisms promoting local persistence rather than wide dispersal and colonization (Hopper et al. 1996; Hopper 1997; 2000). This persistence is thought to be a result of the extended absence of major geomorphological agents of soil disturbance and rejuvenation often associated with volcanism, mountain building and glaciations (Hopper and Goia 2004). However, the flora along wetlands and around rock outcrops (where regular soil disturbance has been prominent) have been known to exhibit better mechanisms for long distance dispersal and colonization (Hopper and Goia 2004).

However, there are very few empirical studies that have tested the low dispersal over local persistence hypothesis. This study aims to address this by directly quantifying patterns of frugivore-mediated LDD in a SWWA fleshy-fruited understory tree species. *Persoonia elliptica* produces large-seeded fruits and exists in the jarrah (*Eucalyptus marginata*) forest of SWWA in populations of very low densities (< 0.01 – 0.34 plants per ha, Chapter Three). Emus are thought to be the key seed dispersal vector within the jarrah forest for *P. elliptica* (Abbott and Van Heurck, 1988), although a number of other species are thought to also play a role in the LDD of *P. elliptica* seed, including the common brushtail possum (*Trichosurus vulpecular*), western brush wallaby (*Macropus irma*), currawong (*Strepera*...........
versicolor) and raven (Corvus coronoides) (Abbott and Van Heurck 1988). The study site within Avon Valley National Park (AVNP), Western Australia has an abundant year round population of emus, and macropods are also present, although in much lower numbers (pers. obs). Given the potentially high mobility of these species (Davies 2002), they provide a capacity for LDD for the plant species on which they feed. The isolation of the study population combined with large distances between neighbouring individuals allows all 450 trees in a large study area (ca. 1,300 ha) to be genotyped, providing a unique opportunity to assess LDD and the tail of the seed dispersal curve through an assignment of parentage to dispersed seed.

This research aims to apply molecular markers and methods of parentage analysis to assign parentage to dispersed seed recovered from fauna scat in a geographically isolated study population P. elliptica, from which long distance seed dispersal can be quantified. Specifically this research has the intent to determine whether:

- *Dromaius novaehollandiae* are a LDD vector of seed for *P. elliptica* in the study site.
- Macropods are a LDD vector of seed for *P. elliptica* in the study site.
- Long distance seed immigration within the study population can be identified and quantified
- LDD of seed is greater than that of pollen for *P. elliptica* in the study site.
- Genetic diversity within the dispersed seed is comparable to that of the adult population.
Chapter Five: Seed Dispersal

5.3 METHODS AND MATERIALS

5.3.1 Study Species

*Persoonia elliptica* R.Br (spreading snottygobble) is an erect spreading shrub or tree (2 – 8 m), with a hard corky grey and somewhat crumbly bark, which survives fire by resprouting (Bell et al. 1993; Florabase 2013). *Persoonia elliptica* occurs largely as well-separated trees in populations of very low densities. It occurs within 50 km of the coast, from Perth to Albany in SWWA and grows in jarrah forest, in sandy soil or laterite (Weston 1995) (Figure 1.1). *Persoonia elliptica* flowers from October to February, with yellow green hermaphroditic flowers. *Persoonia elliptica* is preferentially outcrossing (Chapter Four) and is insect pollinated. Once fertilisation has occurred, the fleshy fruits remain in the canopy until release in August to November (Weston 2003). Fruits are drupes, oval and change from bright green to deep purple when ripe. The fleshy fruits are clearly adapted for animal dispersal and trees can produce large quantities of viable fruit (Abbott 1984; Monaco 2012).

5.3.2 Study Site

The study was conducted within AVNP (-31.57E 116.16S) a 4,800 ha reserve located in SWWA (Figure 4.1). The study plot was located towards the northern edge of AVNP and encompasses ca. 1,300 ha of jarrah forest, where *P. elliptica* occurs more or less continuously throughout, at a density of 0.34 plants per ha (Chapter Three). The eastern side of the study plot is bounded by wandoo forest, the northern side by privately owned semi-cleared land, the southern side by granite outcrops; and the western side by jarrah forest, with no *P. elliptica* present. No *P. elliptica* were evident outside the study plot. The nearest known population of *P. elliptica* is 25 km away to the northeast at Julimar National Park; however there is the possibility of isolated *P. elliptica* trees being present within the remainder of AVNP. *Dromaius novaehollandiae* and species of macropods are found within the study site and in the surrounding national park.

5.3.3 Identification of Seed Dispersal Vectors

Emus (*Dromaius novaehollandiae*) were believed to be a main vector of *P. elliptica* seed dispersal within AVNP due to previous research identifying their importance (Abbott and Van Heurck 1988) and their abundance in the area. However, a thorough search of approximately 200 *D. novaehollandiae* scat within the study area failed to produce any *P.
elliptica seed. Therefore, to determine dispersal vectors of \textit{P. elliptica} and assist in the collection of animal dispersed seed, two Bushnell Trophy Cam Trail cameras (which are triggered by movement and use infrared light wavelengths for recording at night (Bushnell Australia 2011)), were set and focused on two arbitrarily chosen fruiting \textit{P. elliptica} trees for a period of three non–consecutive days in October 2010. Cafeteria experiments were set up using freshly collected drupes to determine what species were consuming and possibly dispersing seeds. Drupes were placed on the ground and a camera was focused on these piles to record any visitation. Cameras were set up to photograph when triggered, with a ten second interval before being able to be triggered again. Camera footage was observed to link any activity to missing or stripped fruits. After photographic identification of potential seed disperser vectors, scat from these species was searched to confirm dispersal.

5.3.4 Sample Collection
All 450 adult \textit{P. elliptica} trees in the ca. 1,300 ha study plot were sampled in 2010 as described in Chapter Four (Figure 4.1). In August 2010 and September 2012, 15 and 20 (respectively) arbitrarily chosen study plots 100 m in diameter were systematically searched for macropod scat. Scat collection was not conducted in 2011 due to site wide reproductive failure of plants in that year. Each scat was broken up to remove endocarps, which if present, were identified using reference endocarps collected from trees in the study area. GPS readings recorded for each endocarps and endocarps were maintained in separate bags.

5.3.5 DNA Extraction
Nuclear DNA was extracted from adult leaf material and seeds using a procedure modified from Jobes et al. (1995), as described in Chapter Two.

5.3.6 Microsatellite Genotyping
Multiplex PCR was performed on DNA extracted from adult leaves and seeds for nine microsatellite loci previously optimised for \textit{P. elliptica} (\textit{PeA102, PeA103, PeA106, PeB103, PeB106, PeB2, PeC104, PeC2} and \textit{Pl15}) (see Table 2.2). PCR amplification, fragment separation and allele size scoring were conducted as described previously in Chapter Two. Positive-control samples were replicated within and between plates to ensure consistency of allele size scoring.
5.3.7 Data Analysis

5.3.7.1 Genetic Diversity

Population genetic diversity statistics were calculated for the adult population and seed collected from 2010 and 2012 scat cohorts. The number of alleles \((N_A)\), observed and expected heterozygosities \((H_O\) and \(H_E\) respectively), fixation indices \((F)\) and number of private alleles \((S)\), for the adult and seed cohorts were calculated using GenAlEx v 6.5 (Peakall and Smouse 2006; 2012). The number of alleles was standardized for sample size (allelic richness) using rarefaction as implemented in FSTAT version 2.9.3.2 (Goudet 1995; 2001). Values were standardized to the sample size of the smallest cohort (2012 seed). A one-way Analysis of Variance (ANOVA) using SPSS (PASW Statistics for Windows, Version 18.0, Chicago) assessed the statistical significance of differences in genetic diversity statistics between adult and seed cohorts.

5.3.7.2 Seed Cohort Genetic Structure

The spatial genetic structure (SGS) of the collected seed was assessed using Spatial Autocorrelation Analysis in GenAlEx v 6.5. The analysis was conducted using all loci and variable distance classes with 12 classes in total. Spatial structure graphs (correlograms) were used to display the correlation between genetic distances of trees by geographic distance. A correlogram was produced with calculated correlation coefficient \(r\) and with upper and lower confidence limits, as generated by 999 random permutations of the data and bound by the 95% confidence interval about the null hypothesis of no spatial structure (Peakall and Smouse 2006). The distance where \(r\) intercepts the x-axis provides an estimate of the spatial extent of genetic structure, beyond which gene flow is no longer effective in connecting populations.

SGS was further quantified by calculating the \(S_p\) statistic using SPAGeDi 1.3 (Hardy and Vekemans 2002). The \(S_p\) statistic was determined as \(b / (1 - F_1)\), where \(b\) is the mean regression slope with natural log of distance (ln) and \(F_1\) is the mean pairwise kinship coefficient for the smallest distance class (Vekemans and Hardy 2004). The \(S_p\) statistic accounts for differences in spatial genetic structure due to variation in sampling schemes by considering average kinship across individuals relative to the extent of the decrease in \(F\) across distance intervals. A low \(S_p\) value means that genetic structure is weak thus; it can be used to compare the extent of spatial genetic structure across species (Vekemans and Hardy...
To increase the power to detect SGS, both dispersed seed cohorts were combined in all SGS analyses.

5.3.7.3 Parentage Analysis

Parentage analysis was conducted on seeds collected from each year to determine dispersal distance. Maximum likelihood parent pairs were assigned using the software CERVUS v 3.0.3 (Marshall et al. 1998; Kalinowski et al. 2007), which uses categorical allocation to assign offspring to the plant pairs that have the highest likelihood of being the sire by calculating likelihood ratios (LOD). CERVUS calculates LOD scores for each candidate parent or parent pair and then evaluates the confidence of the LOD score of the most likely candidate parent pair using the appropriate LOD criteria calculated by simulation. The simulation parameters for CERVUS to assign parentage to the most-likely parent pair with a known level of statistical confidence were as follows: 10,000 cycles of simulation, 450 candidate parents, and 0.01 as the proportion of mistyped loci (as recommended by CERVUS), with confidence levels of 95%, 80% and 50%. Confidence levels are levels of tolerance of ‘false positive’ paternities, or paternities assigned to males who match by chance (Marshall et al. 1998) (i.e. at 80% confidence level, 20% of assignments may be false positives by chance). In addition, the estimated proportion of known potential sires was set at 95% due to the isolation of the population, which enabled exhaustive sampling of all potential parents.

Seed dispersal curves for parentage assignments at both the 80% and 50% confidence levels were constructed by graphing the frequency of seed dispersal events (2010 and 2012 were combined to increase statistical power) against the distance between all plant pairs within the population. Also plotted were the pollen dispersal curves calculated in Chapter Four, for distance classes of 0.50 km. As the 2010 and 2012 pollen dispersal curves were found to be not significantly different (Chapter Four), the two years were combined. The seed dispersal curve, distance frequency of all plant pairs within the population and pollen dispersal curve were then compared using a Kolmogorov-Smirnov two-sample test (Siegel 1956) in SPSS. For each seed assigned parentage, seed dispersal distances were calculated based on the geographic position of the parent pairs within the plot. CERVUS is unable to distinguish maternal from paternal parent, thus each seed to parent distance was included as a potential seed dispersal distance.
Chapter Five: Seed Dispersal

5.4 RESULTS

5.4.1 Identification of Seed Dispersal Vectors

The two motion sensor cameras photographed three fauna species, but only two of these were behaving in a way that could potentially disperse seeds. *Macropus fuliginosus* (western grey kangaroo) (Figure 5.1a) and *M. irma* (brush tail wallaby) (Figure 5.1b) were both observed interacting with seeds during the cafeteria experiments. *Dromaius novaehollandiae* (emu) was photographed by the motion sensor cameras; however, it only triggered the motion sensor in passing and they did not interact with fruits. (Figure 5.1e) The morning after the cafeteria experiment, drupes were observed to be missing and assumed dispersed by both *M. fuliginosus* and *M. irma*.

To confirm seed dispersal by the macropods, arbitrarily chosen macropod scat was searched for *P. elliptica* seed. This preliminary search revealed the presence of *P. elliptica* seed (Figure 5.1c; 5.1d) and thus confirmed that *M. fuliginosus* and *M. irma* are seed dispersal vectors.

5.4.2 Scat Collection

In 2010, macropod scat containing *P. elliptica* seed was found in 11 of the 15 sites (Figure 5.2). From these 11 sites, 80 endocarps containing seeds from 71 macropod scats were found containing at least one *P. elliptica* endocarp. Five scats contained two endocarps and two scats contained three endocarps. In 2012, macropod scat containing *P. elliptica* endocarps was found in 18 of the 20 sites (Figure 5.3). From these 18 sites, 47 seeds were found in 44 scats, with three scats containing two endocarps. The shape and size of *M. fuliginosus* and *M. irma* scat are similar and in this study they were unable to be distinguished from each other. Henceforth, the term macropod will be used to describe both species.
Figure 5.1: a) Photograph of *Macropus fuliginosus* (western grey kangaroo); and b) *M. irma* (brush tail wallaby) consuming fruits of *Persoonia elliptica* in Avon Valley National Park, Western Australia taken by a Bushnell Trophy Cam Trail camera; c) photograph, d) x-ray photograph of *P. elliptica* seed in macropod scat from Avon Valley National Park, Western Australia and e) photograph of *Dromaius novaehollandiae* (emu) passing but not interacting with fruits in Avon Valley National Park, Western Australia taken by a Bushnell Trophy Cam Trail camera.
Figure 5.2: Map of sampled sites searched for macropod scat in 2010, in Avon Valley National Park, Western Australia. Blue dot represents sites where *Persoonia elliptica* seed was found in scat and red dot represents sites where no *P. elliptica* seed was found in scat. Dot sizes correspond to the number of seed found.
Figure 5.3: Map of sampled sites searched for macropod scat in 2012, in Avon Valley National Park, Western Australia. Green dot represents sites were *Persoonia elliptica* seed was found in scat and red dot represents sites were no *P. elliptica* seed was found in scat. Dot sizes correspond to the number of seed found.
5.4.3 Genetic Diversity

In total, 143 alleles were detected across nine loci for the adult and macropod-dispersed seed cohorts of *P. elliptica*. The adult population had a significantly higher mean number of alleles per locus (19.22 ± 2.75) than both the 2010 (10.00 ± 1.27) and 2012 (11.56 ± 1.73) macropod-dispersed seed cohorts (p < 0.01). The number of alleles in the 2010 and 2012 macropod-dispersed seed cohorts, were not significantly different (p = 0.50). However, when standardized for sample size, allelic richness was not significantly different between the adult and macropod-dispersed seed cohorts (p = 0.64). The adult, 2010 and 2012 combined macropod-dispersed seed cohorts displayed comparable levels of observed heterozygosities (p = 0.38), expected heterozygosities (p = 0.80) and fixation indices (p = 0.30) (Table 5.1). Only the adult population displayed private alleles ($S = 5.67 ± 1.32$), strongly suggesting that not all plants contributed parentage to the sampled seeds. The absence of private alleles in the macropod-dispersed seeds shows that there is no evidence that these seeds originated from outside the sampled local population.

5.4.4 Seed Cohort Spatial Genetic Structure

Spatial Autocorrelation Analysis (SAA) identified significant genetic structure for the macropod-dispersed seed cohorts at distances of up to 0.5 km, with $r$ falling outside the upper and lower confidence levels (95%) (Figure 5.4). The $r$ value at 0.50 km ($r = 0.55; p = 0.01$) indicates moderate genetic structure at these distances. Additionally, the correlogram shows a clear trend toward increasingly negative autocorrelation with increasing distance. SAA for the adult population (calculated in Chapter Four) is shown in Figure 5.4b for comparison. The SAA for the macropod-dispersed seed cohort displays a similar trend to the adult population, but it is not a pronounced.

Additional evidence of spatial genetic structure in the macropod-dispersed seed cohorts was supported by the estimate of $S_P$ from SPAGeDi 1.3. The regression slope ($b$) was negative (-0.005) and the kinship coefficient estimate for adjacent individuals in the first distance interval was 0.008. Consistent with this, was the genetic structure among seeds as measured by the $S_P$ statistic ($S_P = 0.005$).
Table 5.1: Genetic diversity parameters for the adult population, the 2010 and 2012 macropod-dispersed seed cohorts for *Persoonia elliptica* in Avon Valley National Park, Western Australia. N: number of individuals, \(N_A\): average number of alleles, \(A_R\): allelic richness (standardised number of alleles), \(H_O\): observed heterozygosity, \(H_E\): expected heterozygosity, \(F\): fixation index, \(S\): average number of private alleles.

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>(N_A)</th>
<th>(A_R)</th>
<th>(H_O)</th>
<th>(H_E)</th>
<th>(F)</th>
<th>(S)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adult</td>
<td>450</td>
<td>19.22 ± 2.75</td>
<td>9.81 ± 1.24</td>
<td>0.75 ± 0.04</td>
<td>0.79 ± 0.04</td>
<td>0.06 ± 0.04</td>
<td>5.67 ± 1.32</td>
</tr>
<tr>
<td>2010 Seed</td>
<td>80</td>
<td>11.56 ± 1.73</td>
<td>9.09 ± 1.33</td>
<td>0.70 ± 0.04</td>
<td>0.76 ± 0.04</td>
<td>0.07 ± 0.04</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>2012 Seed</td>
<td>47</td>
<td>10.00 ± 1.27</td>
<td>8.72 ± 1.06</td>
<td>0.75 ± 0.06</td>
<td>0.76 ± 0.04</td>
<td>0.01 ± 0.07</td>
<td>0.00 ± 0.00</td>
</tr>
</tbody>
</table>
Figure 5.4: a) Spatial autocorrelation analysis correlogram for a) the dispersed seed and b) adult population of *Persoonia elliptica* in Avon Valley National Park, Western Australia showing the genetic correlation coefficient \( r \) for increasing distance class sizes, with 95% confidence intervals about \( r \) as determined by bootstrapping. Distance class option on x-axis is start point.
5.4.5 Parentage Assignment

For the 2010 and 2012 macropod-dispersed seed cohorts, no parent pairs were assigned with 95% confidence. For the 2010 macropod-dispersed seed cohort seven seeds (8.8%) were assigned parent pairs with 80% confidence and for the macropod-dispersed 2012 seed cohort, four seeds (8.5%) were assigned parent pairs. At the 50% confidence level, 100% of seeds were assigned parent pairs for both the 2010 and 2012 macropod-dispersed seed cohorts.

For the 2010 assignments, the seed dispersal distances at the 80% confidence level ranged from 0.18 – 3.88 km, with a mean of 1.61 km (Figure 5.5). At the 50% confidence level, seed dispersal distances in 2010 ranged from 0.06 – 4.35 km, with a mean of 1.56 km. For the 2012 assignments, the seed dispersal distances at the 80% confidence level ranged from 0.72 – 2.00 km, with a mean of 1.50 km (Figure 5.6). At the 50% confidence level, seed dispersal distances in 2012 ranged from 0.02– 4.40 km, with a mean of 1.61 km.

Overall, seed dispersal distances at 50% and 80% confidence displayed a higher fraction of dispersal events at smaller distances, than both plant pair distributions and pollen dispersal distances. Seed dispersal distributions were not significantly different to the plant pair distributions and pollen dispersal distances (Kolmogorov-Smirnov two sample test, p = 0.40 and 0.72, respectively) (Figure 5.7). However, in the 0.50 – 1.50 km distance classes the seed dispersal frequency is noticeable higher than for the inter plant distances and the 2.0 – 2.50 km distance classes the seed dispersal frequency is noticeable lower than for the inter plant distances. Seed dispersal distributions at 50% confidence were not significantly different to the plant pair distributions and pollen dispersal distances (Kolmogorov-Smirnov two sample test, p = 0.99 and 0.98, respectively) (Figure 5.7). Furthermore, seed dispersal distributions were not significantly different for assignments at the 80% and 50% confidence levels (Kolmogorov-Smirnov two sample test, p = 0.99).

For the seven scat collected in 2010 that contained more than one endocarp, four had endocarps with a common parent and the other three did not. For the three scat collected in 2012 that contained more than one endocarp, only one contained endocarps with a common parent. These common pairs are assumed to be the maternal parent. For the four scats containing seeds with assumed known maternal trees, seed dispersal distances ranged from
0.30 – 2.28 km, with a mean of 1.13 km. For the one scat containing seeds with assumed known maternal trees, the seed dispersal distance was 0.51 km. Two of the 2010 scats containing more than one endocarp with a common maternal were found in the same study site and both shared the same maternal parent.
Figure 5.5: Map of *Persoonia elliptica* seed assigned parentage at 80% confidence that were collected in 2010 from macropod scat in Avon Valley National Park, Western Australia. Triangles represent seed and the circles of the corresponding colour represent parent pairs.
Figure 5.6: Map of *Persoonia elliptica* seed assigned parentage at 80% confidence that were collected in 2012 from macropod scat in Avon Valley National Park, Western Australia. Triangles represent seed and the circles of the corresponding colour represent parent pairs.
Figure 5.7: Frequency distribution of observed seed dispersal distances at 80% confidence for *Persoonia elliptica* seeds screened in Avon Valley National Park, Western Australia. Seed dispersal (blue bars), pollen dispersal (orange bars) and frequency distribution of all plant pairs within the population (green bars).
Figure 5.8: Frequency distribution of observed seed dispersal distances at 50% confidence for *Persoonia elliptica* seeds screened in Avon Valley National Park, Western Australia. Seed dispersal (blue bars), pollen dispersal (orange bars) and frequency distribution of all plants pairs within the population (green bars).
5.5 DISCUSSION

Macropods were shown to play an important role in the LDD of \textit{P. elliptica} seed, both in terms of confirming the presence of seed in their scats, but also in terms of the distances these seeds were moved from their maternal origin. Realised seed dispersal distances inferred from parentage assignments showed that macropods consume and move seeds away from the parent tree. Seeds in macropod scats were found to be dispersed between 0.02 – 4.40 km, with a mean of 1.57 km. These findings provide important empirical evidence that doesn’t support the hypothesis that natural selection in SWWA has resulted in mechanisms promoting local persistence rather than wide dispersal (Hopper et al. 1996; Hopper 1997; 2000).

5.5.1 Identification of Seed Dispersal Vectors

Contrary to the prediction that emus are a key seed dispersal vector of \textit{P. elliptica} within the jarrah forest (Abbott 1984b), no evidence for seed dispersal by emus was found in this study. However, macropod species (\textit{M. fuliginosus} and \textit{M. irma}) were found to be effective dispersers of seed in AVNP. A concurrent study on \textit{P. elliptica} using motion sensor cameras (Monaco 2012) also identified \textit{M. fuliginosus} and \textit{M. irma} as major seed dispersal vectors. Macropod species (e.g. \textit{Wallabia bicolor} and \textit{M. rufogriseus}) have also been identified as major seed dispersal vectors of southeastern Australian \textit{Persoonia} species (Rymer et al. 2006; Auld et al. 2007). Auld et al. (2007) found that most of the mature fruits (90%) on the ground below the canopy of plants were removed by \textit{Wallabia bicolor} with 88% of seeds extracted from scats viable and dormant, providing additional evidence that macropods play an important role in moving \textit{Persoonia} seeds away from parent plants.

5.5.2 Realised Seed Dispersal

Seed dispersal assignments at the 50% confidence levels reflected those at the 80% confidence level. Furthermore, seed dispersal distributions were not significantly different to the plant pair distributions. The high mean levels of seed dispersal across large distances (1.55 – 1.61 km) from both years in this study are consistent with the prediction that animal-mediated seed dispersal via endozoochory is associated with LDD of \textit{P. elliptica}. For example, LDD events occur over large distance and they mirror macropod behaviours, in such that they inhabit a home range of 40 to 460 ha (Arnold et al. 1992; Coulson 1993) and show a strong fidelity to their home ranges (Arnold et al. 1992).
The present study did not take into account seed dispersed by other vectors such as the introduced house mouse, the European rabbit and currawongs, all of which have observed consuming *P. elliptica* seed (Monaco 2012). However, it is likely that these species destroy the seeds and as such their role as dispersers is limited. Furthermore, the present study does not take into account gravity dispersed seed that was not removed from below the canopy. Previous studies on southeast Australian *Persoonia* species found that macropods do not remove all fruits (>25 – 90% removed) from below the tree canopies (Rymer 2006b; Auld et al. 2007). As there was little evidence of *P. elliptica* seed below the tree canopy, this could be an indication of the presence of a soil seed bank, as has been observed in other *Persoonia* species (Ayre et al. 2009). Thus short distance dispersal events are not accounted for in this present study, which may be a substantial proportion of all seed and therefore *P. elliptica* may display a more leptokurtic disposal curve than revealed by this study.

However, the macropod-mediated LDD found in this study does provide evidence that conflicts with the hypothesis that natural selection in SWWA has resulted in mechanisms promoting local persistence rather than wide dispersal (Hopper et al. 1996; Hopper 1997; 2000). Furthermore, the level of LDD of seed found in this study (i.e. kilometres not metres) is similar to dispersal distances documented for other frugivore-dispersed tree species. For example, Holbrook et al. (2002) demonstrated very long distance seed dispersal (up to 290 km) in the lowland tropical forests of Cameroon by frugivorous hornbills (*Caretogymna hornbilis* and *C. atrata*). In addition, Fragosa (1997) found that for the Amazonian palm *Maximiliana maripa* large numbers of seeds are ingested and dispersed by tapirs (*Tapirus terrestris*) up to 2 km away. The most likely explanation for these high levels of LDD lie in the behaviours of the seed vectors themselves, as most exhibit idiosyncratic behaviours unique to their species (Choo et al. 2012).

### 5.5.3 Implications of Macropod-Mediated Seed Dispersal

Despite the abundance of macropods in Australia, their role as seed dispersers has been mostly overlooked (but see Clifford and Drake 1985; Calvinõ-Cancela et al. 2008; Calvinõ-Cancela 2011). *Macropus fuliginous* and *M. irma* are mostly grazers (Wann and Bell 1997) and are known to feed on a range of plant species (native and introduced), with a wide overlap of plant species consumed between the two macropods (Wann and Bell 1997). The polyphagous diet for both species indicates an ability to switch diet. Typically, macropods,
like other herbivorous mammals, eat the fruits together with the foliage (Calvinõ-Cancela 2011). Macropods have powerful teeth and chew the food before swallowing, so usually only hard and small seeds (like *P. elliptica*) pass the gut undamaged (Malo and Suárez 1995; Pakeman et al. 2002). Macropods are known to strip the flesh from fruits and chew their food finely often spitting seeds out (Clifford and Drake 1985). Seeds with the flesh removed were observed in the present study and this could account for the low number of seed found within scat, in comparison to the large quantities of fruit produced (ca. 200 fruits per tree) (Abbott 1984b; Monaco 2012). This suggests that only a small proportion of seeds (ca. 5%) are effectively dispersed by macropods.

In addition to the limited number of LDD seed events (in comparison to the quantity of fruit produced), recruitment of *P. elliptica* has failed since ca. 1900 and there is a noticeable absence of seedlings and saplings within all natural populations (Abbott and Van Heurck 1988; pers. obs). Recruitment failure is thought to be caused by seedling herbivory by macropods (Abbott and Van Heurck 1988; Monaco 2012). One possible explanation for this recruitment failure since ca. 1900 could be an increase in macropod numbers over the last century. Macropods are the main large herbivores in Australia (Parsons et al. 2006) and their abundance across Australia has increased over the last century, most likely due to larger water availability from artificial permanent water sources and increases in food availability from farm land (Norbury 1992). Dingoes previously controlled macropod numbers; however they are no longer present within SWAA. The introduced European red fox does provide some control over macropod population size, as foxes are known to attack and kill juvenile macropods (Arnold et al. 1991; Banks et al. 2000). However, in areas where fox management is in place, the survival of juveniles is greater and macropod population control is limited (Banks et al. 2000). Thus even though a higher abundance of macropods will likely result in a higher number of *P. elliptica* seed being dispersed, it will also mean that more *P. elliptica* seedlings will be eaten.

No scat containing an endocarp from *P. elliptica* at the eastern edge of the study was found either year. The eastern side of the study site is bordered by *Eucalyptus wandoo* forest, which is typically more open than jarrah forest (Bell and Havel 1988). However, both *M. fuliginous* and *M. irma* are found in various types of open woodland, scrubland and grassland areas (Burbidge et al. 2008; Morris and Burbidge 2008) and thus as it seems
likely that both macropod species would inhabit the wandoo forest. However, *M. irma* is known to particularly favour open scrubby thickets, which are not characteristic of wandoo forests (Bell and Havel 1988) and show a strong fidelity to their home ranges (Arnold et al. 1992). Thus these behavioural traits suggest LDD of seed might be limited in movement due to vector habitat preference. The absence of scat containing endocarps from *P. elliptica* in eastern edge of the study site also raises intriguing questions about macropods dietary habits and movement, such as how long it takes for an animal to excrete a seed and how far does it travel during that time period? Information on gut retention times would be useful to elucidate such questions; however there is currently no information on gut retention times for these two macropods.

5.5.4 Seed Versus Pollen Dispersal

Seed dispersal distributions were not significantly different to pollen dispersal. This result indicates gene flow by pollinators is comparable to that by seed dispersed by macropods, and that movement of both pollen and seed by vectors contributes equally to LDD in *P. elliptica*. However, pollen is haploid and seeds are diploid and if all else is equal, variance in seed movement contributes twice as much as the variance in pollen movement (Hamilton 1999). In terms of LDD, detected maximum gene flow distances via seed and pollen are very close to the limits of the local population, but dispersal distances via pollen were greater than via seed (5.01 km compared to 4.40 km). This study contrasts to others that have found higher levels of pollen than seed dispersal (e.g. He et al. 2009; Ndiade-Bourobou et al. 2010; Oddou-Muratorio et al. 2010). For example, primary seed dispersal distances for *Banksia hookeriana* seedlings showed a leptokurtic distribution around a median of 5 m, reaching a distance of 36 m, whereas patterns of pollen dispersal departed strikingly from typical near-neighbour pollination (He et al. 2009). In addition, research on dispersal patterns for *Fagus crenata* Blum (Japanese beech) found that seed dispersal distances were markedly lower, than pollen dispersal distances (12.4 m versus 79.0 m) (Oddou-Muratorio 2010). Ndiade-Bourobou et al. (2010) found that gene flow by pollen dispersal ranged from 9.8 to 10.8km, whereas seed dispersal ranged from 4.0 to 6.3 km, for the low density Central African tree, *Baillonella toxisperma* Pierre. These studies suggest that the higher LDD via pollen is a reflection of the pollinators’ ability to travel far distance and/or low density of trees. These conclusions mirror those in Chapter Four where LDD of pollen indicates high mobility of insect pollinators among low density of trees.
5.5.5 Seed Immigration
Seed immigration is unlikely into the study population, as it is geographically isolated from other populations of *P. elliptica* (next nearest is 25 km to the north). In addition, seed immigration is also not likely given that the area of this study site is larger than the home ranges of macropods (Arnold et al. 1992; Coulson 1993) (ca. 1,300 ha compared to 40 to 460 ha) and that macropods show a strong fidelity to their home ranges (Arnold et al. 1992). In support, the absence of private alleles in either the 2010 or 2012 macropod-dispersed seed cohorts strongly suggest they all originated from within the local study population. This is in agreement with studies on southeastern Australian *Persoonia* species where macropod species deposit most seeds within a population (Auld et al. 2007), as evidenced by seed found in macropod scat.

5.5.6 Genetic Diversity
The 2010 and 2012 seed cohorts found within macropod scat displayed high levels of genetic diversity, that were not significantly different to the adult population. Consistent with findings from Chapter Four, there was no evidence of inbreeding (as evident from close to zero fixation indices) in the macropod-dispersed seed cohorts at AVNP. Again, the findings from this study reflect those of other Proteaceae species (He et al. 2004; He et al. 2010) and allows the conclusion that high levels of genetic diversity are maintained due to predominant outcrossing and extensive gene flow through seed and pollen.

In Chapter Three, evidence of heterozygote excess (negative fixation indices) in populations of *P. elliptica* was shown to suggest that this excess could be reflected in future generations and, if so, should be detectable in the seeds. Similar to results reported in Chapter Four for canopy seed, seed found in scat showed no evidence of heterozygote excess (fixation indices were close to zero). This further reinforces that the heterozygote excess found in Chapter Three is most likely a reflection of very low density, rather than self-incompatibility systems and outcrossing breeding systems.

5.5.7 Seed Cohort Spatial Genetic Structure
SGS as measured by the $S_p$ statistic for the sampled macropod-dispersed seed (2010 and 2012 combined) was similar to that reported for the adult population of *P. elliptica* in Chapter Four ($S_p$(seed) = 0.005 and $S_p$(adult) = 0.006). Although $S_p$ indicated overall weak
genetic structure in the macropod-dispersed seed cohorts, there was SGS below 0.50 km. This reflects the fine scale SGS up to 0.50 km reported for the adult population of *P. elliptica* in Chapter Four, which suggests that even with random dispersal of seed, SGS still reflects that of the adult population. Whilst the macropod-dispersed seed cohort and adult populations displayed similar SGS up to 0.50 km, the $r$ value at this distance class is almost twice that of adults, indicating that the strength of SGS for the seed is twice that of adults (i.e. more localized dispersal of seed). Furthermore, the weak within population SGS for the macropod-dispersed seed cohorts of *P. elliptica* at AVNP, supports findings from Chapter Three, in that low density populations show weak SGS within populations (Antonovics and Levin 1980; Loveless and Hamrick 1984). This result highlights the link between the genetic structure of adults, and seed and pollen movement and the role played by these key processes in driving genetic structure in these populations. This result also supports the hypothesis presented in Chapter Three that recruitment limitation contributes to the lack of genetic disjunction between northern and southern populations despite the natural disjunction in the distribution of *P. elliptica*.

### 5.5.8 Conclusion

Utilising a large, geographically isolated population to characterise seed dispersal patterns of a fleshy-fruited tree, this study has demonstrated that macropods move seeds away from the parent tree and over long distances (mean 1.57 km and up to 4.40 km detected). As this study is one of the first that explores the patterns of LDD seed dispersal for flora in SWWA, it has provided novel information that adds to growing body of literature relating to seed dispersal and how it affects genetic variation and its structuring. The LDD of seed found in this study highlights the need for quantification of factors affecting species’ dispersal capacities in order to accurately forecast impacts of global environmental change on biodiversity and to inform management and conservation efforts. In particular this study emphasised the importance of macropods as vectors for LDD of seed for *P. elliptica* and impacts on these macropods will have potential consequences for *P. elliptica*. Evidence of grazing by these macropods on *P. elliptica* seedlings indicates impacts of macropod removal on *P. elliptica* could be both positive (reduced grazing) and negative (reduced seed dispersal).
6.1 INTRODUCTION

Levels of genetic variation and how it is structured are strongly influenced by ecological determinants (Loveless and Hamrick 1984; Ingvarsson 1997). Consequently, understanding the roles and significance of key ecological determinants that shape the levels and spatial structure of genetic variation is a major goal of molecular ecology. In addressing this goal, this thesis focused on three key ecological determinants (plant density, pollen dispersal and seed dispersal) and how they affect genetic variation and its structuring in two co-occurring *Persoonia* species with markedly different population densities.

A thorough review of the literature revealed that there is a paucity of studies that have integrated density and LDD of pollen and seed, especially in sympatric congeners. This thesis represents one the most intensive genetics studies of the *Persoonia* genus. Few genetic studies have been conducted on the genus and even fewer on southwest Australian species. As a result, information was lacking on *Persoonia* population genetic structure and gene flow, via pollen and seed dispersal. This chapter summarises the findings of the preceding chapters, discusses limitations and identifies future research directions.
6.2 KEY FINDINGS

6.2.1 Successful cross amplification of microsatellites for Persoonia elliptica and P. longifolia

In Chapter Two, microsatellite primers developed for the southeast Australian *P. mollis* were screened for amplification in individuals of both *P. elliptica* and *P. longifolia*. The cross amplification of primers was unsuccessful for both study species and supports the hypothesis that these two main centres of radiation (southeastern and southwestern Australia) have been separated for long periods of time (Weston 2003). Microsatellite enriched libraries and 454 GS-FLX shotgun sequencing were then used to develop nine microsatellite loci for *P. elliptica* (one 454; eight cloning) and six loci for *P. longifolia* (three 454; three cloning). This successful cross amplification of some microsatellites in combination with morphological similarity and life history traits, indicates that *P. elliptica* and *P. longifolia* are closely related, but distinct species.

6.2.2 High levels of genetic diversity are maintained in low density species

In Chapter Three, most measures of genetic diversity were found to be similar (except at $< 0.01$ plants per ha) for *P. elliptica* and its relatively high density congener, *P. longifolia*. Furthermore, the low density species did not show signs of elevated inbreeding. This suggests that despite low density, Allee effects are not limiting genetic diversity parameters in populations of *P. elliptica*. However, this study did find significant relationships between density and number of alleles and density and fixation indices. These were driven by very low density *P. elliptica* populations, indicating that density begins to have an effect on number of alleles and fixation indices at a density threshold of 0.01 plants per ha.

Interestingly, whilst the density threshold had a negative impact on number of alleles (i.e. decrease the evolutionary potential of species to adapt to changing environments and reduce fitness), fixation indices reflected a heterozygote excess below the density threshold, which could indicate a heterozygote advantage (Lande and Schemsk 1985; Lessica and Allendorf 1992). Heterozygote excess has several potential causes, including self-incompatibility systems, outcrossed breeding and few reproductive individuals involved in producing the next generation. Results from Chapter Four indicate that *P. elliptica* is predominantly outcrossed, but had high multiplicity of paternity. Furthermore in both Chapters Four and Five, seed cohorts were found to have fixation indices not significantly different to zero. An alternative explanation for heterozygote excess below the density
threshold could be if a population has undergone a recent bottleneck. Unfortunately, such analysis was beyond the scope of this thesis (see Future Directions below).

### 6.2.3 High density populations show greater spatial genetic structure within populations than less dense populations

Chapter Three showed that high density populations displayed stronger spatial genetic structure within populations than less dense populations. On average, *P. longifolia* populations displayed significant positive genetic structure at inter plant distances of up to 25 m, whereas *P. elliptica* populations displayed no significant genetic structure at the within population level. This finding most likely reflects pollinator behaviour. For instance, within a *P. longifolia* population the high density of neighbouring trees allows pollinators to travel the shorter distances among more related trees, acting to produce seeds that have higher kinship than those of plants in less dense populations. On the other hand, within a *P. elliptica* population, pollinators would have to fly considerable distances to find neighbouring sexually reproductive individuals or pollinators do not find the few existing plants at all, resulting in more within-plant movements (de Jong et al. 1993).

### 6.2.4 Low densities species show greater spatial genetic structure among populations than more dense populations

Findings from Chapter Three indicate that the low density populations of *P. elliptica* showed greater among population variation, than the dense populations of *P. longifolia*. Interestingly, no genetic disjunction of northern and southern populations was found for *P. elliptica*, despite the natural disjunction in the distribution. One possible explanation for this could be the life history of *P. elliptica*.

Over the last century the recruitment of *P. elliptica* has ceased and there is a noticeable absence of seedlings and saplings within all natural populations (Abbott and Van Heurck 1988). Furthermore, *P. elliptica* has a slow growth rate and reaches reproductive maturity above a diameter-at-breast-height of 11 cm (Abbott and Van Heurck 1988). This slow growth rate can leave *P. elliptica* seedlings and saplings within a susceptible zone of browsing pressure for an extended period of time, leading to recruitment failure (Abbott and Van Heurck 1988; Monaco 2012). Chapter Three hypothesised that recruitment limitation contributes to the lack of genetic disjunction between northern and southern
populations despite the natural disjunction in the distribution of *P. elliptica*. Chapter Five found that the potential spatial genetic structure was similar to that reported for the adult population of *P. elliptica*. Combined these results provide empirical evidence in support of the above mentioned theory.

### 6.2.5 Native bees facilitate near random mating at a large spatial scale

Pollen dispersal facilitates the exchange of genes within populations (Ouborg et al. 1999) thereby influencing the distribution of genetic variation and enabling plant genes to move through the landscape. An understanding of pollen dispersal vectors can provide insights into the movement of pollen within populations. Chapter Four suggests that native bee species (e.g. species of *Amegilla* and *Megachilidae*) are a major contributor to the striking departure from the predominantly nearest neighbour mating that is typical of insect pollinated plants. Whilst both the introduced honeybee (*Apis mellifera*) and a suite of native bees were observed collecting nectar from *P. elliptica* flowers, the foraging behaviour of native bee species versus feral honey bees, suggests that native bees contribute more to the effective long distance dispersal (LDD) of pollen for *P. elliptica*.

Like most Australian plant species, *P. elliptica* has evolved in the virtual absence of pollinating social insects (e.g. *Apis mellifera*) and have pollination strategies adapted to a unique suite of native insects (Michener 1965). Given this evolutionary context and the current intensity of honeybee visitation, honeybees are likely to displace native pollinators from flowers and/or disrupt pollination services for *P. elliptica* (Paton 1993, 1996; Huryn 1997). The displacement of native bees may adversely affect pollination (Hingston and McQuillan 1999) and have a negative impact on LDD of pollen for *P. elliptica* (however see Future Directions below).

### 6.2.6 Pollen mediated gene flow maintains high levels of genetic diversity and outcrossing rates on the periphery of the study population.

Plant density is expected to decrease towards the periphery of the population, thus affecting pollinator availability and behaviour leading to fewer pollinator visitors. This in turn results in lower pollen flow and lower genetic diversity. In addition, inbreeding is expected to increase as plant density decreases due to reduced mate diversity (Keller and Waller 2002; Biebach and Keller 2010). Thus plants on the periphery of a population are predicted to
display lower genetic diversity and higher inbreeding rates (Lesica and Allendorf 1995) compared to plants in the centre of the population.

In contrast to this prediction, results from Chapter Four failed to detect lower genetic diversity and higher inbreeding rates on the periphery of a population or a species range. Overall, regardless of a maternal tree’s spatial position within the study population there was no significant difference in any parameters measured for the canopy seed. This reinforces that mating and pollen dispersal patterns are robust to position within the population, consistent with highly mobile insect pollinators.

6.2.7 Native macropods contribute to moving P. elliptica seed away from the parent tree and over long distances.

Chapter Five used motion sensor cameras to identify Macropus fuliginosus and M. irma as major seed dispersal vectors for P. elliptica. LDD seed events were found to occur over large distances (mean 1.57 km and up to 4.40km). These LDD events were found to mirror macropod behaviours, in such that macropods inhabit a home range of 40 to 460 ha (Arnold et al. 1992; Coulson 1993) and show a strong fidelity to their home ranges (Arnold et al. 1992).

In comparison to the large quantities of fruit produced (ca. 200 fruits per tree, pers. obs) only a low number of seed was found within macropod scat, suggesting only a small proportion of seeds (ca. 5%) are effectively dispersed by macropods. In addition to the limited number of LDD seed events, recruitment of P. elliptica has failed since ca. 1900 and there is a noticeable absence of seedlings and saplings within all natural populations (Abbott and Van Heurck 1988). Recruitment failure is thought to be caused by seedling herbivory from macropods (Abbott and Van Heurck 1988; Monaco 2012).

Thus this study emphasises the importance of macropods as vectors for LDD of seed for P. elliptica and that impacts on these macropods will have potential consequences for P. elliptica (e.g. grazing by these macropods on P. elliptica seedlings indicates impacts of macropod removal on P. elliptica could be both positive (reduction in grazing) and negative (reduction in seed dispersal).
6.2.8 Long distance dispersal in southwest Australia

The persistence of an extended absence of major geomorphological agents of soil disturbance and rejuvenation in SWWA (Hopper and Goia 2004) has lead to the hypothesis that natural selection has resulted in mechanisms promoting local persistence rather than wide dispersal and colonization (Hopper et al. 1996; Hopper 1997; 2000). Realised seed dispersal distances inferred from parentage assignments in Chapter Five showed that macropods consume and move seeds away from the parent tree (between 0.02 – 4.40 km, with a mean of 1.57 km). These findings provide empirical evidence that conflict with the above hypothesis.

Dispersal events over long distances are vital for species persistence in fragmented landscapes and under a changing climate (Cain et al. 2000; Traktenbrot et al. 2005; Nathan et al. 2008). If these dispersal events are lacking, the ability for the species to persist into the future when faced with global events such as climate change may be compromised. Ecologically, this could result in species decline brought about by a lack of dispersal contributing to other population level issues such as recruitment failure. When seed dispersal is limiting, or ceases, due to disperser decline or loss, the plant population may persist for decades (or centuries in the case of *P. elliptica*) in an apparent healthy state before deleterious demographic consequences may become apparent (McConkey et al. 2011). Thus there is a need for quantification of factors affecting species’ dispersal capacities in order to accurately forecast impacts of global environmental change on biodiversity and to inform management and conservation efforts.
6.3 FUTURE DIRECTIONS

This thesis has produced major new insights into the drivers of population genetic structure and gene flow and the importance of dispersal vectors. However, attention needs to be drawn to some limitations to this present study. These are outlined below, together with potential avenues for further research that will complement the findings of this thesis.

One limitation of this study is that an area in the southwest of the natural range of *P. longifolia* was not genotyped, due to deteriorated plant material from which DNA could not be extracted. Therefore the regional genetic delineation of populations that was detected for *P. longifolia* is most likely a reflection of sampling focus, as the genetic delineation between the two regions corresponds to the gap in the sample distributions. Time constraints meant that re-sampling this area and the subsequent genotyping were beyond the scope of this study. However in the future this work is feasible and would elucidate whether or not this regional genetic delineation is an artefact of sampling.

A further limitation of this study is that without intensive pollen vector surveys and pollen tracing studies, it cannot be indisputably said that native bees are the major contributing vector for long distance pollen dispersal. Future work, utilising pollen vector surveys and pollen tracing would be useful in identifying the main pollen dispersal vectors in the study species. In addition to focusing on the native pollinators, it might be interesting to study whether or not they are at risk of being displaced by feral honey bees.

Another limitation of this study is that a large proportion of seed found within macropod scat were unable to be assigned parentage at the 80% confidence level. This is likely due to the statistical power of the microsatellite markers used. Genotyping additional microsatellite markers would most likely resolve this issue. Microsatellite enriched libraries developed for this species provide a resource for the development of additional microsatellite loci. However time and financial constraints meant that this was beyond the scope of this study.

In addition, this present study did not take into account seed dispersed by dispersal vectors other than macropods and it does not take into account gravity dispersed seed that was not removed from below the canopy. As such a proportion of actual distance dispersal events
are not accounted for in this present study. Further work quantifying this missing portion of dispersal distances would help to further characterise the seed dispersal curve for *P. elliptica*.

Outlier loci have been identified for *P. longifolia* using AFLPs (Stingemore and Krauss 2012). Specifically, markers significantly associated with the climatic variables potential evapotranspiration, solar radiation and minimum temperature were identified. Furthermore, frequency differences at these markers across populations revealed distinct geographic provenances. Thus it would be particularly interesting to identify outlier loci for *P. longifolia* and *P. elliptica* that may be linked to microsatellite markers under the influence of natural selection. Identifying genes that are under natural selection within and among populations is crucial for the understanding of how individuals, populations and species are adapted to their environment (Savolainen et al. 2007; Neale and Kremer 2011; Strasburg et al. 2012). The identification of genes regions that are under selection provides a link between ecology and genetics and can assist the increasingly necessary predictions of how plant species may respond to future climatic change, (Aitken et al. 2008; Wang et al. 2010; Neale and Kremer 2011). One option to identify markers linked to adaptation is with expressed sequence tags (ESTs), which represent expressed genes that often have a known or putative function. The EST database, however, is limited to few well studied species. Another approach that requires no prior knowledge about outlier markers is the landscape genetic approach (Holderegger and Wagner 2008).

Another potential future research avenue could be the development of guidelines for seed sourcing for conservation and restoration activities in *P. longifolia* and *P. elliptica*, as both species of interest for mine site restoration (Mullins et al. 2002). Various guidelines exist to direct practitioners in seed collection strategies that avoid the negative impacts associated with the introduction of non-local material. However, these guidelines still only provide a vague ‘best guess’ and strict application can lead to error in provenance delineation (Krauss and Koch 2004). The greatest limitation to the application of this principle is the extent to what is local? The use of genetic markers in combination with guidelines (Mortlock 1999) is presently the most precise approach available to determine what is local and therefore rapidly delineate the extent of the local genetic provenance (Krauss et al. 2000). The combination of population genetic data (Chapter Three) and outlier loci association with
natural selection (see above) would provide information for the development of conservation and seed sourcing guidelines for *P. longifolia* and *P. elliptica*. 
REFERENCES


Allee WC (1938). The social life of animals. New York: W.W. Norton & Company Inc.


Goudet J (2001). FSTAT, a program to estimate and test gene diversities and fixation indices (version 2.9. 3).


Levin DA and Kerster HW (1975). Gene flow in seed plants. In Evolutionary biology (pp. 139-220). Springer US.


Vigilante T (1996). A study of patch boundaries between forest and rehabilitated bauxite pits, at Jarrahdale, Western Australia (Doctoral dissertation, Honours Thesis, Department of Botany, University of Western Australia).


