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Genetic diversity and gene flow in fragmented populations of the rare shrub, Calothamnus sp. Whicher

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Genetic diversity and gene flow in fragmented populations of the rare shrub, *Calothamnus* sp. Whicher.

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Thesis submitted in partial fulfilment of the requirements for the award of B.Sc. (Biological Sciences) Honours at the School of Natural Sciences, Edith Cowan University, Joondalup.

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Sean Stankowski

3rd November, 2006

Abstract

Calothamnus sp. Whicher (Myrtaceae) is a narrow range endemic shrub restricted to ironstone soils near the town of Busselton in south-western Australia. Due to extensive land clearing for agriculture and mineral exploration, the species is fragmented over the majority of its range. In the present study, microsatellite markers were used to characterise levels of genetic diversity and describe levels of differentiation and gene flow among seven small, isolated road verge populations.

Allelic diversity within the taxon over the six microsatellites was high (A = 17.6 ± 1.6). Diversity within populations was considerably lower (A = 5.19 ± 1.27), and was positively correlated with population size. An excess of homozygotes and high fixation indices in all populations (mean F_{IS} = 0.315 ± 0.13) indicated that inbreeding within populations was high. Estimates of the divergence in allele frequencies between populations (global θ = 0.256) and genetic distance (mean Nei's D = 0.370) revealed a distinct genetic structure within the study sample.

Direct estimates of gene flow, determined by assigning paternity to seed crops from the two largest populations, were low (2.7% and 4%), yet similar to historical estimates derived from the degree of differentiation between populations. However, due to the degree of inbreeding within these populations and their susceptibility to genetic drift, these historical estimates appeared to be a consequence of post fragmentation rates of gene flow rather than reflecting pre-fragmentation rates. Low levels of gene flow into the two largest populations and restricted within population patterns of mating were supported by high global (among population) and mean pairwise (within population) estimates of Φ_{ft}, which represents the degree of differentiation between maternally sampled pollen pools.

The differentiation observed between populations is most likely a result of post-fragmentation processes rather than being driven by mutation and maintained by low levels of historical gene flow. The six natural road verge populations observed in this study were likely to be part of one or more larger, continuous populations similar to those which are located in relatively undisturbed fragments of natural vegetation. Initially, differentiation within these populations probably resulted from their small sizes and the heterogeneous fine scale genetic structure within the larger population(s) from which they originated. Further differentiation appears to have resulted from extensive inbreeding within populations and the increased

vulnerability to drift associated with decreasing population size. Results from other studies, including that conducted on the closely related species *Calothamnus quadrifidus* (Byrne *et al.*, in press), suggest that fragmentation has reduced rates of gene flow from higher historical levels. However, the detection of some gene flow events across the breadth of the study site suggests that isolation itself was not preventing gene flow. Rather, the loss of natural vegetation may have reduced the abundance of bird pollinators. The conservation and evolutionary implications of these findings are discussed.

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'There is no way to determine the importance of gene flow in natural populations because there is no direct way to estimate levels of gene flow.'

Slatkin (1981).

Chapter 1: Thesis Overview

1.1 The global loss of biodiversity

Current species extinction rates are between 100 and 1000 times those associated with pre-human existence (Pimm *et al.*, 1995). With species disappearing at such an alarming rate, which itself is expected to increase (Pimm *et al.*, 1995; Pimm & Raven, 2000), conservation biologists and ecologists are faced with the monumental task of maintaining a representative portion of the biological diversity which has evolved over the past three billion years (Redford & Richter, 1999; Myers *et al.*, 2000; Brooks *et al.*, 2001). Biological diversity, or biodiversity, is the total variability of life on earth; it includes genes, species, populations, and ecosystems (UNEP, 1995). There are many reasons for conserving biodiversity, with these ranging from spiritual to purely scientific (Gatto & Giulio, 2000). Perhaps the most compelling reason for conserving biodiversity is the fact that our subsistence, as a species, depends on it (Daily *et al.*, 1997; Gatto & Giulio, 2000). It provides us with resources (i.e., food, pharmaceutical products, timber and fuels) and ecosystem services (i.e., air and water purification, the replenishment of soil nutrients and climate regulation) which, at present, cannot be acquired by other means (Daily *et al.*, 1997; Gatto & Giulio, 2000). As we are uncertain to how much biodiversity is required to maintain these resources and services in the long-term (Walker, 1992), we should make every attempt to conserve what remains.

1.1.1 Threats to global biodiversity

There are many human activities which threaten global biodiversity (Trakhtenbrot *et al.*, 2005). These include the pollution of air and water (UNEP, 1995; Estes, 1998), emission of greenhouse gasses (Brereton *et al.*, 1995; Beaumont & Hughes, 2002) and introduction of alien species into ecosystems (Akinyemiju, 1987; Keighery, 1992; Philips *et al.*, 2003). However, the clearing of natural vegetation (a process which will be referred to hereafter as 'land clearing') is considered the most significant. This is due to the number of species lost and the pervasive nature of land clearing with regards to the number of terrestrial ecosystems currently affected (UNEP, 1995; State of the Environment, 2001). Natural vegetation, which can range in structure from open grasslands to rainforest (Goodall pers. comm.), provides habitat for a myriad of terrestrial organisms, both plant and animal (Pimm & Raven, 2000). As a result, land clearing can significantly alter the distribution and abundance of organisms within a landscape (Myers *et al.*, 2000; Brooks *et al.*,

2001). The most obvious consequences of land clearing are the death of countless organisms during the disturbance event (Cogger *et al.*, 2003) and the loss of the conditions and constituents (i.e., habitat) they require to complete their lifecycle (Root, 1998).

However, neither of these effects, the direct loss of organisms or loss of habitat, is considered to be the key driver of species extinction (Pimm *et al.*, 1995; Pimm & Raven, 2000). This is because landscapes rarely experience complete clearing, and the majority of species which were present within a landscape prior to disturbance, will be represented in the patches of habitat that remain (Pimm *et al.*, 1995; Pimm & Raven, 2000). Landscapes such as these, which are comprised of discrete patches of habitat set within a highly altered landscape matrix, are referred to as fragmented (Saunders *et al.*, 1991; Hobbs & Yates, 2003). Fragmented landscapes are often associated with what Tilman *et al.* (1994) refer to as an 'extinction debt'. This term results from the observation that a large proportion of species within fragmented landscapes do not persist in the long term. Broadly speaking, these delayed extinctions are a result of significant alterations in landscape level characteristics and processes which result as a consequence of landscape fragmentation (Saunders *et al.*, 1991; Hobbs & Yates, 2003).

1.2 The biological consequences of fragmentation

The biological consequences of fragmentation have been explored in detail by a number of authors (Saunders *et al.*, 1991; Hobbs & Yates, 2003; Lowe *et al.*, 2005). The most commonly sited consequences are those which have arisen from the principles of island biogeography. In particular, the observation that the size of an island is positively correlated with the number of species which it can accommodate (MacArthur & Wilson, 1967; Simberloff & Abele, 1982). More recently, authors have concentrated on identifying how fragmentation affects populations. Hobbs and Yates (2003) recognise four direct consequences of fragmentation which can influence the persistence of populations by altering organism abundance. These are: (i) the creation of small habitat patches, (ii) the alteration of landscape processes, (iii) increased isolation between patches and (iv) reductions in population size (Figure 1.1).

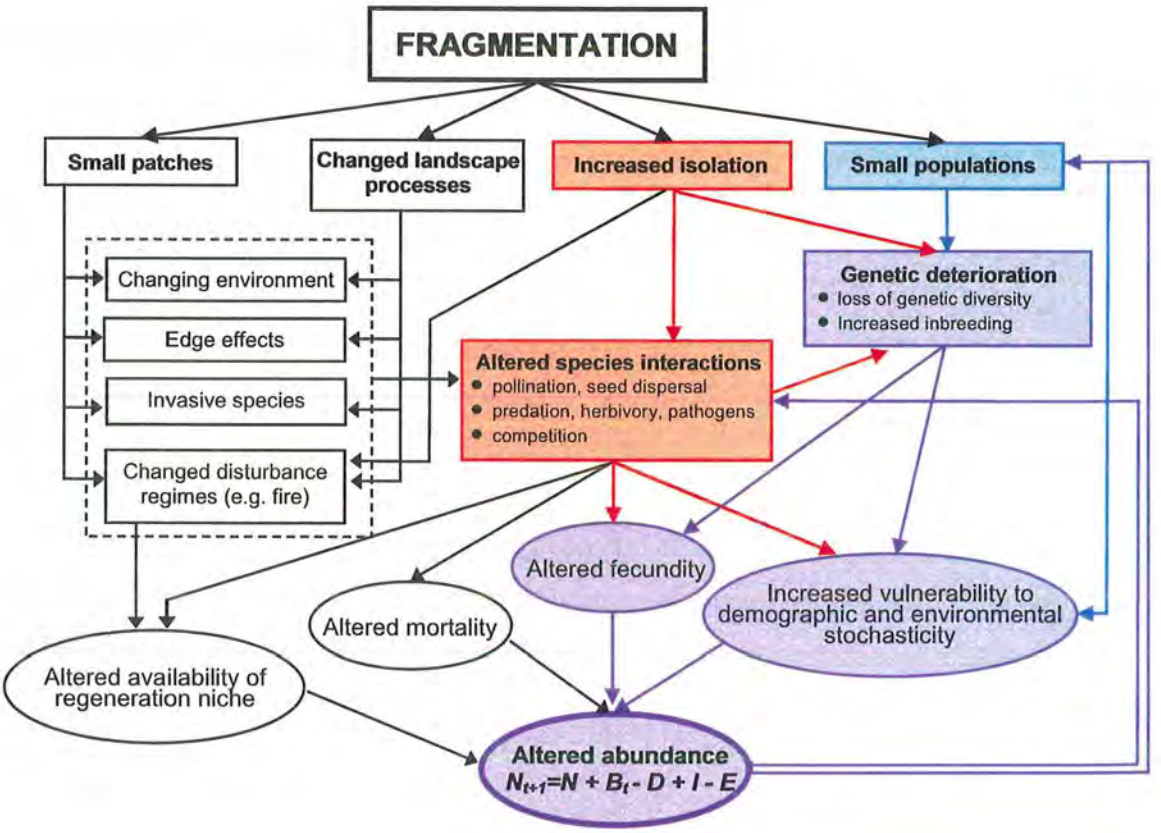


Figure 1.1 The biological consequences of landscape fragmentation. Fragmentation results in four consequences which have implications for the persistence of populations. These are (i) the creation of small patches (ii) changed landscape processes (iii) increased isolation between populations and (iv) reduced population size. Of these, increased isolation and small populations can have direct impacts on the genetic structure of populations. These can operate independently (red or blue shading) or synergistically (purple shading), though both result in genetic erosion which ultimately results in altered organism abundance (N = population size; B number of births; D = number of deaths; I number of immigrants; E number of emigrants). Adapted from Hobbs & Yates (2003).

1.2.1 The genetic consequences of fragmentation

Although it is widely appreciated that fragmentation has had a profound impact upon biodiversity at the species level, a number of authors suggest that the genetic impacts of fragmentation are considerably worse (Tillman *et al.*, 1994). While some species will continue to persist in fragmented landscapes, it is likely that they will experience considerable genetic erosion as a result of the altered population characteristics and ecosystem processes which accompany fragmentation (Saunders *et al.*, 1991; Hobbs & Yates, 2003). Of the four consequences of fragmentation described above, the latter two (increased isolation between patches and reductions in population size) have the greatest potential to impact upon the genetic structure of populations (Saunders *et al.*, 1991; Hobbs & Yates, 2003).

1.2.1.1 The effects of reduced population size

Reductions in population size (i.e. the number of organisms within a population) can influence the genetic structure of populations in a number of ways, though all act to reduce genetic diversity and organism fitness, the consequence of which is altered organism abundance (Charlesworth & Charlesworth, 1987; Ellstrand & Ellam, 1993; Figure 1.1). The most direct consequence of reduced population size is the loss of genetic diversity which accompanies the loss of individuals (Ellstrand & Elam, 1993; Hamrick & Godt, 1996). Because the amount of diversity which can be maintained within a population at a given time is a function of the number of individuals within it, any reduction in size will reduce the maximum diversity that it can maintain (Ellstrand & Elam, 1993). In addition, reduced population size can result in increased inbreeding, which leads to reduced heterozygosity, the fixation of recessive lethal alleles and diminished reproductive fitness (Charlesworth & Chralesworth, 1987; Keller & Waller, 2002). Finally, reductions in size leave populations highly susceptible to stochastic processes (Ellstrand & Elam, 1993). Random events will ultimately influence the genetic structure of all populations to some degree. However, the extent of this influence is a function of population size (Ellstrand & Elam, 1993). In large populations (i.e. 1000 individuals), the loss of a few individuals is unlikely to alter the total diversity and abundance of alleles at any given locus. In contrast, the loss of a single individual in a small population (i.e. 10 individuals) may have a profound impact upon population diversity (Ellstrand & Elam, 1993).

1.2.1.2 The effects of increased spatial isolation

Unlike the effects of reduced population size, which have received considerable research attention in the past 50 years, few authors have focused on the way that increased spatial isolation influences the genetic structure of populations. There is, however, a growing body of evidence which suggests that increases in spatial isolation influence the genetic structure of populations by influencing gene flow (Kearns *et al.*, 1998; Hobbs & Yates, 2003).

1.3 Gene flow

Gene flow, which is the movement of genes from one population to another (Slatkin, 1985), is one of the major factors determining the genetic structure of populations (Slatkin, 1985; Hamrick & Godt, 1996; Whitlock & McCauley, 1999). Limited gene flow often results in increased genetic differentiation between populations through the fixation of advantageous alleles, the long-term consequence of which may be speciation (Slatkin, 1985). In contrast, extensive gene flow has a homogenising effect, resulting in populations which share a similar genetic structure (Slatkin, 1985).

1.3.1 Mechanisms for gene flow

Gene flow can be mediated by a number of mechanisms, some of which include the migration of individuals, the spread of gametes and the dispersal of reproductive propagules (Slatkin, 1985; Lowe *et al.*, 2004). Due to the different life-history strategies employed by species, the primary mediator of gene flow can differ markedly between them. For terrestrial animals, most of which are capable of moving significant distances over short time scales, migration is considered the chief mediator of gene flow (Slatkin, 1985; Lowe *et al.*, 2004). Due to their mobility, animals are often capable of traversing large expanses of habitat, which may be of varying quality, in order to reach neighbouring populations (Slatkin, 1985; Washitani *et al.*, 2005). In contrast, terrestrial plants are sessile and, therefore, incapable of direct migration (Washitani *et al.*, 2005). As a result, land plants must rely solely on gamete or propagule dispersal for gene flow (Washitani *et al.*, 2005). For the majority of plant species, particularly those where seed dispersal is limited, gene flow is restricted to the dispersal of pollen (Kearns *et al.*, 1998; Washitani *et al.*, 2005).

1.3.1.1 Pollen dispersal vectors

Pollen dispersal can be facilitated by a range of abiotic and biotic agents (Richards, 1986; Kearns *et al.*, 1998). However, the pollination syndrome employed can vary markedly within and between taxonomic groups (Richards, 1986; Ackerman, 1999). Abiotic agents, such as wind and water, are considered the primary vectors for pollen dispersal in some plant groups (i.e., wind mediated dispersal in *Pinus* spp.), but the vast majority of the world's angiosperm species (approximately 80%, or 200 000) rely solely on biotic vectors for pollen dispersal (Kearns *et al.*, 1998). Most of these species rely on insect pollination (Kearns *et al.*, 1998), but birds and mammals are important dispersal agents for many taxa (Richards, 1986; Kearns *et al.*, 1998).

1.3.2 The effects of fragmentation on pollen mediated gene flow

For biological pollen vectors, the plants that they visit represent an energy source (with the exception of some deceptive plant species such as members of the Orchidaceae) (Richards, 1986; Kearns *et al.*, 1998; Cresswell & Osborne, 2004). Thus, there are a number of factors that influence whether a pollinator will visit a given population (Cresswell & Osborne, 2004). These include, the distance of the population relative to others, the quality of the interlying habitat which connects populations and the size of the energy resource relative to the energy spent gaining it (Richards, 1986). Fragmentation has the potential to increase spatial isolation between populations, reduce the quality of interlying habitat, and reduce the size of plant populations within a landscape (Saunders *et al.*, 1991; Hobbs & Yates, 2003). If any of these factors are reduced below a threshold which will be specific to the biological vector in question, the result may be a loss of, or a reduction in, pollination services (Richards, 1986; Kearns *et al.*, 1998).

1.4 Estimating pollen mediated gene flow

Because of the importance of pollination in determining the genetic structure of populations (Slatkin, 1985; Richards, 1986), in addition to the sensitivity of biotic pollination syndromes to anthropogenic disturbance (Kearns *et al.*, 1998), many authors have attempted to describe patterns of pollen dispersal for a range of species (See Richards, 1986 and Lowe *et al.*, 2004). Depending upon the methods used, studies can be placed into one of three categories: observational, indirect genetic and direct genetic (Lowe *et al.*, 2004).

1.4.1 Observational methods

Past estimates of pollen-mediated gene flow have generally been inferred from observations of pollinator behaviour or by tracking the movement of marked pollen grains, or pollen-analogous particles (i.e., fluorescent dye particles) (Richards, 1986; Adler & Irwin, 2005). Studies using such methods indicate that the distances travelled by pollinators are generally short, with the frequency of pollination events decreasing in a leptokurtotic fashion as distance from the donating flower increases (Slatkin, 1985; Richards, 1986; Austerlitz *et al.*, 2004). Many authors have therefore suggested that pollen dispersal is unlikely to result in significant levels of long-distance gene flow (Levin & Kerster, 1974; Slatkin, 1985).

These observational methods are associated with a number of shortcomings which may result in inaccurate gene flow estimates. Firstly, the methodologies associated with observational studies are clearly biased towards dispersal events which occur close to the pollen source (Lowe *et al.*, 2004). It is highly likely that rare, long-distance, biologically important dispersal events will be missed, resulting in an underestimate of gene flow (Lowe *et al.*, 2004). Secondly, pollinator visits to flowers do not always result in pollen delivery (Richards, 1986; Maki & Masuda, 1993), nor does the deposition of pollen onto a receptive stigma always result in fertilisation (Richards, 1986; Maki & Masuda, 1993). This is particularly important for species that employ mechanisms to reduce rates of self fertilisation or inbreeding (Newbigin *et al.*, 1994), as genetic relatedness generally decreases with increasing distance from an individual (Jones *et al.*, 2006). Thus, in these species, it is likely that observational techniques overestimate rates of effective pollination close to the pollen source.

1.4.2 Indirect estimates from population genetic structure

Gene flow can also be estimated indirectly from molecular data (Slatkin, 1985). Indirect estimates are derived from the degree of genetic differentiation between populations (Ouborg *et al.*, 1999). Although a number of differentiation coefficients can be converted to estimates of gene flow (Lowe *et al.*, 2004), Wright's (1951) F_{ST} is most commonly used. $F_{ST,}$ which is a measure of the standardised interpopulation variance in allele frequencies, is calculated as:

$$F_{ST} = \frac{\sigma_p^2}{[p(1-p)]} \qquad \text{(Equation 1)}$$

Where σ_p^2 is the variance in allele frequencies among populations and p is the frequency of the ith allele (Wright, 1951). Based on the assumptions of Wright's (1931) island model of migration, Slatkin (1987) described the non-linear relationship between gene flow, as a measure of the number of migrants entering a population per generation (Nm), and F_{ST} in the following equation:

$$F_{ST} = \frac{1}{(4Nm+1)} \qquad \text{(Equation 2)}$$

Where N is the effective size of each population, and m is the migration rate between populations. Thus one can solve for Nm by inverting equation two:

$$Nm = \frac{(1-F_{ST})}{4F_{ST}} \qquad \text{(Equation 3)}$$

Indirect methods of gene flow taken from population genetic structure, such as those derived from F_{ST}, are associated with a number of shortcomings (Whitlock & McCauley, 1999). Firstly, there are many other mechanisms besides gene flow which influence the genetic structure of populations. Indirect estimates of gene flow assume that these mechanisms, some of which include selection and mutation as well as stochastic events, have not contributed to any observed differences in population genetic structure; any observed differentiation between populations is assumed to be a result of reduced reproductive connectivity (Whitlock & McCauley, 1999; Lowe *et al.*, 2005).

Second, indirect genetic estimates are generally based upon mathematical models of migration which are unlikely to exist in nature (Whitlock & McCauley, 1999; Lowe *et al.*, 2005). For example, estimates of Nm derived from F_{ST} are generally based on the assumptions of the Wright's (1931) island model of gene flow. This model assumes that all populations contain the same number of diploid individuals, and that each population donates and receives migrants from all other populations at the same rate (Wright, 1931). It is unrealistic to assume that natural populations will be comprised of the same number of individuals. Furthermore, populations are set in space, and their spatial arrangement is likely to affect rates of migration between them (Ouborg *et al.*, 1999). For example, it is widely appreciated that dispersal rates are a function of distance, with dispersal occurring most frequently over

shorter distances (Trakhtenbrot *et al.*, 2005). Thus, one would expect higher rates of migration between populations which are close together and lower rates between those which are located further apart (Trakhtenbrot *et al.*, 2005). The island model does not take this 'distance effect' into account (Whitlock & McCauley, 1999). Nor does it consider other spatial variables such as the quality of habitat connecting populations, or ecological (i.e. source to sink dispersal in metapopulations) and physical scenarios (i.e. prevailing winds or steep topography) that may result in non random patterns of dispersal (Whitlock & McCauley, 1999). While a number of other authors have proposed models of gene flow that attempt to overcome the simplicity of the island model (Wright, 1940; Kimura, 1953), all are associated with their own inherent limitations (Whitlock & McCauley, 1999). The consequence of these limitations is that indirect estimates are unlikely to represent actual rates of gene flow occuring between natural populations.

Another significant limitation is that, depending on the lifecycle of the organism, patterns of gene flow may not be representative of those associated with the time of sampling (Whitlock & McCauley, 1999; Lowe *et al.*, 2005). Climatic variability, changes in the demographic structure of populations and human induced disturbance are all factors which are likely to lead to variation in patterns of gene flow on a range of temporal scales (Manel *et al.*, 2003; Kenta *et al.*, 2004). Thus, indirect estimates of *Nm* may be representative of gene flow which has occurred over a broad timescale, as organisms will persist in the environment long after the gene flow events have occurred (Lowe *et al.*, 2005). For example, in an intact landscape, gene flow may have occurred between two populations, but may have ceased following landscape fragmentation. In this situation, indirect estimates of *Nm* made subsequent to fragmentation would suggest that gene flow was still occurring between these populations.

1.4.3 Direct estimates from molecular markers

Due to the limitations of indirect genetic estimates described above, as well as advances in molecular and statistical methods, it is now possible to estimate gene flow directly using genetic markers (Ouborg *et al.*, 1999). Direct estimates differ from indirect estimates in that the source of dispersal events is determined by the genotypes of propagules or progeny, as opposed to established individuals (Manel *et al.*, 2003; Austerlitz *et al.*, 2004; Lowe *et al.*, 2005). As a result, direct estimates of dispersal represent patterns of gene flow at the time of sampling, rather than

indirect methods which may be confounded by historical dispersal events (Ouborg *et al.*, 1999). Also, unlike observational methods, direct methods only measure realised gene flow (that which results in progeny) rather than dispersal which is often used as a surrogate to describe gene flow (Ouborg *et al.*, 1999).

1.4.3.1 Paternity assignment

The most commonly used direct method to measure pollen dispersal is paternity assignment. The aim of paternity assignment is to identify which father, from a sample of potential fathers, contributed the gamete which resulted in a given fertilisation event (Jones & Arden, 2003; Austerlitz *et al.*, 2004). As assignment is based on male-haplotype information, the technique requires that the genotypes of all potential fathers are known (Lowe *et al.*, 2004). Also, the mother's genotype is required prior to assignment, in order to reveal the paternal contribution (Lowe *et al.*, 2004). Paternity assignment techniques are well suited to studies of plants, because seed is often retained on the plant. Therefore, the identity of the mother plant is known at the time that seed is sampled (Austerlitz *et al.*, 2004). After progeny are genotyped and the maternal haplotype identified, paternity can be assigned via a number of methods. Most common is the exclusion method, which eliminates potential fathers based on a genetic mismatch (Wilmer *et al.*, 1999). Due to laboratory genotyping errors, and the high mutation rates associated with neutrally selective genetic markers, other more conservative assignment techniques have also been developed. For example, the maximum likelihood approach of Marshal *et al.* (1998) allows mismatches and, based on the frequency of alleles within the total sample, assigns paternity to the 'most likely' male within a confidence interval which can be established by the user.

1.4.3.2 TwoGener analysis of pollen pool structure

The biggest drawback in the use of paternity analysis is the need to exhaustively sample all potential fathers in the study area. As a result, paternity analysis is not suited to the study of gene flow in species which are numerous within a landscape. In contrast, the TwoGener analysis of Smouse *et al.* (2001) does not attempt to assign paternity to offspring, and therefore does not require exhaustive sampling of potential fathers. Rather, it is a variation of analysis of molecular variance (AMOVA; Excoffier *et al.*, 1992) that compares pollen pools sampled by female plants; seeds collected from plants represent replicates, while the maternal plants represent strata for comparison. Male haplotype variation within and between plants is

converted to an interclass correlation measure of pollen pool structure referred to as Φ_{ft}. Thus, Φ_{ft} is similar to Wright's (1951) F_{ST}, though enables within population comparisons to be made.

1.4.3.3 Suitable genetic markers for direct gene flow estimates

Both of the above techniques are dependent upon molecular markers to provide a basis on which individuals and their genetic contributions can be identified (Ouborg *et al.*, 1999; Austerlitz *et al.*, 2004). Generally, two different types of markers are used. The majority of studies, particularly earlier ones, utilised isozymes. Isozymes, which are neutrally selective structural variants of functional enzymes, are relatively inexpensive to assay and can be applied to most species (Nybom, 2004). However, because polymorphism is generally low, with two or three structural variants existing for each enzyme, a high number of markers must be assayed to provide the exclusion probabilities required to confidently assign paternity, or reveal pollen pool differentiation in the TwoGener analysis (Nybom, 2004).

More recently, authors have turned to the use of microsatellite markers when making direct estimates of gene flow (Ouborg *et al.*, 1999; Nybom, 2004). This is for a number of reasons. Firstly, they are located in the non coding regions of the genome and, as a result, are highly polymorphic. Levels of diversity exceeding 15 alleles per locus are routinely reported in the literature (Elliot & Byrne, 2005; Fitch *et al.*, 2005; Revaldaves *et al.*, 2005; Spies *et al.*, 2005). These high levels of polymorphism have produced the highest exclusion probabilities achieved in studies involving paternity assignment (Burczyk *et al.*, 2004). In addition, different microsatellite alleles are characterised by differences in their length, as opposed to other DNA markers which differ only in sequence (Ouborg *et al.* 1999). As a result, alleles can be distinguished using standard electrophoretic techniques (Ouborg, *et al.*, 1999). The disadvantage associated with microsatellite markers comes with the need to develop 'species specific' primers which enable their amplification via the polymerase chain reaction (Nybom, 2004). This process is both time consuming and expensive.

1.5 Hotspots of biodiversity

Biodiversity, at the level of the species, is not distributed homogenously across the globe (Brooks *et al.*, 2001). Rather, the vast majority of species are concentrated within 25 global biodiversity 'hotspots' (Myers *et al.*, 2000; Brooks *et al.*, 2001).

Collectively, these hotspots once covered 12% of the Earth's surface. Now, after extensive land clearing, they account for only 1.4% of the land (Brooks *et al.*, 2001). Despite this, between 50% and 75% of all threatened plants and 57% of threatened animals are hotspot endemics (Brooks *et al.*, 2001). One of these biodiversity hotspots is the Southwest Australian Floristic Region (Myers *et al.*, 2000; Hopper & Gioia, 2004).

1.5.1 The Southwest Australian Floristic Region

The Southwest Australian Floristic Region (SAFR), which is located in the southwest corner of Western Australia, is unique in that it has extremely high floral diversity and endemism (Hopper, 1979; Hopper *et al.*, 1996; Hopper & Gioia, 2004), yet exhibits few of the characteristics thought to be synonymous with high levels of botanical richness (Hopper & Gioia, 2004). The region is essentially flat, with few significant topographical features (Hopper & Gioia, 2004) and is associated with nutrient deficient soils and simple rainfall patterns (Hopper & Gioia, 2004). However, a number of authors have identified key geohistorical features of the SAFR which largely explain current patterns of diversity (Beard *et al.*, 2000; Hopper & Gioia, 2004). Firstly, a lack of geological activity and past variation in rainfall patterns has resulted in the formation of a complex soil mosaic comprised mainly of nutrient deficient sands and laterites (Beard *et al.*, 2000; Coates, 2000; Hopper & Gioia, 2004). Specialisation of flora to edaphic heterogeneity, which is maintained even at the local scale (Hopper & Gioia, 2004), has resulted in high floral β-diversity, and, consequently, many endemic species (Hopper, 1979; Hopper & Gioia, 2004). These evolutionary patterns have been made more evident by a lack of catastrophic disturbance events (such as glaciation, volcanism, and mountain uplifting) which are typically associated with mass extinction (Coates, 2000).

1.5.2 Fragmentation in the Southwest Australian Floristic Region

Land clearing has been practised in south-western Australia since 1845 (Main, 1993). The intensity of clearing reached its peak in the mid 20th century (Main, 1993) resulting in the loss of approximately 89% of natural vegetation in the SAFR (Myers *et al.*, 2000). As a result of this habitat loss, and the high degree of endemism within the region, many taxa are no longer represented within pristine habitat and are now fragmented across their distributions (Coates, 2000; Myers *et al.*, 2000; Brooks *et al.*, 2001).

1.5.3 The species: Calothamnus sp. Whicher

One species that fits this profile, in that it is a narrow endemic which persists in a highly fragmented landscape, is the woody shrub, *Calothamnus* sp. Whicher (B.J. Keighery & N. Gibson) (Myrtaceae). Although it was previously classified as the common, widespread species *Calothamnus quadrifidus*, *C. sp.* Whicher was recently declared a distinct taxonomic unit based on morphology (Western Australian Herbarium, 2006). The species is restricted to a small area of south-western Australia near the town of Busselton (Figure 1.2), and is represented in approximately 25 populations, the size of which ranges from thousands of individuals to three individuals (Western Australian Herbarium, 2006). More than half of these populations are small (<150 individuals) and are confined to road verges where little or no remnant vegetation is present (Western Australian Herbarium, 2006). Because of its demographic situation and narrow geographic range, the conservation status of *C.* sp. Whicher has been upgraded to priority four, which is reserved for species that are rare, but are not immediately threatened by any identifiable factors (Western Australian Herbarium, 2006).

Like *C. quadrifidus*, *C.* sp. Whicher grows to a height of 2-3 meters and flowers profusely between June and December (winter – spring) (Western Australian Herbarium, 2006; Figure 1.2). The hermaphroditic flowers are semi-tubular, pendulous, born in cauliflorous inflorescences, and are generally confined to one side of the stem (Ford *et al.*, 1979; Western Australian Herbarium, 2006; Figure 1.2). While pollination has not been formally studied in *C.* sp. Whicher, the pollination mechanism has been described as unspecialised across the genus (Western Australian Herbarium, 2006). However, flower colour (red) and structure are indicative of bird pollination (Ford *et al.*, 1979). This has been confirmed by a number of studies, particularly those which have concentrated on *C. quadrifidus.* (Collins *et al.*, 1984; Hopper and Burbidge, 1986; Yates *et al.*, in press A). Of the many bird species that visit members of the genus, honeyeater species (family Meliphagidae) are most commonly observed (Hopper and Burbidge, 1986; Yates *et al.*, in press A), and studies have demonstrated that these birds are capable of carrying considerably more pollen than other biological vectors. Collins *et al.* (1984) have demonstrated that pollination can also result from honey possums (*Tarsipes rostratus*) and European honey bees (*Apis mellifera*).

No study of mating system has been conducted on *C.* sp. Whicher, yet the species is expected to have a mixed mating system similar to that observed in *C.*

quadrifidus (Yates *et al.*, in press A), as well as a number of other myrtaceous species (Hopper & Moran, 1981; Sampson *et al.*, 1995; Millar *et al.*, 2000). Thus, the species is expected to be predominately outcrossing with low to intermediate rates of self fertilisation. Seed is held on the plant in dehiscent woody capsules which persist for many years after flowering (Western Australian Herbarium, 2006; Figure 1.2). The seed itself is minute, non-endospermous and has no morphological characteristics which may aid in dispersal by wind or water (Western Australian Herbarium, 2006).

1.6 Thesis aims

Most authors now agree that, as well as having an understanding of the demographic status of a species, an understanding of its genetic characteristics is essential when forming an effective conservation plan (Hamrick & Godt, 1996). This thesis aims to describe the genetic scenario associated with seven isolated, road verge populations of *Calothamnus* sp. Whicher. The primary aim is to illustrate patterns of gene flow among these isolated populations. This will be achieved via paternity assignment techniques and a TwoGener analysis of pollen pool structure. The molecular data required for these analysis will be obtained from microsatellite markers. This data will also enable an investigation into patterns of genetic diversity and differentiation among the study populations.

In addition to having direct outcomes for the conservation of *C.* sp. Whicher, the outcomes of this thesis will also have implications for the management of other species in fragmented landscapes. To date, most direct investigations into plant gene flow have concentrated on rainforest tree species which rely on insect or wind pollination (see review by Burczyk *et al.*, 2004). As plants species exist at a variety of scales and employ varying breeding systems and pollination syndromes, it is necessary to investigate how fragmentation affects gene flow in species with differing life history strategies. This study of *C.* sp. Whicher will further contribute to our general understanding of patterns of pollen dispersal in bird pollinated species. Thus, the conclusions drawn in this thesis may be extrapolated to other species with similar life-histories.

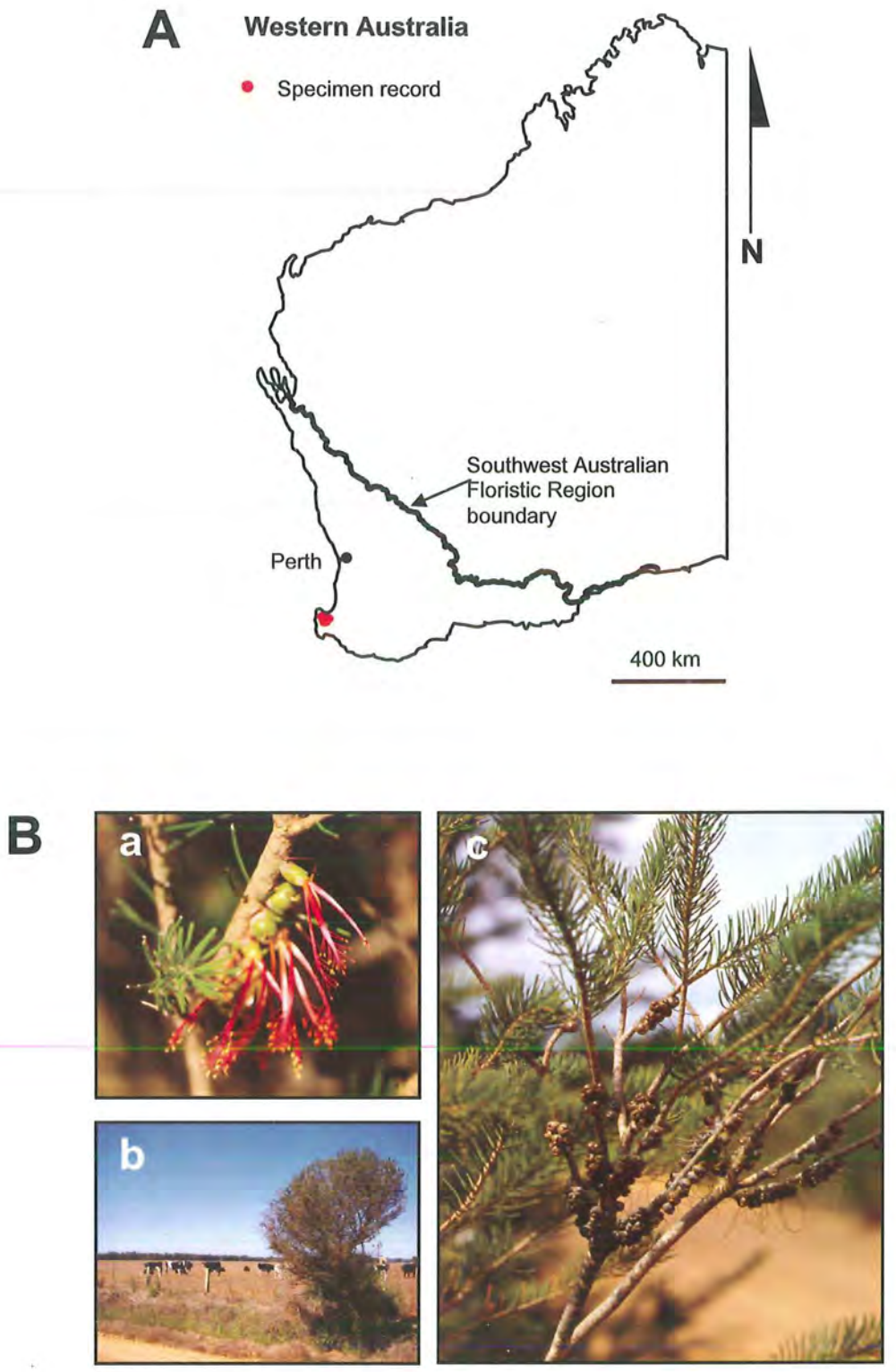


Figure 1.2 (A) The distribution and (B) morphology of *Calothamnus* sp. Whicher (B.J. Keighery & N. Gibson), including (a) floral morphology, (b) growth habit and (c) seed capsules. Figure A adapted from the Western Australian Herbarium (2006).

1.7. Thesis structure

This thesis has a conventional structure (Figure 1.3). The following chapter (Chapter 2) describes the conceptual structure of the project and the materials and methods which were associated with sample collection, DNA extraction and microsatellite characterisation. The only methods which are not included in this chapter are those associated with the analysis of data; these are included in later chapters.

Chapter 3 is concerned with describing the genetic structure of the study populations. All populations were expected to have similar levels of diversity, with the exception of very small populations (< 10 individuals), where diversity was expected to be much lower as a result of sampling effect. Genetic differentiation between populations was also expected to be low considering their close geographical proximities to each other.

Chapter 4 is dedicated to describing patterns of pollen dispersal within and between the study populations. The mating system of *C.* sp. Whicher is also described. Given the findings of gene flow and mating system studies performed by Byrne *et al.* (in press) on *Calothamnus quadrifidus*, gene flow into the two assayed populations was expected to be high. In addition, *Calothamnus* sp. Whicher was expected to have a mixed mating system with similar rates of outcrossing to that exhibited by *C. quadrifidus.* Also, patterns of within population dispersal were expected to reflect those observed in other bird pollinated species.

The final chapter, chapter 5, consists of a general discussion, the aim of which was to discuss the genetic diversity and gene flow components of the study together. It is here that the results of the study will be considered in light of habitat fragmentation. In addition, the evolutionary and conservation implications of the work are discussed. The thesis concludes with recommendations for future research.

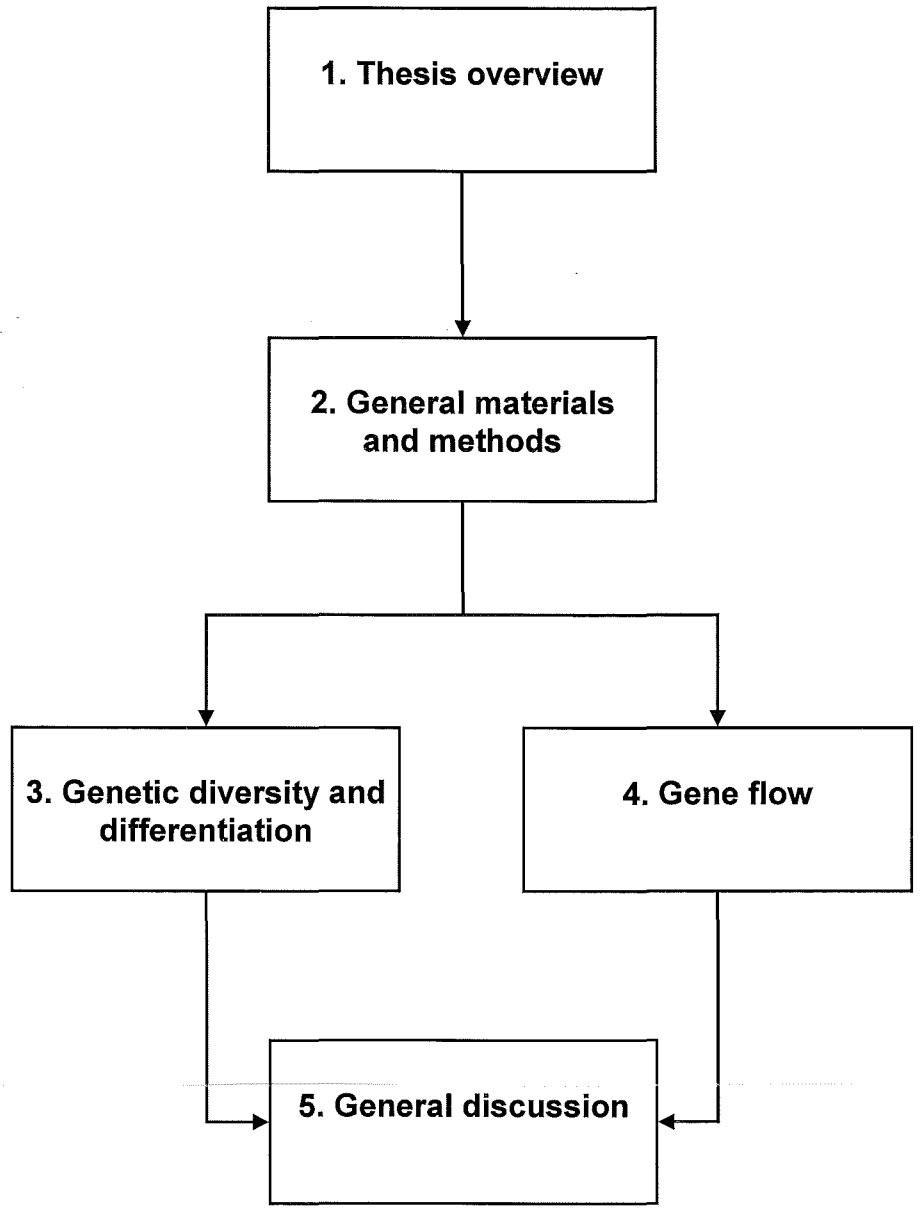
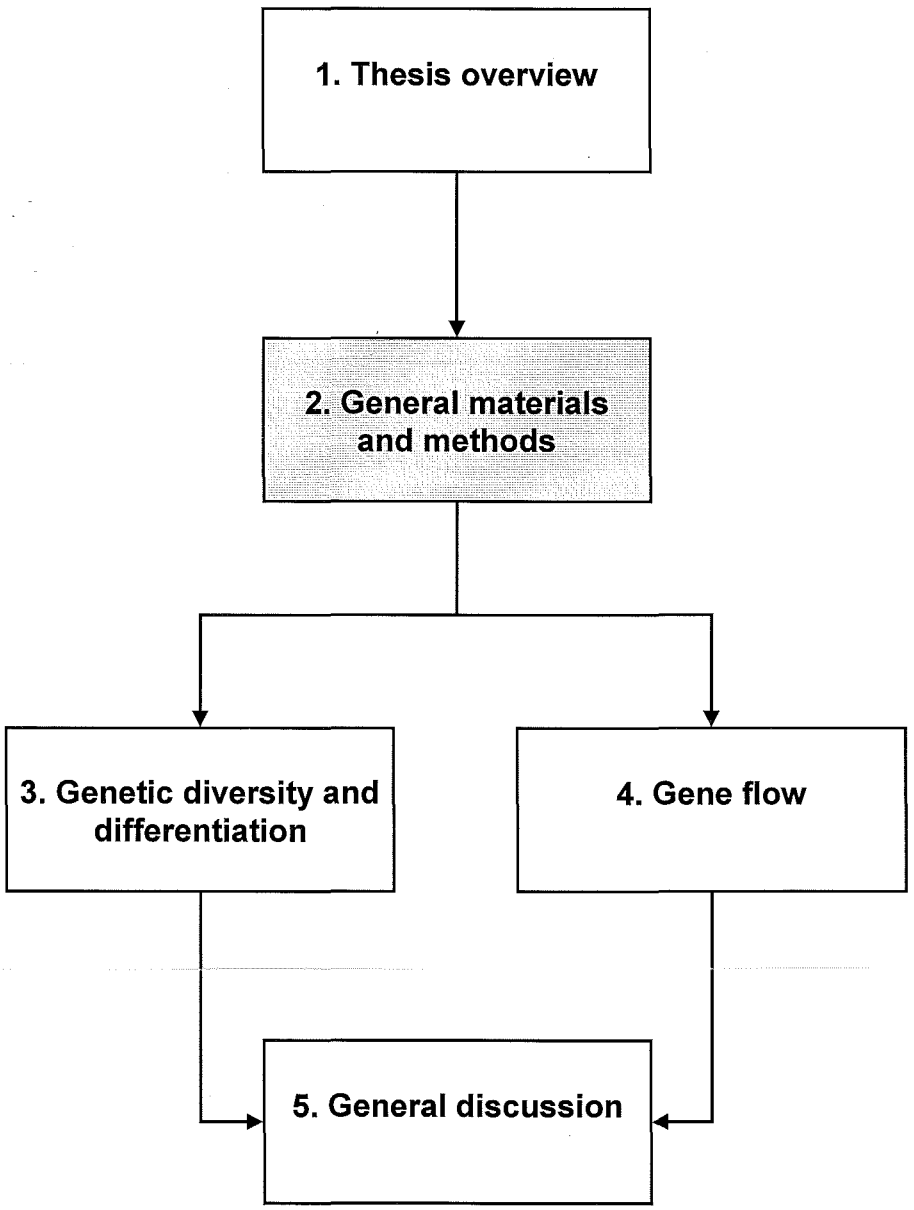


Figure 1.3 A conceptual diagram outlining the structure of this thesis.

Chapter 2: General materials and methods



Chapter 2: General materials and methods

2.1 Study site and population descriptions

The study was undertaken approximately 300 km south of Perth, Western Australia, within 15 km of the town of Busselton (Figure 2.1). Climate in the area is Dry Mediterranean with cool, wet winters and hot, dry summers. The mean annual rainfall in Busselton is 864 mm, with the wettest period being from May to September (Bureau of Meteorology, pers comm).

The study site was located in an area referred to as the Busselton Ironstones, which consists of approximately 1 200 hectares of ironstone soils which are unique, as they have only been observed at three locations on the Swan Coastal Plain. They are characterised by a shallow heavy clay or sandy layer over an impermeable layer of ferricrete which can be several meters thick (Tille & Lantzke, 1990; Gibson *et al.*, 2000). As a result, they are prone to waterlogging in periods of heavy rainfall. In addition to their unique physical and hydrological characteristics, these soils are also associated with a unique flora (English, 1999; Gibson *et al.*, 2000) consisting of a number of endemic species (11 recorded to date; Gibson *et al.*, 2000). Extensive land clearing for agricultural production and mineral exploration has seen over 90 % (1080 hectares) of their associated vegetation cleared in the last century (English, 1999). What remains exists as remnant islands surrounded by a matrix of agricultural production.

Seven populations of *Calothamnus* sp. Whicher, a priority species endemic to the Busselton Ironstones, were selected for use in this study (Figures 2.1-2.6; Table 2.1). A population was defined as a discrete group of individuals which was isolated from conspecifics by more than 50 m. The number of reproductive individuals in the study populations ranged from three (Doyle Small and Hairpin Small) to 83 (Doyle Large), with straight line distances between populations ranging from 150 m to 5.8 km. Six of the populations were located on disturbed road-verges where weeds were prolific and there was little or no natural vegetation. Individuals were present at much higher densities in these populations than in other populations in larger vegetation remnants which were surveyed prior to the study. In most cases, the foliage of plants formed a single canopy. The single population located in native vegetation, Ambergate, was located in Ambergate Reserve, which occupied an area of approximately 75 ha. Habitat type and quality varied markedly throughout the reserve, though *C.* sp. Whicher was located over a small area which had an

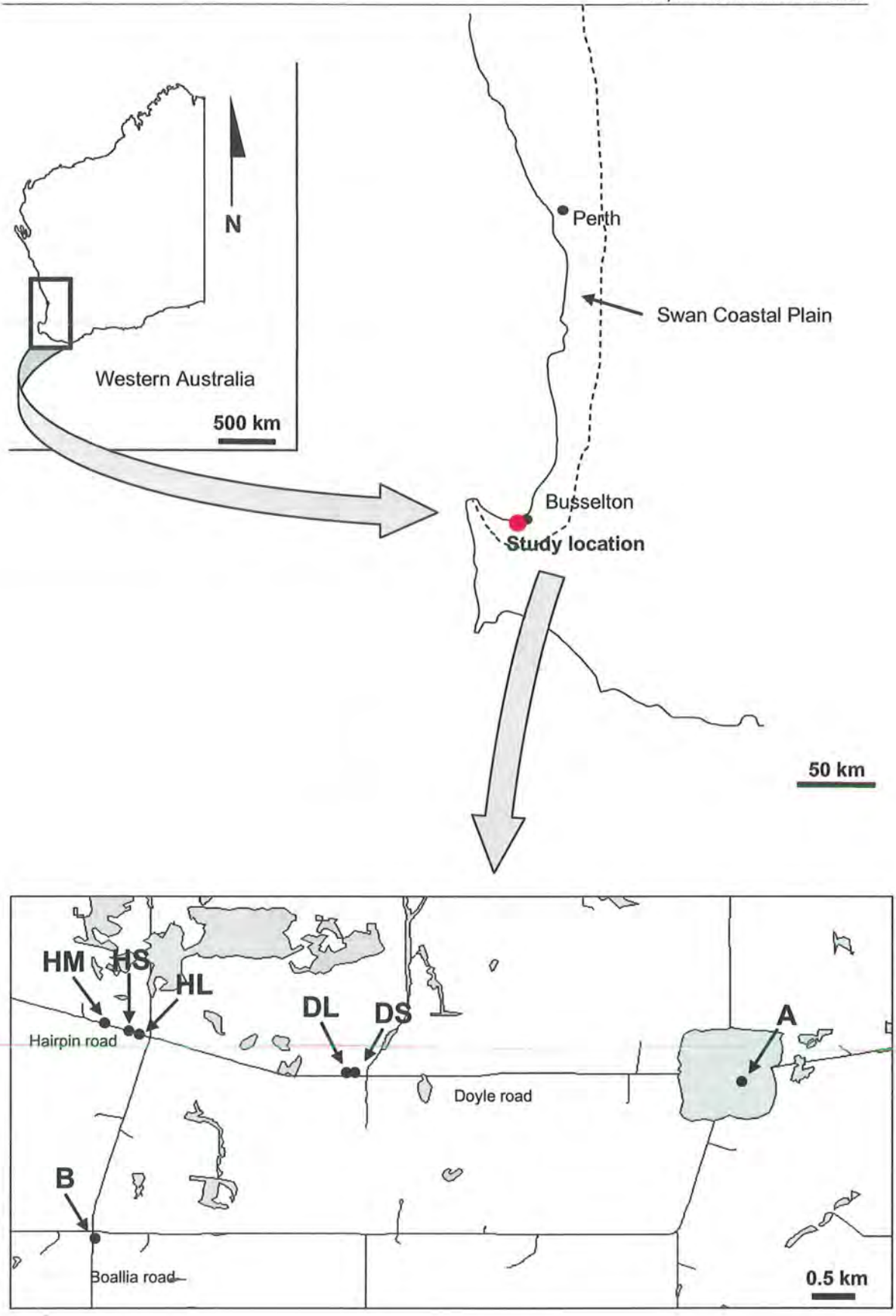


Figure 2.1 Locations of the seven populations of *Calothamnus* sp. Whicher sampled in the study. HM, Hairpin Medium; HS, Hairpin Small; HL, Hairpin Large; DL, Doyle Large; DS, Doyle Small; A, Ambergate; B, Boallia. The Grey shaded areas in the site map represent remaining native vegetation.

Table 2.1 The characteristics of the seven populations of *Calothamnus sp.* Whicher examined.

Population	Latitude	Longitude	Number of samples collected	Habitat type	Seedling Recruitment?	Seed collected?
Hairpin Medium	33° 43' 49.8"	115° 15' 50.2"	12	Roadside	N	N
Hairpin Small	33° 43' 50.8"	115° 15' 53.8"	3	Roadside	N	N
Hairpin Large	33° 43' 53.0"	115° 16' 00.5"	99	Roadside	Y	Y
Doyle Large	33° 44' 13.4"	115° 17' 22.2"	44	Roadside	N	Y
Doyle Small	33° 44' 13.3"	115° 17' 28.7"	3	Roadside	N	N
Ambergate	33° 44' 24.4"	115° 29' 30.9"	67	Native vegetation	N	N
Boallia	33° 45' 02.6"	115° 16' 0.76"	41	Roadside	N	N

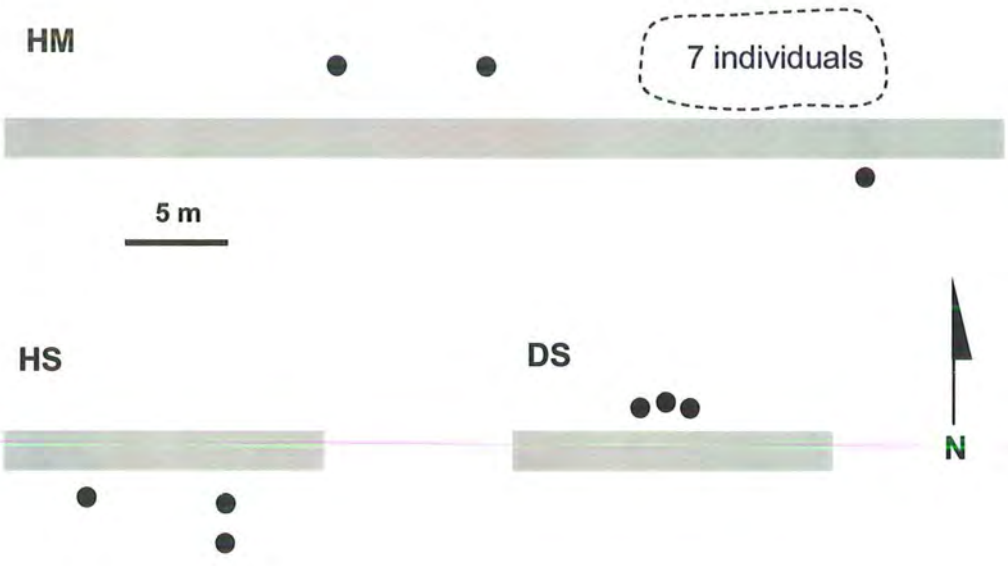


Figure 2.2 Photograph of plants in Hairpin Medium and maps of the Hairpin Medium (HM), Hairpin Small (HS) and Doyle Small (DS) populations of *Calothamnus* sp. Whicher. The photograph is representative of all three populations. Black dots represent the locations of individual plants, while the dashed oval represents multiple, tightly packed plants. The grey line represents the road.

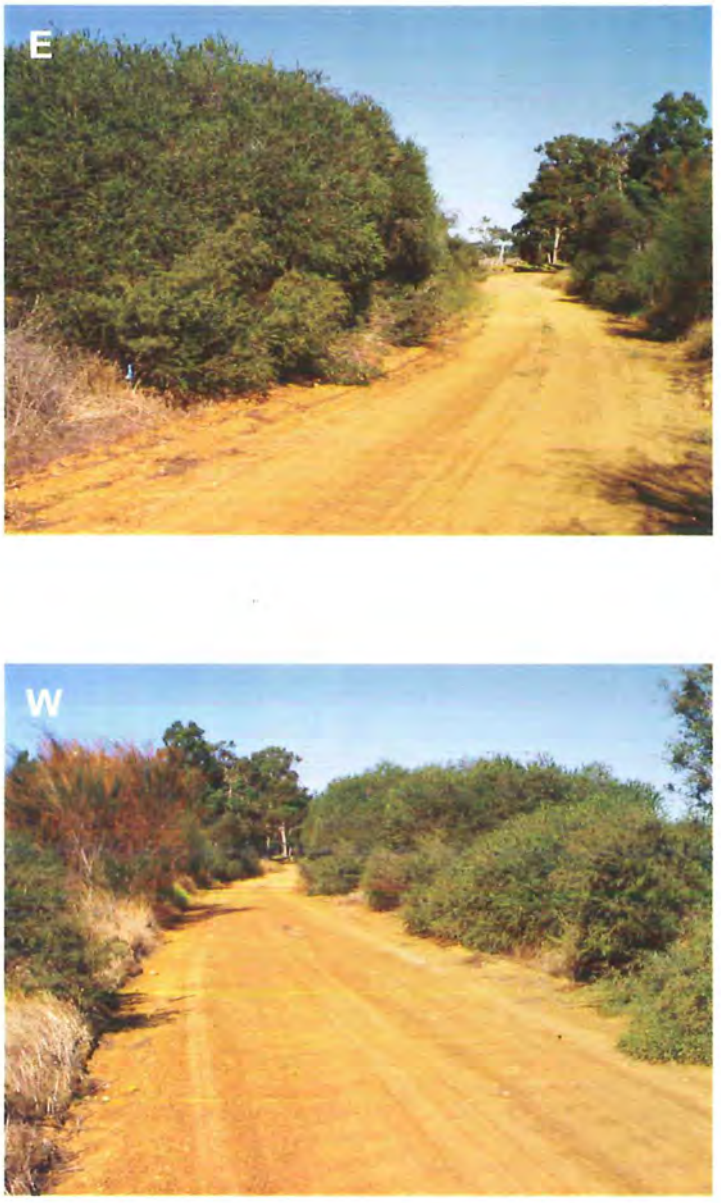


Figure 2.3 Photographs (top: east end; bottom: west end) and a map of the Hairpin Large population of *Calothamnus* sp. Whicher. Black dots represent the locations of individual plants, while the dashed oval represents multiple, tightly packed plants. The grey line represents the road.

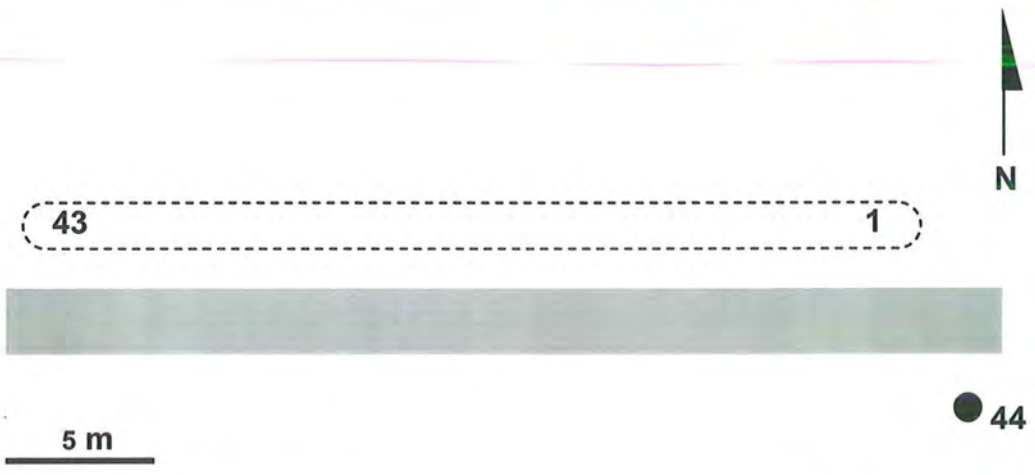


Figure 2.4 Photographs (top, plant 44; bottom, east end) and a map of the Doyle Large population of *Calothamnus* sp. Whicher. Black dots represent the locations of individual plants, while the dashed oval represents multiple, tightly packed plants. The grey line represents the road.

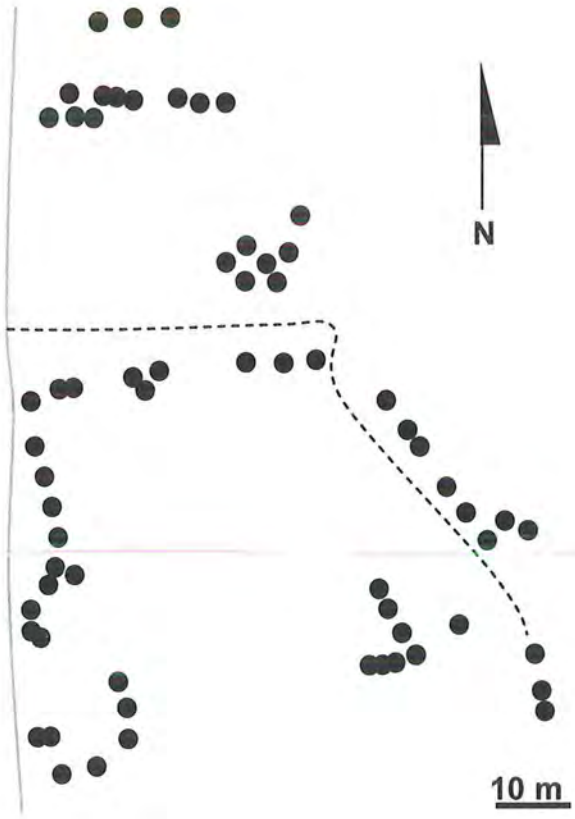


Figure 2.5 Photograph and a map of the Ambergate population of *Calothamnus* sp. Whicher. Black dots represent the locations of plants. The grey line represents the road, while the dashed line represents a walking path.

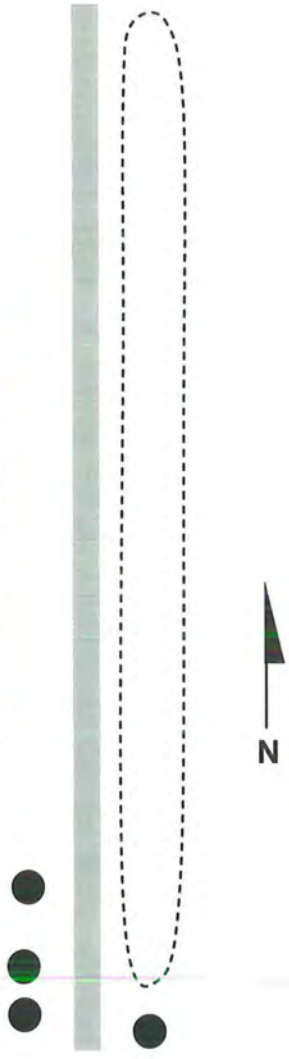


Figure 2.6 Photograph (from north end) and a map of the Boallia population of *Calothamnus* sp. Whicher. Black dots represent the locations of individual plants, while the dashed oval represents multiple, tightly packed plants. The grey line represents the road.

overstory of *Eucalyptus marginata* with a diverse understorey of woody and herbaceous taxa. Individuals were present at a much lower density in Ambergate than in the other sampled populations, and other remnant populations surveyed during the study. This observation, the difference in soil type in Ambergate, the proximity of plants to walking paths (Figure 2.5) and an atypical vegetation community all suggest that it is a planted population (M. Byrne & N. Gibson, pers. comm.).

2.2 Collection of parental leaf material and seed samples

Leaf material was collected from all reproductive plants in the seven study populations on the 28th and 29th of March (autumn), 2006. Reproductive plants were identified in each population by the presence of seed capsules on the plant. Each plant was allocated a number and marked with flagging tape to enable subsequent identification. Approximately 20 g of leaf material was cut from each and placed in a sealable plastic bag with the air removed. Samples were kept cool (~4 °C) in the field by storing them in an insulated container with ice. As it was not always possible to determine which rootstock stems originated from, some 'individuals' were sampled multiple times to ensure that all potential fathers had been sampled (Table 2.1). On arrival at the laboratory, 120 mg of leaf material was taken from each sample and stored at -80 °C prior to DNA extraction.

Seed was collected from ten randomly selected mother plants in Hairpin Large and Doyle Large by collecting capsules from multiple branches (approximately ten) at a range of heights and positions to avoid sampling bias. Capsules were only collected from the most terminal position on the branch in an attempt to obtain a sample that reflected seed produced in the previous year (the assumption being that all plants flowered in the previous year). Each sample was placed in a seed envelope which was stored in dark, dry conditions for ten days, and then placed in an oven for 24 hours at 27 °C to accelerate the drying process. To ensure that all seed was liberated from the open capsules, each sample was transferred to a glass beaker and shaken vigorously. The capsules were then discarded, and the seed transferred to a second envelope, which was stored in a dark, cool environment.

2.3 Seedling establishment

Approximately 50 seeds from each mother plant were germinated in a temperature controlled greenhouse (set at 25-30 °C) on a soil mix comprised of equal parts of Bailey's premium potting mix, perlite and white sand. Approximately one month

after germination, 20 seedlings from each tray were transferred to individual 100 ml pots containing Bailey's premium potting mix.

2.4 Preparation of plant material and DNA extraction

DNA was extracted from adult and seedling material (fifteen seedlings from each of the 20 mothers) using Qiagen DNeasy™ plant mini extraction kits with a modified protocol. Each 120 mg sample was ground in liquid nitrogen using a mortar and pestle until a fine powder was obtained. The ground material was then transferred to an eppendorf tube containing 400 µl of Qiagen cell lysis buffer 'AP1'. Three µl of Qiagen 'RNase A' was also added, and the sample vortexed and placed in a 65 °C water bath for 10 minutes to facilitate cell lysis. During incubation, each sample was mixed by inversion at three minute intervals. After adding 120 µl of Qiagen precipitation buffer 'AP2' each sample was placed on ice for 10 minutes to precipitate proteins and polysaccharides. Precipitates were then pelleted by centrifugation (5 minutes at 13 000 rpm) and the lysate transferred to the Qiagen 'QIAashredder mini spin column' which was then centrifuged (2 minutes at 13 000 rpm) to remove remaining precipitates and cell debris. The flow through captured in each QIAashredder collection tube was then transferred to a new eppendorf tube, and 1.5 volumes of Qiagen DNA precipitation buffer 'AP3' was added. This mixture was then applied to the same Qiagen 'DNeasy mini spin column' in two separate 650 µl aliquots (this was the maximum capacity of the column) and centrifuged at 8 000 rpm. The flow through was discarded and the DNeasy mini spin column transferred to a new collection tube. Two wash steps were then conducted, each of which involved pipeting 500 µl of Qiagen buffer 'AW' into the column. The column was then centrifuged (8 000 rpm for one minute for the first wash and 13 000 rpm for two minutes on the second wash) and the flow through discarded. DNA was eluted twice (in two separate tubes) by transferring the DNeasy mini spin columns to a 1.5 ml eppendorf tube and applying 50 µl of Qiagen elution buffer 'AE' directly onto the spin column membrane to which the DNA was bound. After incubating at room temperature for 5 minutes, the columns were spun at 8 000 rpm. Extracted DNA was stored at –20 °C.

The concentration of all extracted parental and seedling DNA samples was determined using a DyNA Quant™ 200 fluorometer (Hoefer Pharmacia Biotech) using the low range assay (designed for samples with a concentration between 10 and 500 ng/µl) and an 85 ng/µl DNA standard. Samples with a concentration over 25 ng/µl were diluted to 20 ng/µl using sdH_2O, while the samples under 25 ng/µl

were used in subsequent procedures undiluted. The quality of extracted DNA was determined by subjecting a random selection of parental and seedling samples, along with 5 µl aliquots of 100 ng/µl Hind III/Lambda DNA molecular weight marker (GeneWorks), to electrophoresis on 1% agarose gels for 1 hour at 80 volts (V) using a Bio-Rad PowerPac^{TM} 300. Gels were stained for 20 minutes in a 1 µg/ml solution of ethidium bromide, de-stained in DH_20 and viewed under ultraviolet light.

2.5 *Microsatellite amplification and characterisation of alleles*

Six microsatellite loci were amplified via the polymerase chain reaction (PCR) using primers developed for *Calothamnus quadrifidus* (Elliot and Byrne, 2005; See Table 2.2 for primer sequences). Each 15 µl PCR reaction contained the following constituents: 50 mM KCl, 20mM Tris HCl (pH 8.4), 0.2 mM of each DNTP, 0.3 mM forward and reverse primer, 0.5 units of *Taq* polymerase, either 1 or 1.5 mM $MgCl_2$ (Table 2.2) and 20 ng of *Calothamnus* sp. Whicher DNA (except for samples which had a concentration lower than 25 ng/µl where 1 µl of DNA was added). Amplification of all loci was achieved using a single PCR programme comprised of the following steps: an initial denaturation period of 96 °C for 2 min: 20 cycles of 30 s at 94 °C (denaturation), 30 s at 69.5 °C with a step down of 0.5 °C per cycle (annealing), 30 s at 72 °C (extension), and 10 cycles of 30 s at 94 °C (denaturation), 30 s at 60 °C (annealing), 30 s at 72 °C (extension) with a final extension period of 2 minutes at 72 °C.

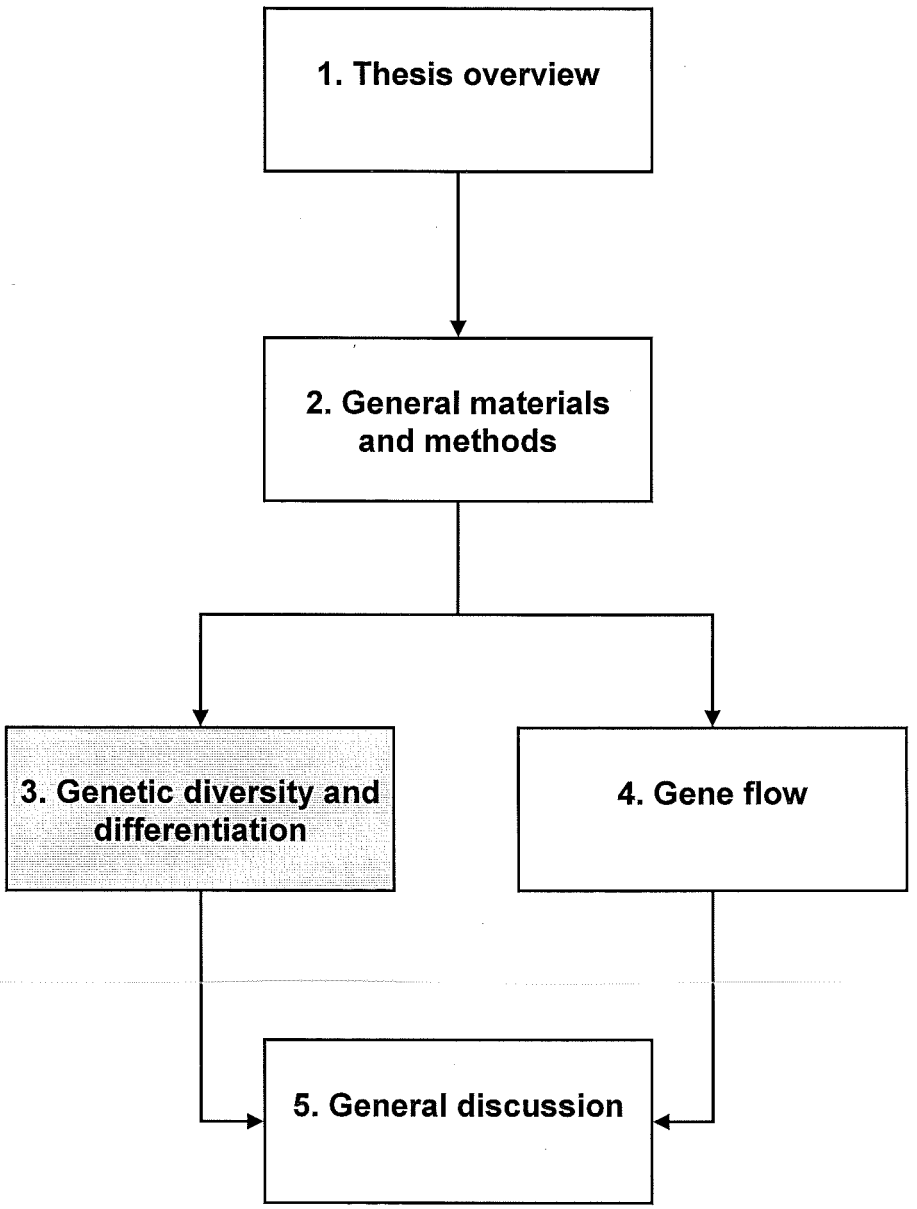
Table 2.2 Details of primer sequence, amplification conditions and dye set characteristics for six microsatellie loci designed for *Calothamnus quadrifidus* (Elliot & Byrne, 2005) as used in the present study of *Calothamnus* sp. Whicher.

Locus	Primer sequences (5'-3')	$MgCl_2$ (mM)	Dye colour (5' forward primer)	Multiplex set
CQ 1.7	F: CCGCAGTATCACTCCTTTATCC R: CTCCCCAAACCTGCCTATTC	1.0	VIC (green)	1
CQ 1.10	F: TGCCCACATACTTCCAGAAC R: CTAAACCGTCCCAAGACTCC	1.0	VIC (green)	2
CQ 4.3	F: CGTGAGTTCAGGGGAGCTATG R: CCGATTTTCGTTTCTTCAGG	1.5	6-FAM (blue)	1
CQ 5.11	F: CGCACAACAGAGGTCAGAAG R: TCCATAGCATCCAGGAAACCC	1.5	NED (yellow)	1
CQ 6.1	F: GCGTCAACGCTTCACTTTAC R: ATTTGTTGAAGGCGACGAAC	1.0	NED (yellow)	2
CQ 6.7	F: CAAGACTTGGCCTTTTGCTC R: AACACGACCTGCAAAACCAG	1.0	6-FAM (blue)	2

To ensure that DNA amplification conditions were optimal, preliminary products were combined with 3 µl of 5 x loading buffer and electrophoresed on 8%, non-denaturing polyacrylamide gels for three hours at 400 V. Power for electrophoresis was provided by a Gibco BRL PS3002 power supply. Gels were stained for 20 minutes in a 1 µg/ml solution of ethidium bromide, de-stained for 20 minutes in de-ionised water and viewed under UV light. The size of fragments was estimated by comparing them to 5 µl aliquots of GeneWorks® pUC19/HpaII DNA molecular weight marker.

To enable products to be analysed on Applied Biosystems 96-well capillary sequencer at Murdoch University, subsequent PCR reactions were conducted with fluorescently labelled forward primers (Table 2.2). Three different dyes were used, 6-FAM (CQ4.3 and CQ6.7), VIC (CQ1.7 and CQ1.10) and NED (CQ5.11 and CQ6.1), to enable products to be combined and analysed in sets. Each well on the 96-well sequencer plate contained 12.85 µl of formamide, 0.15 µl of GS 500-250 Liz size standard (Applied Biosystems) and 1 µl of three different PCR products (CQ1.7, CQ4.3 & CQ5.11 in analysis set one; CQ1.10, CQ6.1 and CQ6.7 in analysis set two). Chromatograms were viewed using ABI Gene Mapper software (Applied Biosystems) and microsatellite alleles were determined for each individual.

Chapter 3: Genetic diversity and differentiation



Chapter 3: Genetic Diversity and Differentiation

3.1 Introduction

Assessing levels of genetic diversity within and among natural populations has been a major focus of population genetics since its conception (Hamrick & Godt, 1996). This is a consequence of two observations. Firstly, the genetic structure of populations is one of the key factors governing their ability to persist over long timescales as high levels of genetic variability are associated with outcrossing and high levels of morphological and physiological plasticity (Ellstrand & Elam, 1993; Frankham, 1995; Newman & Pilson, 1997). It is this plasticity which enables populations to adapt following shifts in environmental conditions (Amos & Harwood, 1998). Secondly, the distribution of diversity within and between populations can provide insight regarding the historic evolutionary processes that have shaped, and may continue to shape, current population relationships (Hamrick *et al.*, 1992; Krauss, 1997). Some of these processes include founder events, genetic bottlenecks and the presence of reproductive barriers which result in inter-population divergence (Hamrick *et al.*, 1992; Amos & Harwood, 1998).

Population genetic structure can be influenced by a number of intrinsic and extrinsic factors (Hamrick *et al.*, 1992; Amos & Harwood, 1998). Intrinsic factors (some of which include life form, fecundity and mating system) are those which are an inherent feature of a given species (Hamrick *et al.*, 1992; Marcelo *et al.*, 2002). In contrast, extrinsic factors (including population size, population structure and any number of ecological processes and interactions) are those associated with the surrounding environment (Hamrick *et al.*, 1992; Amos & Harwood, 1998).

One extrinsic factor which appears to be a significant determinant of genetic structure in woody plants is the breadth of their geographical range (Hamrick *et al.*, 1992). Because they encompass a broad geographical area, common species are more likely to exhibit high diversity and differentiation as a result of adaptation to a range of environments, or as a consequence of isolation by distance (Hamrick *et al.*, 1992; Coates *et al.*, 2003). In contrast, endemic species, which are characterised by having narrow geographical distributions, would be expected to exhibit low genetic variation as a result of adaptation to a narrow range of environmental conditions (Hamrick *et al.*, 1992; Coates *et al.*, 2003).

These hypotheses have held in many studies which have compared levels of diversity in narrow range endemic species and their more common congeners (Hamrick & Godt, 1996; Gitzendanner & Soltis, 2000; Coates *et al.*, 2003). However, there are also examples where narrow range endemics and common species have been associated with similarly high levels of diversity (Edwards & Wyatt, 1994; Young & Brown, 1996; Coates *et al.*, 2003). Thus there appears to be no definitive relationship between the geographic range occupied by a species and the levels of genetic diversity maintained. In addition, the effects of landscape fragmentation further confound diversity estimates reported in the literature, as many studies have also demonstrated decreased genetic diversity and increased differentiation in species following land clearing (see review by Young *et al.*, 1996).

The major aims of this chapter were to quantify genetic diversity within and among remnant populations of *C.* sp. Whicher, and to characterise the pattern of genetic diversity and interpopulation differentiation. As a result of the geographic proximity of populations, and assuming a mixed mating system similar to that observed in the closely related species *Calothamnus quadrifidus*, two hypotheses were proposed: (i) that similar levels of diversity would be observed within and among populations, with the exception of the very small populations (<10 plants) where diversity was expected to be significantly lower due to sampling effect, and (ii), that differentiation between populations would be low, with no significant relationship between genetic distance and geographical distance.

3.2 Materials and Methods

The genotypes of all reproductive plants in all seven study populations were determined for six microsatellite loci. All sampling and microsatellite characterisation techniques were described in chapter two.

Allelic diversity parameters, including the mean number of alleles per locus (A), percentage polymorphic loci (P), observed heterozygosity (H_O), expected heterozygosity (H_E) and Wright's (1978) fixation index (F_{IS}), were estimated using the program POPGENE (Yeh *et al.*, 1997). Wright's (1951) F statistics, including, F, an overall inbreeding coefficient (analogous to F_{IT}), θ, divergence in allele frequencies among populations (analogous to F_{ST}) and f, degree of inbreeding within populations (analogous to F_{IS}), were calculated across populations as described by Weir and Cockerham (1984) using FSTAT (Goudet, 2001); ninety-five

percent confidence intervals for each parameter were estimated by bootstrapping over loci (1000 bootstraps). Pairwise estimates of θ were obtained between all populations using the same program. The relationship between pairwise genetic distance and geographic distance was explored via linear regression, and the significance of the relationship tested using a Mantel (1967) randomization test (999 permutations) in GENALEX 6 (Peakall & Smouse, 2006). Unweighted pair group method with arithmetic average (UPGMA) hierarchical cluster analysis was performed on Nei's (1978) unbiased estimates of genetic distance with the program GDA (Lewis & Zaykin, 2001), and the results illustrated as a dendogram using TREEVIEW (Roderic, 2001).

As Lavene's test indicated that variances were heterogenous, and this could not be rectified via transformation, the mean number of private alleles (those observed in only a single population) and rare alleles (which were defined as having a global frequency of less than 5%) were compared across populations using the non-parametric Kruskal-Wallis test for three or more independent samples. Pearson's correlation coefficient was used to explore the relationships between population size and the mean number of alleles per locus and population size and observed heterozygosity. The relationships were initially investigated for all seven study populations, and the analyses repeated following the removal of the Ambergate population, which was believed to be a planted population from an unknown seed source, and then once more without Doyle Small and Hairpin Small in order to remove bias associated with their small sample sizes. Significant relationships were further explored via regression analysis. All routine data analysis was performed using SPSS version 11. Results were considered statistically significant when outside 95% confidence intervals ($P < 0.05$).

3.3 Results

3.3.1 Resolution of individuals and genetic diversity

In total, 271 tissue samples were assayed to reveal a total of 245 distinct genotypes. Identical genotypes were observed for 26 samples. Twenty-two of these were observed between samples which were closely located in the field and suspected to be from the same individual at the time of collection. The vast majority of these matches were in Hairpin Large, where samples 1 to 19 appeared to be collected from a single plant regenerating from ground coppice (Figure 3.1). Other matches that were from immediately adjacent stems included Ambergate samples 38 & 39, and Boallia samples 5 and 6, and 13 and 14. These matches were

assumed to represent the same individuals and only one of each was retained for further analysis. The remaining four pairs of identical genotypes (Ambergate 3 & 56, Boallia 2 & 25, 12 & 33, 31 & 42) were from plants not adjacent in the field. Thus they were likely to represent full sibs and were retained. This left a total of 250 genotyped individuals for subsequent analysis.

Figure 3.1 A single multi-stemmed individual of *Calothamnus* sp. Whicher in Hairpin Large. As it was not possible to determine if all of the stems were originating from the same root stock, 19 separate tissue samples were collected and assayed. All samples had the same genotype over six microsatellite loci.

In total, 106 alleles were observed over the six microsatellite loci. All loci exhibited high variability, with the number of alleles per locus ranging from 13 (CQ1.7 and 6.1) to 22 (CQ5.11) with a mean of 17.6 ± 1.6 (Table 3.1). The mean number of alleles per locus (*A*) varied markedly between the six natural populations, with estimates ranging from 1.83 ± 0.31 in Doyle Small to 7.50 ± 1.06 in Doyle Large and a mean of 4.33 ± 0.78 (Table 3.2). Diversity was considerably higher in Ambergate than in any of the natural populations (11.67 ± 1.35). Statistically significant relationships were observed between the mean number of alleles per locus and population size when all populations were included (r = 0.785; P = 0.037), when Ambergate was excluded (r = 0.949; P = 0.004) and following the further exclusion of Doyle Small and Hairpin Small (r = 0.953; P = 0.047) (Figure 3.2). All loci were polymorphic within populations, except for CQ 6.1 which was monomorphic in the Boallia population (Table 3.2). Observed heterozygosity was considerably lower (mean 0.349 ± 0.05) than expected heterozygosity (mean 0.530

± 0.05) in all populations, translating to high fixation indices for all populations (mean F_{IS} 0.315 ± 0.13). No significant relationships were observed between observed heterozygosity and population size ($P > 0.05$; Figure 3.3).

Table 3.1 Allelic diversity characteristics for six microsatellite loci in *Calothamnus* sp. Whicher. *n*, number of samples assayed; *A* number of alleles observed; standard error in parenthesis.

Locus	*n*	*A*	Allele size range (base pairs)
CQ 1.7	271.0	13.0	90-120
CQ 1.10	267.0	20.0	90-128
CQ 4.3	268.0	21.0	102-152
CQ 5.11	271.0	22.0	133-185
CQ 6.1	248.0	13.0	87-129
CQ 6.7	263.0	17.0	104-152
Mean		17.6 (1.6)	

3.3.2 Population genetic structure and differentiation

For all loci, the most frequent allele(s) varied between the seven populations (Figure 3.4). For example, five out of the seven populations had the same dominant allele at locus 1.7 (allele 7), while a different dominant allele was observed in the two remaining populations (Hairpin Medium and Ambergate). There was no consistent pattern in the presence of dominant alleles between populations over all six loci. Ambergate generally had most variability spread over a large number of alleles which were present at low frequencies, while most other populations had distinct dominant alleles (Figure 3.4). The number of private alleles per locus differed between populations (X^2 = 22.57 df = 6; P = 0.01), with mean values ranging from 0.33 ± 0.21 to 1.80 ± 0.79 for the six natural populations, while Ambergate had a mean value of 5.33 ± 0.8. The same trend was observed for the mean number of rare alleles (X^2 = 22.59 df = 6; P = 0.01) with the mean number being three times greater in Ambergate than in any other population (value of 8.33 ± 1.96 for Ambergate vs. mean of 1.55 ± 0.32 for the remaining 6 pops; Figure 3.5).

A Weir and Cockerham (1984) estimate of divergence in allele frequency across populations revealed high genetic differentiation within the study sample (θ = 0.256; Table 3.3). Marked genetic differentiation was also evident from pairwise estimates of θ between all populations, with values ranging from 0.104 between Doyle Large and Doyle Small to 0.348 between Hairpin Medium and Doyle Small, with a mean

Table 3.2 Genetic diversity statistics for seven populations of *Calothamnus* sp. Whicher based on six microsatellite loci. *N*, population size; *n*, number of individuals genotyped; *A*, Mean number of alleles per locus; *P*, percentage polymorphic loci; H_O, Observed heterozygosity over all loci; H_E, Hardy-Weinberg expected heterozygosity over all loci; F_{is}, fixation index (Wright 1978); standard error in parenthesis.

Population	*n*	*A*	*P*	H_O	H_E	F_{IS}
Hairpin Medium	12.0	4.00 (0.52)	100.0	0.317 (0.06)	0.499 (0.08)	0.331 (0.07)
Hairpin Small	3.0	3.16 (0.17)	100.0	0.444 (0.70)	0.711 (0.04)	0.226 (0.14)
Hairpin Large	83.0	7.50 (1.06)	100.0	0.523 (0.68)	0.644 (0.05)	0.178 (0.08)
Doyle Large	44.0	4.83 (0.70)	100.0	0.429 (0.07)	0.626 (0.03)	0.331 (0.13)
Doyle Small	3.0	1.83 (0.31)	100.0	0.111 (0.11)	0.389 (0.13)	0.485 (0.23)
Ambergate	62.0	11.67 (1.35)	100.0	0.315 (0.01)	0.539 (0.13)	0.476 (0.73)
Boallia	43.0	4.66 (0.88)	83.3	0.302 (0.09)	0.382 (0.12)	0.176 (0.05)
Mean	35.7 (11.7)	5.19 (1.27)	92.8 (5.2)	0.349 (0.05)	0.530 (0.05)	0.315 (0.13)

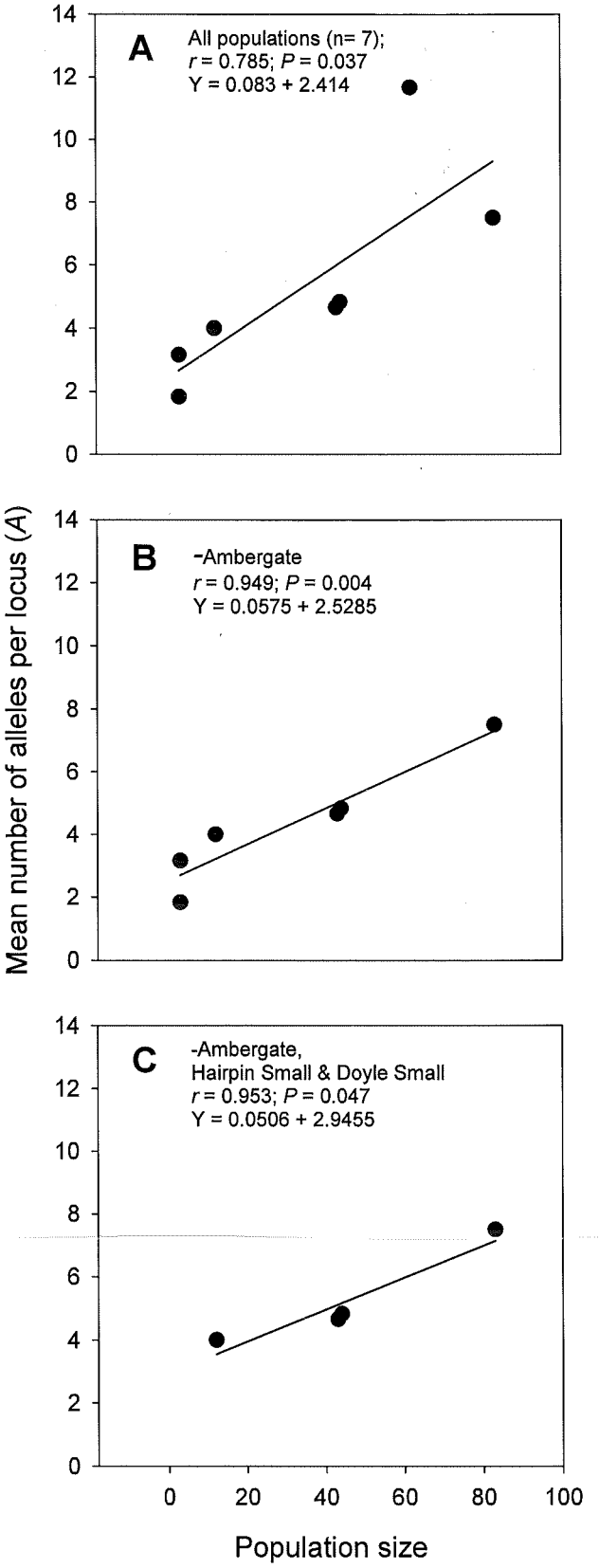


Figure 3.2 The relationship between allelic diversity (mean number of alleles per locus) and population size for seven populations of *Calothamnus* sp. Whicher. Correlations performed for (a) all populations, (b) with Ambergate removed and (c) with Ambergate and Doyle Small and Hairpin Small removed

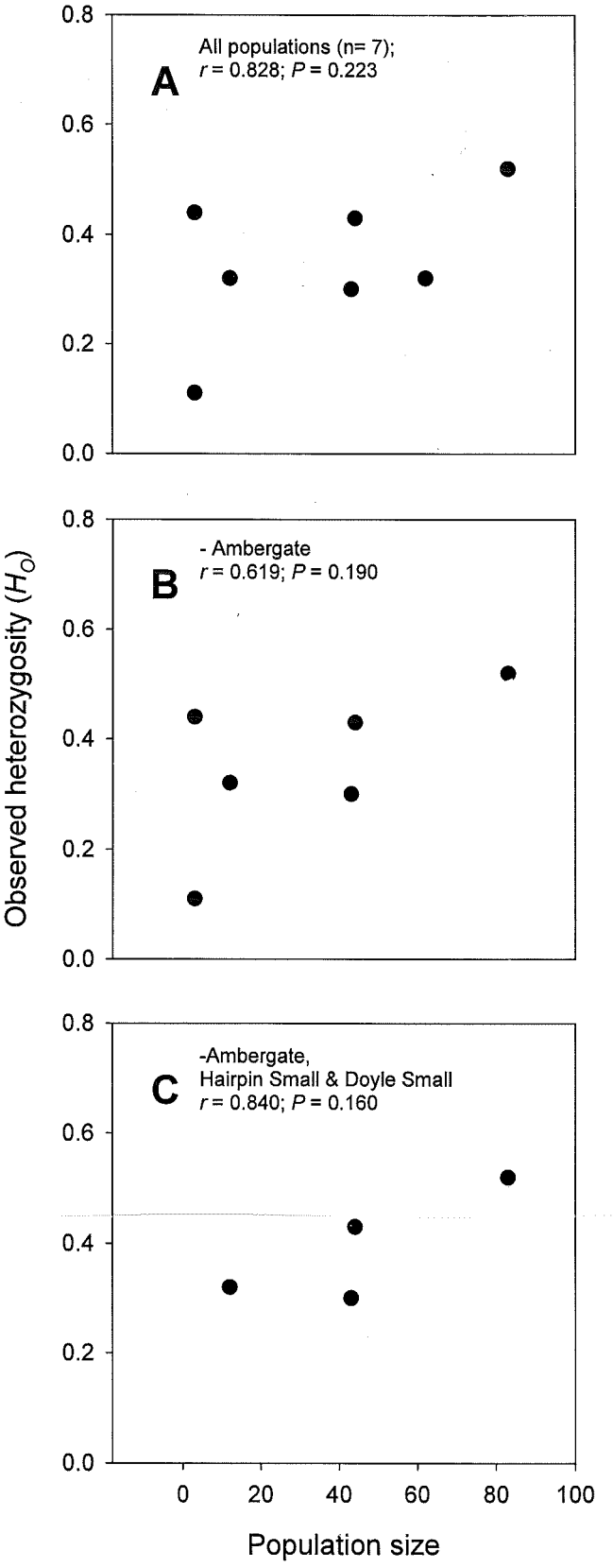


Figure 3.3 The relationship between mean observed heterozygosity and population size for seven populations of *Calothamnus* sp. Whicher. Correlations performed for (a) all populations, (b) with Ambergate removed and (c) with Ambergate and Doyle Small and Hairpin Small removed.

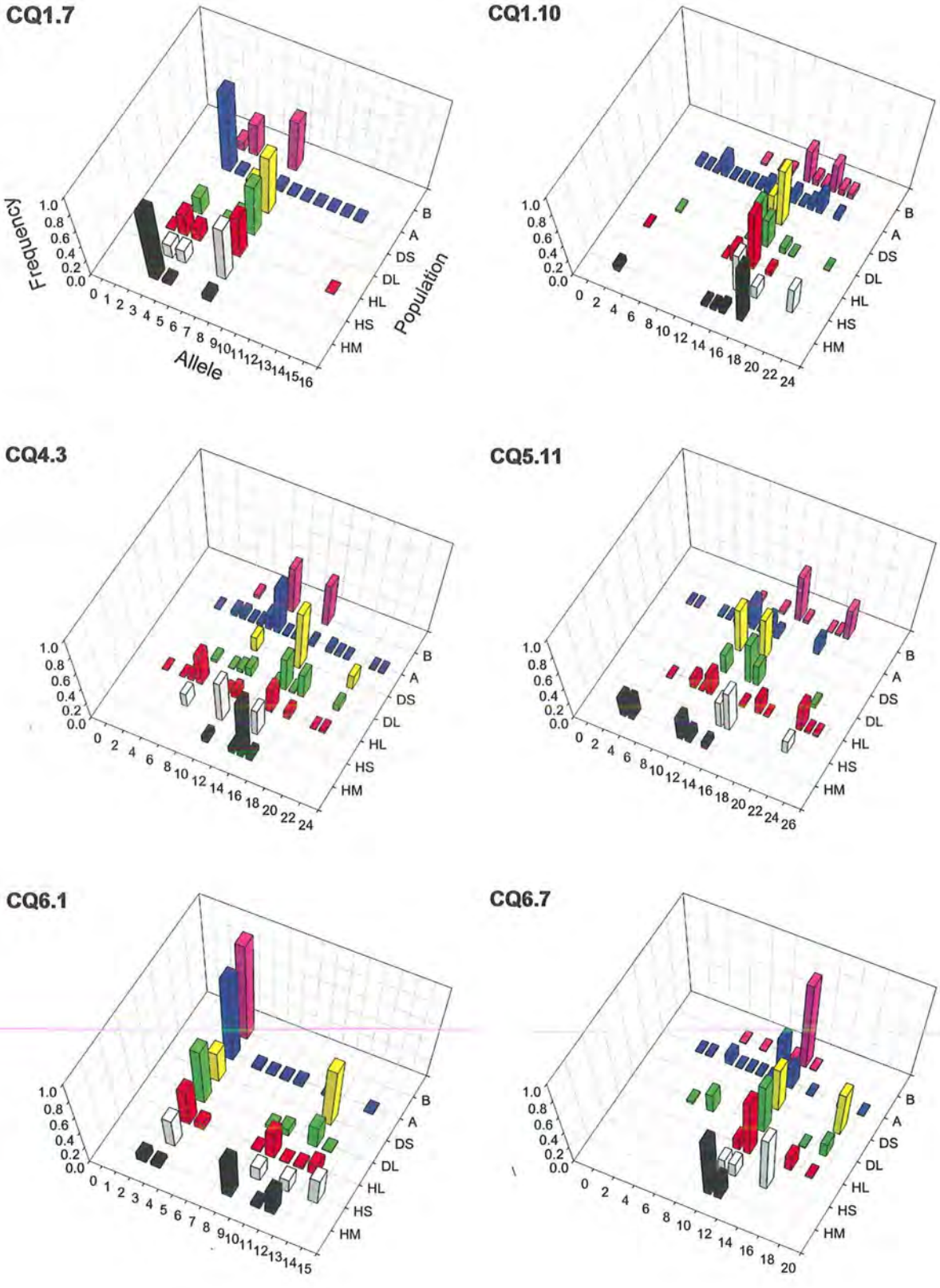


Figure 3.4 Allele frequencies at six microsatellite loci for seven populations of *Calothamnus* sp. Whicher. Alleles are ordered according to their size. HM, Hairpin medium; HS, hairpin Small; HL, Hairpin Large; DL, Doyle Large; DS, Doyle Small; A, Ambergate; B, Boallia. Axis titles on plot one hold for all plots.

estimate of 0.287 ± 0.10 (Table 3.3). Although there was a modest relationship between this pairwise differentiation and geographic distance when the four largest populations were examined (r^2 = 0.56) it was not statistically significant according to a Mantel randomization test (P = 0.130; Figure 3.6).

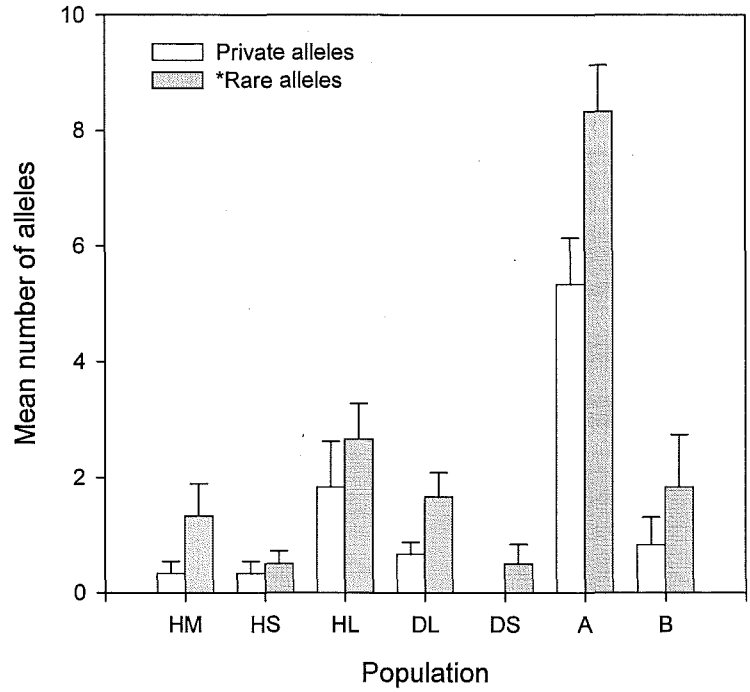


Figure 3.5 The mean number of private alleles and rare alleles over six microsatellite loci for seven populations of *Calothamnus* sp. Whicher. The means were significantly different within treatments based on non-parametric analysis (Kruskal-Wallis X^2 = 22.57 and 22.59, df = 6 for private alleles and rare alleles respectively; P = 0.01 for both treatments). Vertical bars represent the standard error around each mean. *Rare alleles were defined by having a frequency lower than 5% over all populations. HM, Hairpin Medium; HS, Hairpin Small; HL, Hairpin Large; DL, Doyle Large; DS, Doyle Small; A, Ambergate; B, Boallia.

Table 3.3 Differentiation and inbreeding estimates calculated as described by Weir & Cockerham (1984) among seven populations *Calothamnus sp.* Whicher, based on six microsatellite loci. F, Overall inbreeding coefficient (analogous to F_{IT}), θ, divergence in allele frequencies among populations (analogous to F_{ST}), f, degree of inbreeding within populations (analogous to F_{IS}). Ninety-five percent confidence intervals are presented below indices.

F	θ	f
0.469	0.256	0.349
0.398 – 0.545	0.220 – 0.300	0.191 – 0.375

Unbiased estimates of pairwise genetic distance (Nei's D, 1978) also indicated distinct genetic differentiation between populations of *C.* sp. Whicher. Estimates derived from the four large populations were high, ranging from 0.366 between

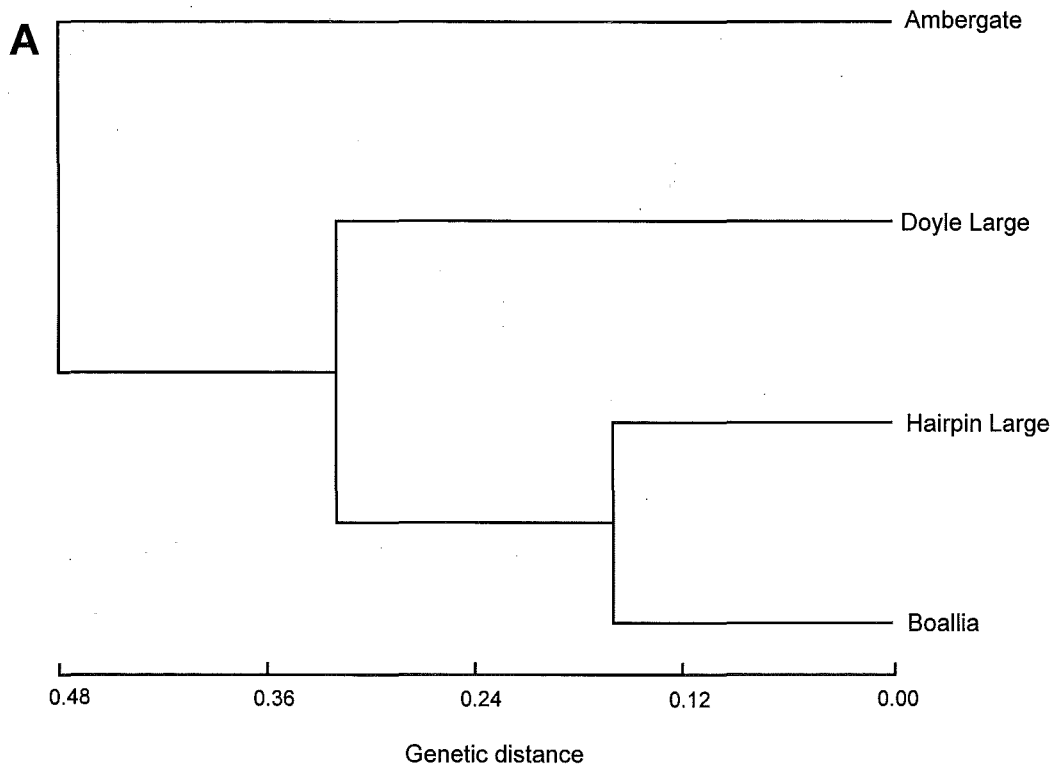
Hairpin Large and Boallia to 0.876 observed between Doyle Large and Ambergate, with a mean distance of 0.37 ± 0.59. A UPGMA dendogram of population relationships is presented in Figure 3.6. This indicates that Ambergate was distinct from the natural populations.

Table 3.4 Differentiation matrix based on pairwise estimates of θ, divergence in allele frequencies among populations (analogous to Wright's (1931) F_{ST}), between seven populations of *Calothamnus* sp. Whicher over six microsatellite loci. Estimates with an asteric include one or more populations with a sample size lower than 12.

	HM	HS	HL	DL	DS	A	B
HM	0.000						
HS	0.232*	0.000					
HL	0.201*	0.105*	0.000				
DL	0.206*	0.116*	0.088	0.000			
DS	0.348*	0.260*	0.195*	0.104	0.000		
A	0.167*	0.205*	0.171	0.185	0.328*	0.000	
B	0.341*	0.178*	0.141	0.147	0.227*	0.219	0.000

3.4 Discussion

The principal aims of this chapter were (i) to characterise the levels of genetic diversity within and among seven populations of *Calothamnus* sp. Whicher and (ii) to determine the amount of differentiation between them. Although global allelic diversity was high over all six loci (17.6 ± 1.6), allelic diversity in the six natural populations was lower (mean A = 4.33 ± 0.77), but similar to levels observed in other woody species with restricted geographical distributions. For example, England *et al.* (2002) observed 3.46 ± 0.20 alleles per locus (six microsatellites) over seven populations of *Grevillea macleayana*, while Jones *et al.* (2005) observed 6.3 alleles per locus (six microsatellites) over three populations of *Eucalyptus morrisbyi*. However, these levels were much lower than those reported for microsatellite studies of woody taxa with broader distributions. For example, Rossetto *et al.* (1999) observed a mean of 38.7 alleles across three populations of the widespread *Melaleuca alternifolia* (Myrtaceae), while Holman *et al.* (2003) observed an average of 16.3 alleles over five microsatellite loci in a *Eucalyptus populnea* – *E. brownii* hybrid zone. Geographic range has been described previously as a factor affecting levels of allelic diversity in species. For example, in an extensive review of the allozyme literature (322 woody taxa), Hamrick *et al.* (1992) found that endemic species maintained approximately 70% less diversity



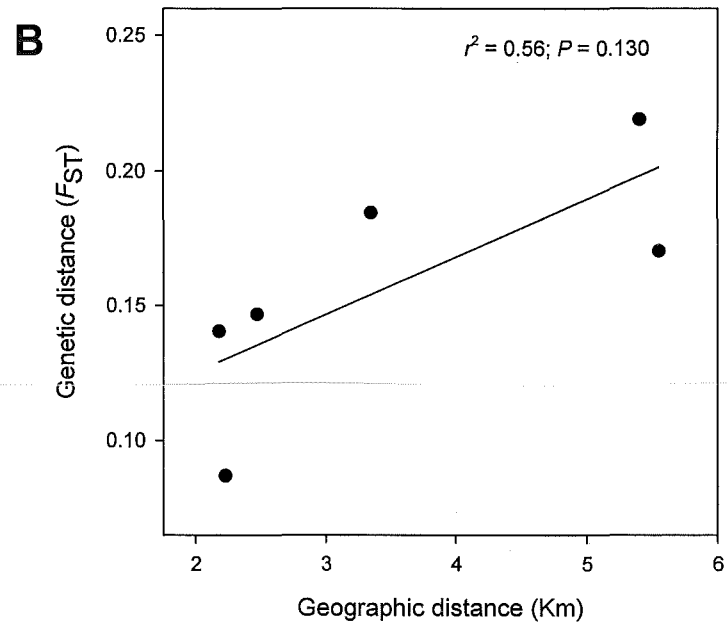


Figure 3.6 (A) UPGMA dendogram based on Nei's (1978) unbiased genetic distance for the four largest populations of *Calothamnus* sp. Whicher over six microsatellite loci and (B) the relationship between pairwise estimates of θ, divergence in allele frequencies between populations (Weir and Cockerham, 1984), and pairwise geographic distance for the same four populations. The significance of the regression in plot B was determined using a Mantel (1967) randomisation test (999 permutations).

than widespread species. These observations appear to be a consequence of the low environmental heterogeneity experienced by species which have limited geographic distributions (Hamrick *et al.*, 1992). Hamrick *et al.* (1992) also suggest that geographic distribution is good predictor of within population diversity, as endemics often occur in small isolated populations where gene flow is limited and the effects of drift are amplified.

Population size does appear to be a factor influencing allelic diversity in this species. The relationship between population size and allelic diversity is widely appreciated and has been observed in a number of plant species. For example, Coates *et al.* (unpublished data) found a positive correlation between the mean number of alleles per locus and population size in *Calothamnus quadrifidus* and *Eucalyptus wandoo*, both of which were within a fragmented landscape. Similarly, Luijten *et al.* (2000) observed a positive relationship between allelic diversity and population size in *Arnica montana* (Asteracea) (see Young *et al.,* 1996 for more examples). According to Ellstrand and Elam (1993), population size affects levels of genetic diversity because the effects of genetic drift and inbreeding increase with decreasing population size. But levels of diversity in very small populations can also be low as a result of sampling effect, as population size dictates the maximum level of allelic diversity that a population can maintain (Amos & Harwood, 1998). In this study, levels of allelic diversity were correlated with population size, even when the very small populations (Hairpin Small and Doyle Small) were excluded. Thus, this relationship suggests that smaller populations had lower allelic diversity, presumably as a result of sampling.

In contrast, levels of observed heterozygosity (H_O) were not associated with population size. Although a number of authors have observed a positive correlation between H_O and population size in plants (Fisher *et al.*, 2000; Paschke *et al.*, 2002), this has not been the case for the majority of studies (Luijten *et al.*, 2000). According to Amos & Harwood (1998), levels of heterozygosity are less sensitive to changes in population size than levels of allelic diversity. This is because heterozygosity is not directly dependent upon the number of individuals in a population, as is the case with allelic diversity (Amos & Harwood, 1998). Instead, heterozygosity is controlled by patterns of mating (i.e. rates of outcrossing and self fertilisation), though these are influenced by population size to some degree.

Considering the close proximities of the study populations, global and pairwise estimates of differentiation were high. Generally, differentiation of this nature is observed between populations over vast geographical distances, and is a consequence of limited gene flow, diversifying selection in response to variation in local environmental conditions or founder events (Amos & Harwood, 1998; Hamrick *et al.*, 1992). However, the levels of differentiation and genetic distance observed between populations in this study were similar to those observed in other threatened species in fragmented landscapes (Les *et al.*, 1991; van Trueren *et al.*, 1991; Dolan, 1984; Raijman *et al.*, 1994; Godt *et al.*, 1996; Fischer & Matthies, 1998). For example, Hoebee & Young (2001) observed high genetic differentiation (F_{ST} = 0.204 ± 0.040) and intermediate distance (Nei's D = 0.15) between populations of the endangered shrub, *Grevillea iaspicula*. England *et al.* (2002) also found similar patterns of diversity (F_{ST} = 0.204; Nei's, 1978 D ranging from 0.118 – 0.713) in another rare *Grevillea* species, *Grevillea macleayana.* Not only do these species have similar life history strategies to *Calothamnus* sp. Whicher, in that they are long-lived, bird-pollinated woody shrubs, the study populations were similar in size and located over a similar geographical distance to those in the present study. The extent of differentiation and genetic distance observed in these studies has been attributed to limited gene flow. This also seems to be the case for the populations in this study, as the observed differentiation is unlikely to be a result of adaptation to local conditions considering their close proximity and restriction to a specific soil type. However, the pattern of differentiation was not explained by a simple isolation by distance model.

In addition to limited gene flow between the study populations, the high levels of differentiation between them appears to be a consequence of high levels of self fertilisation or bi-parental inbreeding, as high F_{IS} values were observed within and among populations. Some selfing would be expected considering the mixed mating systems exhibited by myrtaceous species. For example, Yates *et al.* (in press A) observed low (0.10) to moderate (0.45) rates of self fertilisation in the closely related shrub, *Calothamnus quadrifidus*, while Sampson *et al.* (1995) observed similar rates in the bird-pollinated mallee, *Eucalyptus rameliana*. However, the high fixation indices obtained in this study indicate that rates of selfing may be higher in these populations.

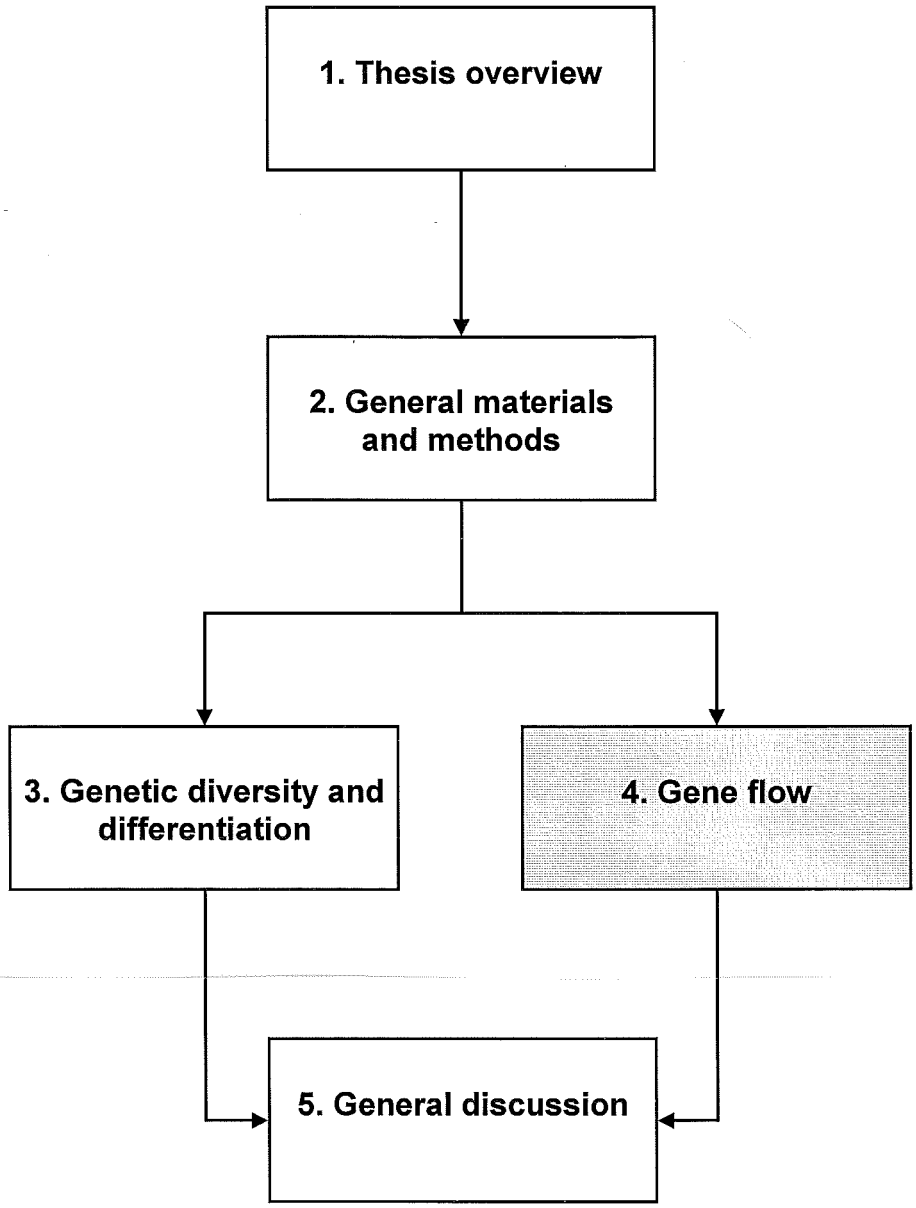
It is also likely that the effects of genetic drift have played a major role in the observed patterns of differentiation. All of the populations (with the exception of

Ambergate) are located on severely degraded roadsides which are surrounded by expanses of agricultural matrix. Based on their locations (> 250 m apart), the heavily modified state of the interconnecting 'habitat', and the large areas occupied by other populations which exist in natural vegetation, it is also likely that the three smallest populations were once connected to the nearby larger populations (Hairpin Small and Medium with Hairpin Large, and Doyle Small with Doyle Large). Through a combination of drift and inbreeding, it is possible these populations may have diverged as a consequence of land clearing.

The data obtained in this study also enables a test of the hypothesis that the population in Ambergate reserve was planted. The high allelic diversity in Ambergate, and the high degree of divergence from other populations, does support this hypothesis. Moreover, the high number of private alleles in this population suggests that the seed used to establish the population was collected outside of this study area.

The results of this study indicate that the selected populations of *Calothamnus* sp. Whicher had high levels of among population diversity, yet considerably lower levels of diversity within populations. In addition, considerable genetic differentiation was observed between populations. This differentiation appears to be a consequence of long-term reproductive isolation between populations, considerable levels of inbreeding and the effects of random genetic drift. Such a scenario was not expected for these populations considering the life history strategy of this species. However, it would appear that bird pollination and the long life span of adults have not buffered these populations against genetic change.

Chapter 4: Gene flow



Chapter 4: Gene flow

4.1 Introduction

Gene flow is a major factor that impacts upon the genetic structure of populations and species (Slatkin, 1985). Although this concept has been central to studies of population genetic structure for decades, paternity assignment techniques have recently broadened our understanding of gene flow and the factors that affect it (Adams *et al.*, 1992).

A large proportion of the paternity assignment studies performed on plants have focused on estimating pollen dispersal between economically important species and their wild relatives (Wang *et al.*, 2004; Chen *et al.*, 2004; Otero-Arnaiz *et al.*, 2005). More recently, studies have focused on patterns of pollen-mediated gene flow in natural populations. These studies (White *et al.*, 2002; Dunphy *et al.*, 2004; Oostermmeijer & De Knegt, 2004; Byrne *et al.*, in press) have demonstrated patterns of gene flow which contradict a number of the existing paradigms associated with pollen dispersal. It appears that for many species, pollen travels significantly further than originally estimated. For example, White *et al.* (2002) observed gene flow events over a maximum distance of 4.5 km in the insect pollinated tropical forest tree, *Swietenia humilis*; an estimate ten times greater than those determined for insect pollinated tropical trees from pollinator observations. A number of studies also suggest that increased spatial isolation has little impact upon pollen immigration rates (Trapnell & Hamrick, 2005; Byrne *et al.*, in review).

The vast majority of these studies have investigated patterns of gene flow in common species which are associated with broad geographical distributions (Lowe *et al.*, 2004). Those which have focused on rare species have mainly examined insect-pollinated tropical trees (Lowe *et al.*, 2004). Tropical trees are characterised by low density distributions and rarely form clumped populations which are common in temperate species (Lowe *et al.*, 2004). Thus, one would expect the pollen dispersal vectors associated with tropical species to be well adapted for long distance dispersal (Lowe *et al.*, 2004).

In Western Australia, many bird pollinated species rely on honeyeaters as pollen vectors (family Melaphigidae). These species are considered generalists as they are known to visit many plant species. For example, Hopper and Burbridge (1986) observed the New Holland and Brown Honeyeaters feeding from over 65 native

taxa. A number of studies also report that honeyeater abundance is associated with flower abundance (Paton, 2000). More specifically, more honeyeaters were observed in areas where there were more flowers (Paton, 2000). Because rare endemic species are often geographically restricted and are represented by fewer individuals, this may influence the way that they are visited by avian pollinators.

This chapter has three aims: (i) to describe patterns of gene flow into and between the two study populations in which seedlings were assayed, (ii) to describe the mating system employed by this species and (iii) to describe and compare patterns of pollen dispersal within the two study populations. Given the findings of mating system and gene flow studies performed on the closely related species, *Calothamnus quadrifidus*, the following three hypothesis were formed: (i) that gene flow into and between the two sampled populations would be similarly high, (ii) that *Calothamnus* sp. Whicher would exhibit a mixed mating system with similar rates of outcrossing to that exhibited by *C. quadrifidus* and (iii) that patterns of within population dispersal would reflect those observed in other bird pollinated species.

4.2 Materials and Methods

The genotypes of all plants in all seven study populations were determined for six microsatellite loci. In addition, seed samples were collected in Hairpin Large and Doyle Large from ten randomly selected mother plants. Fifteen seedlings from each mother were then genotyped at the same six microsatellite markers. All sampling and microsatellite characterisation techniques are described in chapter two.

Paternity for each seedling was assigned using the maximum likelihood approach in CERVUS version 2.0 (Marshall *et al.*, 1998). The log-likelihood statistic, Δ (Delta), was obtained using the default simulation settings. Confidence intervals for stringent and relaxed assignment were set at 95 and 80% respectively. Outcrossing rate was determined as 1 - s where s is the proportion of selfed progeny. The level of pollen immigration in each population, t_o, was determined as 1 - (t_i + s) where t_i is the proportion of outcross events resulting from pollen intrinsic to the populations. The number of migrants entering each population per generation (*Nm*) was estimated as t_o x (2n) where n is the number of reproductive plants in the population. A historical estimate of gene flow was also made indirectly as the number of migrants (*Nm*) between populations using the F_{ST} method (Slatkin, 1987) in GENALEX 6 (Peakall & Smouse, 2006).

Differentiation of maternally sampled pollen pools was assessed by calculating global, within population and pairwise among population estimates of, Φ_{ft}, using the TWOGENER analysis of Smouse *et al.* (2001) in GENALEX 6. Probability values based on the deviation of estimates from zero were obtained via 999 permutations. The relationship between pollen pool differentiation and geographic distance in Hairpin Large was investigated via linear regression, and the significance of the relationship tested via a Mantel (1967) randomization test (999 permutations) using the same program. Mean pairwise pollen pool differentiation between Hairpin Large and Doyle Large was compared via a t-test using SPSS version 11. Prior to performing the t test, data were tested for normality and homogeneous variances using the Shapiro-Wilks and Lavene's tests, respectively.

Comparisons of the mean percentage of progeny resulting from self-fertilisation, internal outcrossing and pollen immigration between the two populations, as well as the mean number of outcrossed seedlings with internal fathers and the mean number of different fathers per mother, were also made using t tests. Results were considered significant when outside 95% confidence intervals ($P < 0.05$).

4.3 Results

Microsatellite characteristics, paternity assignment and outcrossing rates

Polymorphic information content was high over the six microsatellite loci, with values ranging from 0.514 for CQ 6.1 to 0.872 for CQ4.3, with a mean of 0.683 ± 0.056 (Table 4.1). Individual exclusion probabilities ranged from 0.385 for CQ1.7 to 0.767 for CQ4.3. The total exclusion probability obtained over all six markers was 0.991. The frequency of null alleles at each locus ranged from 0.178 to 0.415 with a mean of 0.293 ± 0.038 (Table 4.1).

Of the 285 progeny analysed, paternity assignment identified a pollen source for 246 (86%) within 80% confidence intervals. Of the 40 unresolved progeny, 31 (78%) had paternal haplotypes which were consistent with self fertilisation. For these samples, paternity was assigned to the mother plant. For the remaining nine progeny (6%), paternity was assumed to have arisen from the pollen parent with the highest likelihood score.

Outcrossing rates were highly variable between mother plants with values ranging from 7% to 100% percent (Table 4.2), with a mean of 44.4 ± 6.3 across all 20 mothers. The internal outcross rate was higher in Hairpin Large (51.3 ± 9.9) than in

Doyle Large (37.6 ± 7.6), though this was not significantly different ($t_{df18} = 1.102$; $P = 0.285$).

Table 4.1 Polymorphic information content (PIC), exclusion probabilities (Excl) and estimated frequency of null alleles (Null) for six microsatellite markers over seven populations of *Calothamnus* sp. Whicher.

Locus	PIC	Excl	Null
CQ 1.7	0.587	0.385	0.415
CQ 1.10	0.756	0.592	0.322
CQ 4.3	0.872	0.767	0.178
CQ 5.11	0.777	0.635	0.365
CQ 6.1	0.514	0.347	0.285
CQ 6.7	0.519	0.411	0.193
Mean	0.683 (0.056)		0.293 (0.038)
Total		0.991	

4.3.1 Gene flow

Direct estimates of gene flow into both populations were very low (Figure 4.1; Table 4.2). For Hairpin Large only 2.7% of pollinations were a result of pollen immigration, with less than 1% of pollination events resulting from pollen originating from Hairpin Small (158 m), Boallia (2.18 km), Doyle Large (2.23 km) and Ambergate (5.5 km) (Figure 4.1). Although pollen was received from fewer populations and over shorter distances, 4% of pollinations within Doyle Large resulted from immigrant pollen, with 2% originating from Doyle Small (148 m), 1% from Hairpin Large (2.23 km) and 1% from Boallia (2.47 km). Rates of pollen immigration did not differ significantly between the two populations. ($t_{df18} = -0.483$; $P = 0.635$).

The mother plants which received extrinsic pollen were generally located on the periphery of the population which was closest to the pollen source. For example, mother number 99, which was the eastern most plant in Hairpin large, received pollen from Doyle Large which was located to the east. Similarly, mother number 56, which was located on the southern periphery of Hairpin Large, received pollen from Boallia, which was located directly south.

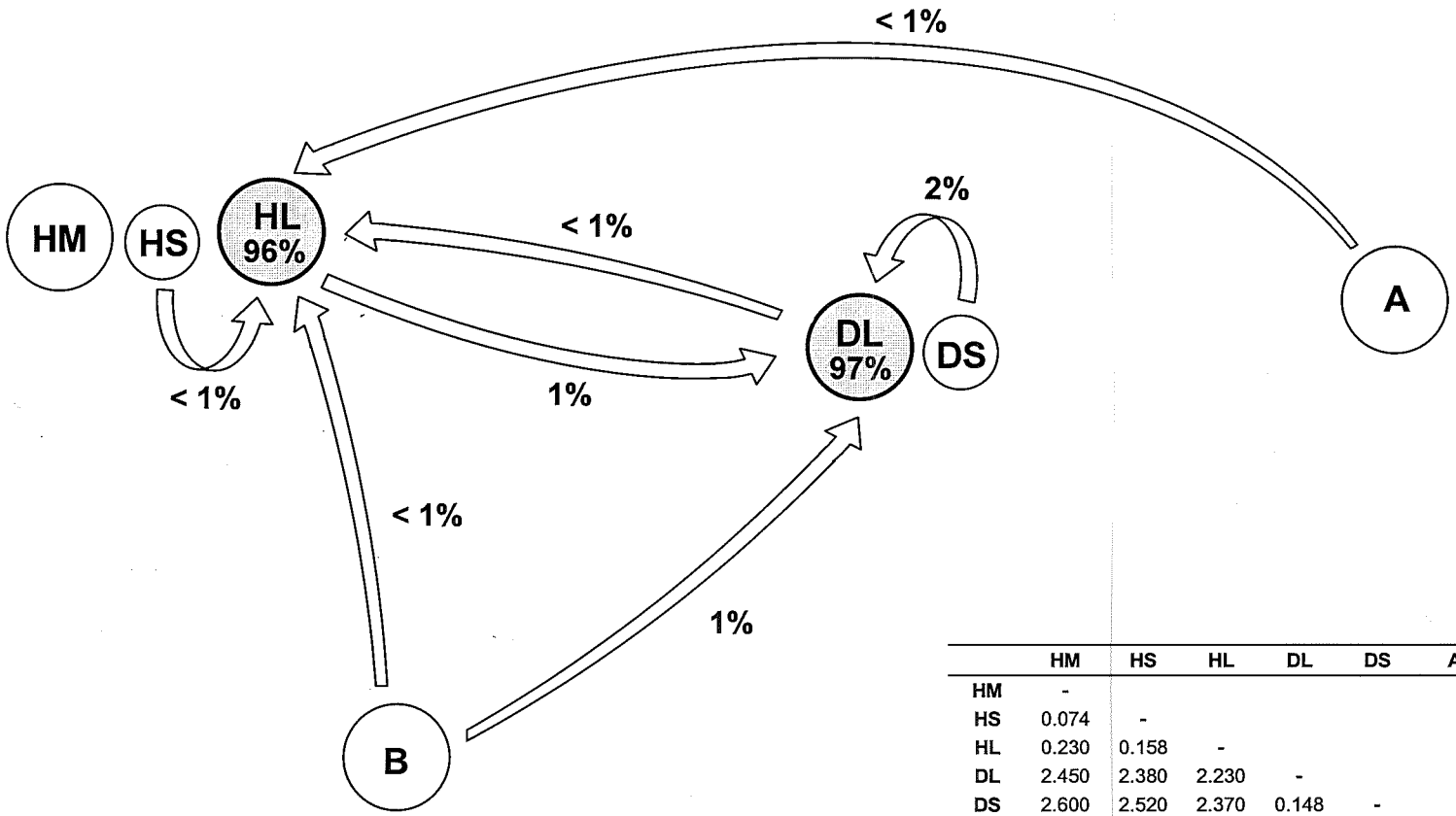
Table 4.2 Selfing and outcrossing rates for seed crops from two populations of *Calothamnus* sp. Whicher as determined via maximum likelihood paternity assignment. n = number of seedlings assayed.

Population	Mother	n	Selfing rate (%)	Internal outcross rate (%)	Apparent pollen immigration (%)
Hairpin Large	1	15	93	7	0
	22	15	53	40	7
	28	13	54	46	0
	35	15	67	33	0
	45	15	33	67	0
	49	15	7	93	0
	56	15	47	40	13
	62	15	0	100	0
	79	15	27	73	0
	99	14	79	14	7
Mean		14.7 (0.2)	46.0 (9.4)	51.3 (9.9)	2.7 (1.5)
Doyle Large	2	15	67	33	0
	4	15	53	47	0
	8	15	7	80	13
	12	15	60	20	20
	17	14	71	22	7
	26	15	60	40	0
	29	15	80	20	0
	36	15	67	33	0
	38	4	25	75	0
	44	15	93	7	0
Mean		13.8 (1.1)	58.3 (8.0)	37.6 (7.6)	4 (2.25)

Indirect estimates of the number of migrants between the seven study populations were indicative of moderate historical gene flow (Table 4.3). Pairwise estimates between the six natural populations ranged from 0.556 between Boallia and Hairpin Medium to 3.438 between Doyle Large and Doyle Small, with a mean value of 1.551 ± (0.158). The mean estimate of *Nm* between the three largest populations was 2.160 ± 0.365. This was similar to the estimates of *Nm* calculated from direct pollen immigration rates for Hairpin Large and Doyle Large (4.40 and 3.52 respectively).

4.3.2 Within population patterns of dispersal

For both of the assayed populations, the majority of pollinations (97 and 96% for Hairpin Large and Doyle Large, respectively) resulted from pollen of an intrinsic



	HM	HS	HL	DL	DS	A	B
HM	-						
HS	0.074	-					
HL	0.230	0.158	-				
DL	2.450	2.380	2.230	-			
DS	2.600	2.520	2.370	0.148	-		
A	5.780	5.712	5.555	3.350	3.208	-	
B	2.270	2.240	2.180	2.470	2.586	5.405	-

Figure 4.1 Patterns of pollen dispersal among seven populations of *Calothamnus* sp. Whicher illustrated by assigning paternity to progeny propagated from seed collected from the two shaded populations, HL (n = 147) and DL (n = 138). The values within the circles represent the percentage of pollinations resulting from a parent within the population including selfed events. Values next to the arrows represent the percentage of pollinations resulting from gene flow into populations. Map not to scale, but distances between populations (km) are provided in the tri-matrix. HM, Hairpin Medium; HS, Hairpin Small; HL, Hairpin Large; DL, Doyle Large; DS, Doyle Small; A, Ambergate; B, Boallia.

Table 4.3 Pairwise estimates of indirect gene flow, as the mean number of migrants between populations per generation (*Nm*), calculated using the F_{ST} method (Slatkin, 1987), for six natural populations of *Calothamnus* sp. Whicher. HM, Hairpin Medium; HS, Hairpin Small; HL, Hairpin Large; DL, Doyle Large; DS, Doyle Small; B, Boallia. Mean *Nm* = 1.551 ± 0.158; mean *Nm* between the three large populations (estimates denoted in bold) = 2.16 ± 0.365.

	HM	HS	HL	DL	DS	B
HM	0.000					
HS	0.826	0.000				
HL	0.983	2.048	0.000			
DL	0.970	2.112	**2.890**	0.000		
DS	0.748	1.248	1.748	3.438	0.000	
B	0.556	1.310	**1.762**	**1.828**	0.816	0.000

source (Figure 4.1; Table 4.2). The mean number of outcrossed seedlings was higher in Hairpin Large (8.0 ± 1.44) than in Doyle Large (5.1 ± 1.16) (Table 4.4). However, due to the variation observed between mothers, no statistically significant difference was observed (t_{df18} = 1.570; P = 0.134). Similarly, no significant difference was observed when the percentage of outcrossed seedlings with internal fathers (Hairpin Large = 91.3% ± 4.03; Doyle Large = 92.7% ± 4.01; t_{df18} = -0.246; P = 0.808) or the number of different fathers contributing pollen to each mother were compared (Hairpin Large = 4.6 ± 0.65; Doyle Large = 3.1 ± 0.43; t_{df18} = 1.91; P = 0.072) (Table 4.4).

Analysis of the dispersal events within Hairpin Large over distance classes revealed that the vast majority of pollination events (76%) resulted from a pollen parent located less than 10 m from the mother plant (Figure 4.2). Of these 106 progeny, 67 (63%) were a result of self fertilisation, while the remaining 39 (37%) were outcrossed. Although longer distance dispersal events were recorded across the 50 m breadth of the population, the number of pollinations generally decreased with increasing distance between plants (with the exception of the 21-30 m class which was associated with more pollinations than the 11-20 m class) (Figure 4.2).

For Hairpin Large, the number of near neighbour matings as a percentage of outcrossed events was highly variable among the ten Hairpin Large mothers, with values ranging from 0 to 70%, and a mean value of 36.1% ± 8.8. However, when

assessed over all events, this mean value decreased to 23% ± 6.6, with individual values ranging from 0 to 60% (Table 4.5).

Table 4.4 Percentage of seedlings with fathers internal to the population and the number of different fathers for each mother plant in two populations of *Calothamnus* sp. Whicher.

Population	Mother	Number of outcrossed seedlings	Percentage of outcrossed seedlings with internal fathers	Number of different fathers
Hairpin Large	1	1	100	1
	22	7	86	3
	28	6	100	5
	35	5	100	4
	45	10	100	5
	49	14	100	6
	56	8	88	6
	62	15	100	7
	79	11	73	7
	99	3	66	2
	mean	8.0 (1.44)	91.3 (4.03)	4.6 (0.65)
Doyle Large	2	5	100	3
	4	7	100	4
	8	14	86	6
	12	6	66	4
	17	4	75	3
	26	6	100	2
	29	3	100	3
	36	5	100	3
	38	3	100	2
	44	1	100	1
	mean	5.1 (1.16)	92.7 (4.01)	3.1 (0.43)

4.3.3 Pollen pool differentiation

Pollen pool differentiation among all 20 mothers from both populations was high and significantly different from zero (Φ_{ft} = 0.505; P = 0.001) (Table 4.6). Significant differentiation was also observed within populations, though differentiation was lower in Hairpin Large than in Doyle Large (0.358 vs 0.543; P = 0.001 for both estimates). Pairwise differentiation between mothers within populations was also variable with values in Hairpin Large ranging from 0.046 to 0.624, while those in Doyle Large ranged from 0.028 to 0.808. Mean pairwise Φ_{ft} was significantly higher in Doyle Large than in Hairpin Large (0.515 ± 0.028 vs. 0.344 ±

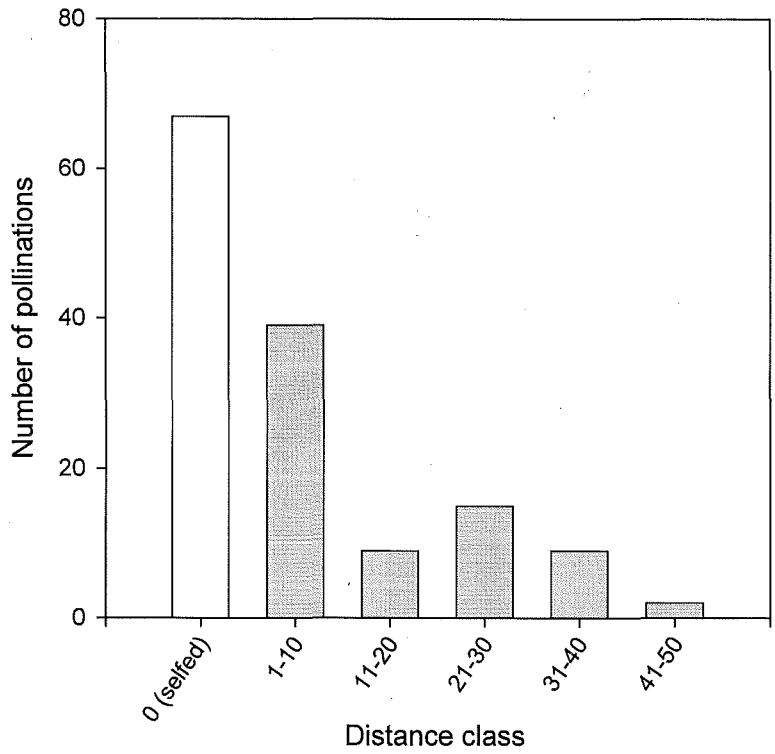


Figure 4.2 The frequency of pollination events as a function of distance between pollen parents and mother trees in the Hairpin Large population. n = 141.

Table 4.5 Number of near neighbour mating events within the Hairpin Large population of *Calothamnus* sp. Whicher.

Mother	n	Near neighbour matings over outcrossed events (%)	Near neighbour matings over all events (%)
1	15	0	0
22	15	57	29
28	13	67	31
35	15	0	0
45	15	70	47
49	15	36	33
56	15	38	20
62	15	60	60
79	15	0	0
99	14	33	7
mean	14.7 (0.21)	36.1 (8.8)	22.7 (6.6)

± 0.019; t = 2.538 $_{df}$18; P = 0.021) (Table 4.4). Although differentiation was lower at all scales (globally, within populations and mean pairwise) when selfed progeny were removed from the analysis, mean pairwise estimates of Φ_{ft} still differed significantly between the two study populations. No statistically significant relationships were observed between pairwise estimates of Φ_{ft} between mother plants in Hairpin Large and geographic distance for all pollination events (r^2 = 0.055; P = 0.062), or when selfed progeny were removed (r^2 = 0.001; P = 0.380; Figure 4.3).

Table 4.6 Global, among mother (within populations) and mean pairwise (within populations) differentiation in maternally sampled pollen pools (Φ_{ft}) for two populations of *Calothamnus* sp. Whicher.

	Population	
	Hairpin Large	Doyle Large
Global Φ_{ft}	0.505*	
Global Φ_{ft} outcross events only	0.364*	
Among mothers Φ_{ft}	0.358*	0.543*
Among mothers Φ_{ft} outcross events only	0.267*	0.349*
[a]Mean pairwise Φ_{ft}	0.344 (0.019)	0.515 (0.028)
[a]Mean pairwise Φ_{ft} outcross events only	0.231 (0.019)	0.348 (0.025)

*Values differ from 0 at the 0.001 alpha-level.
[a] Mean pairwise values were significantly different (P < 0.000).

4.4 Discussion

The six hyper-variable microsatellite markers, which were designed for the closely related species, *Calothamnus quadrifidus*, have enabled the assignment of paternity to the majority of progeny with a high level of confidence in *Calothamnus* sp. Whicher. The multilocus exclusion probability of 0.991 obtained here was similar to that obtained in other studies of paternity which have employed microsatellite markers (White *et al.*, 2002, 0.983; Otero-Arnaiz *et al.*, 2005, 0.960; Byrne *et al.*, in press, 0.998). Moreover, it exceeded the total exclusion probabilities obtained in studies which employed approximately twice the number of isozyme markers (Dunphy *et al.*, 2004, 0.940 using 11 markers; Schuster & Mitton, 1999, 0.880 using 10 markers). Such data clearly emphasize the superiority of microsatellite markers in studies of parentage analysis. However,

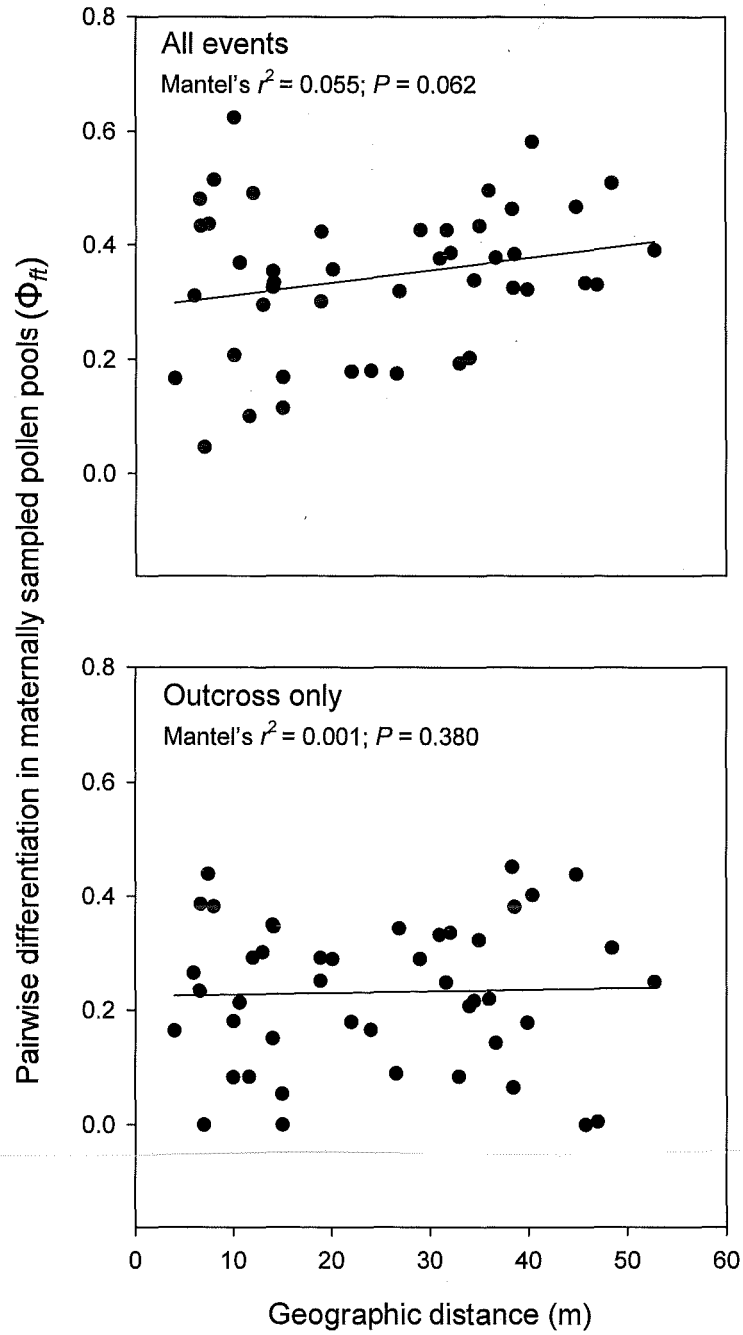


Figure 4.3 The relationship between pairwise differentiation in maternally sampled pollen pools (Φ_{ft}) and geographic distance for ten individuals of the woody shrub, *Calothamnus* sp. Whicher in the Hairpin Large population. Pairwise estimates of Φ_{ft} in the first plot were based on the genotypes of 13 to 15 progeny for each mother over six microsatellite loci. Estimates in the second plot were derived from outcrossed events only. The significance of the regression in each plot was tested using a Mantel (1967) randomization test (999 permutations). n = 45 in both plots.

even with the high exclusion probability obtained, the maximum likelihood assignment approach was unable to assign paternity to a number of progeny that had genotypes consistent with self fertilisation. This was a consequence of the high levels of genetic similarity observed between plants within populations (see chapter 3). Given the high levels of self pollination within the assigned progeny, it was assumed that the progeny with unassigned paternity but with genotypes consistent with self fertilisation resulted from self pollination, rather than pollination from another plant. Therefore, outcrossing rates may be underestimated by 2.7% in Hairpin Large and 20% in Doyle Large. Similarly, ambiguous paternity could not be resolved for nine outcrossed progeny in Doyle Large. The assumption that paternity arose from the parent with the highest likelihood score is most conservative, and in all but one situation, paternity was assigned to a pollen parent intrinsic to the population. However, if pollination actually occurred from an outside pollen source, this assumption may have led to an underestimate of pollen immigration into Doyle Large by up to 6%.

Maximum likelihood assignment of pollen parents to seed crops revealed limited gene flow into the two assayed populations, with pollen immigration rates of 2.7% and 4% for Hairpin Large and Doyle Large, respectively. These rates are considerably lower than those reported in most other direct studies of gene flow, including those determined for tropical (White *et al.*, 2002; Dunphy *et al.*, 2004; Ward *et al.*, 2005) and temperate (Byrne *et al.*, in review) insect pollinated trees, herbaceous species (Otero-Arnaiz *et al.*, 2005) and the bird pollinated orchid, *Laelia rubescens* (Trapnel & Hamrick, 2005). In addition, rates of gene flow observed here are considerably lower than those observed in the closely related species *Calothamnus quadrifidus* (mean pollen immigration rate of 30% over three populations; Byrne *et al.*, in press). While this was largely unexpected, there are a number of possible explanations for the observed difference. The first is associated with the size of the populations examined in each study. Theory predicts that the influence of extrinsic pollen on pollination events in a population will be largely determined by the number of flowers which are visited subsequent to the arrival of a pollinator into a population (Cresswell & Osbourne, 2004). This is a consequence of the fact that extrinsic pollen brought into a population will be rapidly depleted during the pollinator's initial floral visits (Ellstrand & Elam, 1993; Cresswell & Osbourne, 2004), after which time all subsequent pollinations will be a result of pollen intrinsic to the population (Ellstrand & Elam, 1993; Cresswell & Osbourne,

2004). Thus, as the number of flower visits increases, the ratio of extrinsic pollinations to intrinsic pollinations is continually reduced (Cresswell & Osbourne, 2004; demonstrated by Broyles *et al.*, 1994; White *et al.*, 2002; Trapnell & Hamrick, 2005). Many factors influence the number of flowers visited by a pollinator within a population or patch, a phenomenon referred to as pollinator residence (Cresswell & Osbourne, 2004). Of these, Cresswell and Osbourne (2004) suggest that the most important is the number of flowers available within the patch. In most cases, the number of flowers within a population would be expected to increase with increasing population size (Cresswell & Osbourne, 2004). One may therefore expect higher pollinator residence within populations that contain a greater number of flowering individuals, or plants with larger canopies (Pyke, 1984; Cresswell & Osbourne, 2004). In their study of *C. quadrifidus*, Byrne *et al.* (in press) concentrated on populations which ranged in size from one plant to 23 plants. In this study, paternity assignment was performed on progeny derived from populations of 44 and 83 individuals. Thus, the larger populations here may have lead to greater pollinator residence, and consequently a lower proportion of progeny resulting from pollen immigration. However, this hypothesis is not supported by the positive relationship between population size and pollen immigration observed by Byrne *et al.* (in press) in *C. quadrifidus*.

Although not investigated in this study, another possible explanation for the limited gene flow is a low abundance of avian pollinators in the study area. Birds, particularly honeyeaters, have been described as the primary pollen vectors within the genus *Calothmanus* (Ford *et al.*, 1979; Collins *et al.*, 1984; Yates *et al.*, in press A). Of all biological pollen vectors, birds are considered one of the most effective with regards to their ability to mediate gene flow, as they are capable of travelling large distances over sub-optimal habitat (Low *et al.*, 2004; Trapnell & Hamrick, 2005). The presence of some long dispersal distance events in this study (up to 5.55 km) suggests that birds are capable of traversing the distance between any pair of the study populations despite extensive fragmentation. This is also supported by previously recorded observations of honeyeater flight between fragments that were isolated by up to 12.5 km (Ford *et al.*, 2000). A decrease in the abundance of birds may be linked to lack of suitable habitat in the study area. This is supported by a number of ecological studies of bird-pollinated species. For example, Paton (2000) observed significantly fewer honeyeaters in heavily fragmented areas of South Australia compared to areas which were largely undisturbed. The study also demonstrated significant pollinator limitation in these

fragmented areas for a number species (primarily *Astroloma* and *Grevillea*) which were naturally bird pollinated (Paton, 2000). Similarly, Watson *et al.* (2003) observed a reduction in species richness and functional groups of birds in a fragmented woodland in south eastern Australia. In the present study, neither of the sampled populations was connected to natural vegetation, and there was very little remnant vegetation in the immediate study area, providing little shelter or food for large bird populations. Ambergate Reserve represented the only significant vegetation remnant in the study area and, apart from a small population of *C.* sp. Whicher, there were few mass flowering, bird pollinated species present in the reserve. This is a very different scenario to that associated with the study performed by Byrne *et al.* (in press) on *C. quadrifidus*. Although their study was also conducted in a fragmented landscape, the area contained many populations of the study species, some of which were large with more than 1000 individuals (Byrne *et al.*, in press; Yates *et al.*, in press A). If *Calothamnus* sp. Whicher did represent a primary food resource for bird species in the study area prior to fragmentation, the clearing of the species would have removed an important food resource, resulting in reduced honeyeater abundance.

Although a number of authors have suggested that indirect estimates of gene flow (based on population genetic structure) are unlikely to represent those actually occurring between natural populations (Whitlock & McCauley, 1999; Lowe *et al.*, 2005), estimates of the mean number of migrants between populations described here are in some agreement with rates of *Nm* estimated from the pollen immigration rates revealed by paternity analysis (Hairpin Large = 4.4; Doyle Large = 3.52). Most estimates, including the mean pairwise estimate between the three large populations (2.16 ± 0.365), fall within the range of 1 – 4 migrants per generation, where theory predicts a moderate to slow rate of homogenisation over time (Wright, 1931). In contrast, one-third of values were less than zero, where theory suggests differentiation over time (Wright, 1931). These results should be interpreted with caution, as the populations and data obtained in this study violate some assumptions of Wright's (1931) island model of migration. These include differences in the size of populations studied (with some containing only three individuals) as well as significant variation in the distances between populations. Otherwise, these low indirect rates do suggest that the rates of gene flow obtained directly over a single year are representative of historical patterns.

As expected, the patterns of mating revealed in this study indicate that *C.* sp. Whicher employs a mixed mating system similar to that exhibited by a number of other self compatible myrtaceous species (Sampson *et al.*, 1995; Burczyk *et al.*, 2002; Millar *et al.*, 2000; Yates & Ladd, 2004). The mean outcrossing rate obtained for *C.* sp. Whicher (0.44) was considerably lower that that observed across populations of other myrtaceous species, including *Eucalyptus marginata* (0.81; Millar *et al.*, 2000), *Eucalyptus regans* (0.84; Burczyk *et al.*, 2002), *Eucalyptus rameliana* (0.83; Sampson *et al.*, 1995) and *Eucalyptus wandoo* (0.74; Byrne *et al.*, in review). In addition, outcrossing rates in *C.* sp. Whicher were lower than those obtained in a study of mating system of *Calothamnus quadrifidus* (0.71; Yates *et al.*, in press A). Considering the many parallels between these two taxa, with regards to their morphology and the ecological settings of the studied populations (as both were situated in fragmented landscapes), there is no obvious reason why *C.* sp. Whicher was associated with higher rates of self-fertilisation. Yates *et al.* (in press A) included a broader range of population sizes in their study of the mating system of *C. quadrifidus* (22-2014 individuals), yet no relationship between population size and outcrossing rates were observed.

One possibility that may explain the high rates of self fertilisation observed in this study is that post-zygotic seed abortion mechanisms are not present in *C.* sp. Whicher. Post-zygotic seed abortion is a feature of many myrtaceous species, though is not ubiquitous among the family (James & Kennington, 1993). Such seed abortion systems result in a mating system which is more outcrossing than would be expected from pollinator observations (James & Kennington, 1993), as heterozygous seed is selected for within the capsule. However, a number of authors have suggested that, in eucalypts, the post-zygotic selection against homozygotes may be restricted to mass flowering species, as opposed to those which produce solitary flowers (James & Kennington, 1993; Sampson *et al.*, 1995; Sampson, 1998). If this holds for other members of family Myrtaceae, one would expect that both *C. quadrifidus* and *C.* sp. Whicher would exhibit similar rates of post-zygotic selfed seed abortion, as they are closely related and are both mass flowering. An investigation of seed production in *C. quadrifidus* by Yates *et al.* (in press B) observed a decrease in seed production with decreasing population size, which is indicative of post-zygotic seed abortion following increased rates of inbreeding. A similar investigation will be undertaken in *C.* sp. Whicher with the expectation that similar seed abortion rates will be observed (Yates, pers comm).

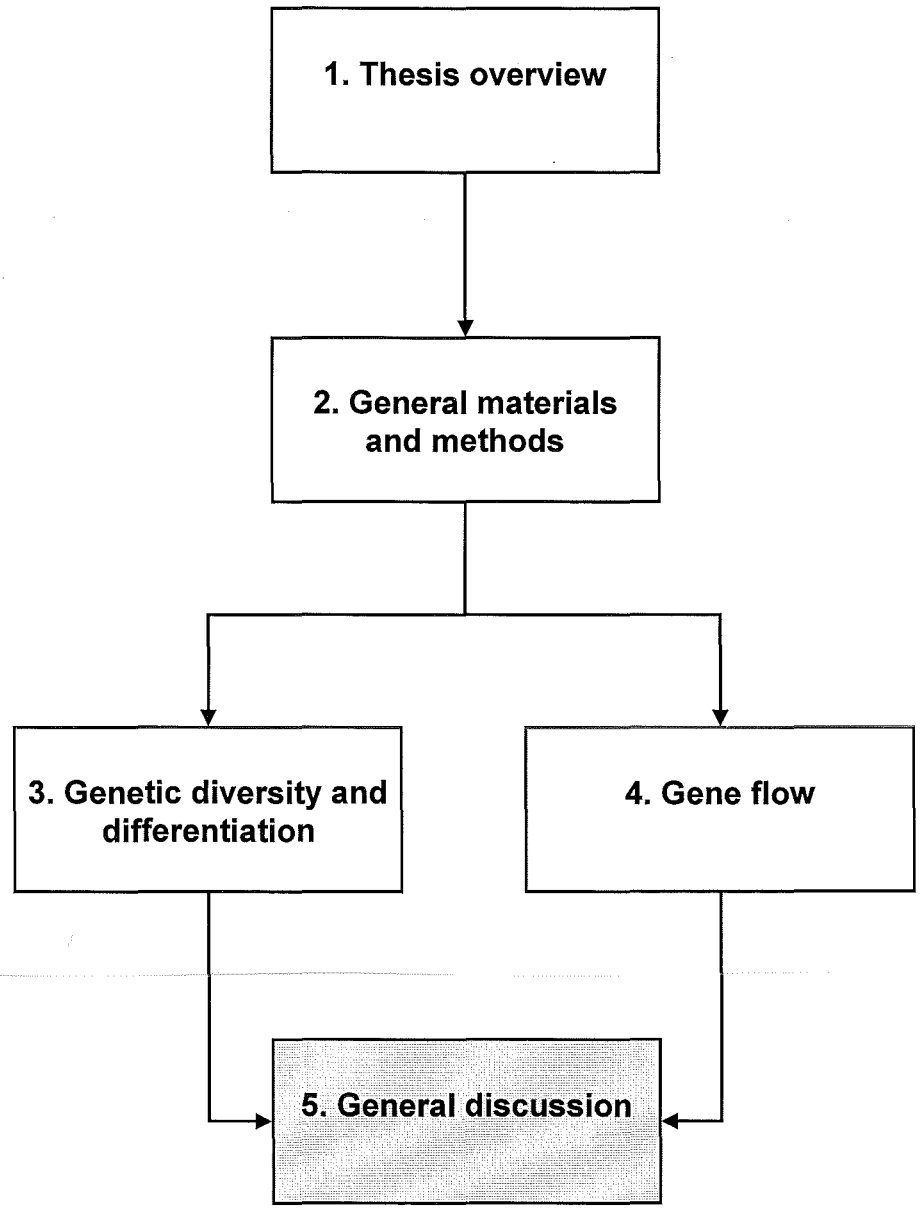
It is more likely that the low rates of outcrossing observed in this study are a consequence of extensive pollination by insects – primarily European bees (*Apis mellifera*). In addition to pollination by birds, it has been demonstrated that effective pollination in *Calothamnus* can also result from foraging by bees (Collins *et al.*, 1984). The patterns of plant mating associated with these two different pollen vectors differ markedly (Richards, 1986; England *et al.*, 2001). Studies of honeyeater foraging behaviour report extensive between plant movement, the result of which is a plant mating system with high levels of outcrossing (Richards, 1986; Paton, 2000). Such foraging patterns have even been observed in populations where plant density is high and within plant or near neighbour movement would be expected assuming an optimal pattern of foraging (England *et al.*, 2001). For example, in a pollination study of the bird pollinated species, *Eucalyptus stoatei*, Hopper and Moran (1981) report that most intertree bird movements were between trees farther apart than nearest neighbours. In contrast, bees are typically associated with large bouts of within plant activity, followed by migration to a nearby plant (Richards, 1986; England *et al.*, 2001). These patterns are often associated with high levels of selfing (Richards, 1986). High level of pollination by bees is also supported by the patterns of pollen dispersal observed in Hairpin Large. The vast majority of pollination events resulted from self pollination (48%), or from a pollen parent within 10 m of the pollen parent (28%). Many authors have suggested significant disruption of plant mating systems by exotic bees, both in Australia (Paton, 1993; 2000) and at a global scale (Huryn, 1997), and recent studies have aimed at quantifying these impacts. For example England *et al.* (2001) examined the influence of bees on *Grevillea macleayana*, a rare, naturally bird-pollinated shrub in eastern Australia. Their results suggest that the sheer abundance and high activity of exotic bees greatly outnumbered the pollen contributions made by honeyeater species – even though bees were far less effective at mediating effective pollination due to floral morphology. In addition, Paton (1993; 2000) suggests that masses of foraging bees are capable of removing up to 80% of floral resources from species which are naturally bird pollinated. In one case, this resource decline was linked to a reduced density of the New Holland Honeyeater (Paton, 1993). High activity by bees combined with reduced abundance of avian pollinators may explain the within population patterns of mating observed in these populations of *C.* sp. Whicher. While the occurrence of pollination events across the 50 m breadth of the population suggests that birds still play a role in the dispersal of pollen within populations, these longer distance dispersal events may be a result of pollen carryover by bees.

The observation that global pollen pool differentiation was higher among populations than within them indicates that mothers are not receiving pollen randomly from a global pool. Rather, these values suggest that mothers are primarily receiving pollen from within their respective populations. In addition, within population values of Φ_{ft} were also high, suggesting that pollen movement within populations was restricted. These observations are in strong agreement with the patterns of pollen dispersal revealed by paternity analysis. While the removal of selfed genotypes did reduce the global (0.364), within population (Hairpin Large = 0.267; Doyle Large = 0.349) and mean pairwise estimates of Φ_{ft}, (Hairpin = 0.231; Doyle = 0.348) differentiation was still higher than that observed in other studies of myrtaceous species (*Eucalyptus wandoo*: mean Φ_{ft} over four populations. = 0.097; Byrne *et al.*, in review; *Calothamus quadrifidus:* mean Φ_{ft} over 3 populations = 0.224; Byrne *et al.*, in press). Moreover, the degree of pollen pool differentiation between mothers was not related to geographic distance in Hairpin Large (with and without selfed events). Therefore, closer mothers do not appear to be sampling more similar pollen pools as would be expected considering the restricted patterns of within population mating revealed by paternity analysis. A more likely explanation for the high pairwise estimates of Φ_{ft} is asynchronous flowering among individuals. Asynchronous flowering would result in heterogeneity in flowering time and intensity, both among individuals and populations. This is a common feature of many mass-flowering myrtaceous species (Law *et al.*, 2000; Keatley *et al.*, 2004). Floral induction in *C.* sp. Whicher mainly occurs between the months of July and December (Western Australian Herbarium, 2006) and flowering intensity and periodicity vary markedly between individuals. Moreover, low intensity flowering has been observed as late as March (Stankowski, pers obs).

Within population and mean pairwise estimates of pollen pool differentiation were significantly higher in Doyle Large than in Hairpin Large. This may be a consequence of the linear shape of Doyle Large, where all but 1 plant was confined to single side of the road. In Hairpin Large, where plants lined both road verges, 30% of internally outcrossed events arose from pollen originating from the opposite side of the road. Thus, it appears that the linear shape of the Doyle Large population has resulted in more restricted patterns of mating than those observed in Hairpin Large. This is also supported by a lower mean number of fathers associated with the seed produced in Doyle Large.

In conclusion, the results of this chapter have revealed limited gene flow into and between the two assayed populations. This may be a consequence of high pollinator residence, or a lack of avian pollinators in the study area. High global self-fertilisation rates suggest that pollination by insects (most probably European bees) may be occurring at high levels. This is supported by within population patterns of mating, as the vast majority of outcross events were a result of pollen donated from a parent within 10 m of the mother plant. These patterns of pollen dispersal are supported by high global, within population and mean pairwise estimates of pollen pool differentiation which are indicative of limited gene flow and restricted within population pollen dispersal.

Chapter 5: General discussion



Chapter 5: General Discussion

The results of this study have revealed low levels of within population genetic diversity, high genetic differentiation and limited gene flow between the study populations. Such a scenario is contrary to what was expected considering the geographical arrangement of these populations and the pollination syndrome of the species. Extensive gene exchange between populations was expected due to their close proximities to one another, and the mobility of bird pollinators. As a consequence, genetic divergence between the populations was expected to be low.

5.1 A consequence of fragmentation?

In addition to being an endemic species with a narrow geographical distribution, *Calothamnus* sp. Whicher has been fragmented over most of its range (Western Australian Herbarium, 2006). Of the remaining 25 populations, the six selected for this study are located in the area where fragmentation is most severe (Western Australian Herbarium, 2006). None of the populations are in direct contact with significant amounts of remnant vegetation and there is little native vegetation in the study area. As a result, it is likely that landscape scale fragmentation has played a primary role in shaping the current genetic structure of these populations. But as there are no records of genetic diversity or gene flow for these populations prior to land clearing, it is not possible to directly determine the effects of fragmentation. However, these effects can be hypothesised by discussing the results of this study in light of others which have examined the effects of fragmentation on genetic structure and gene flow.

5.1.1 Influence on patterns of genetic differentiation

The literature is replete with studies which have investigated the effects of fragmentation on levels of genetic diversity and patterns of differentiation (Hogbin *et al.*, 1997; Luijten *et al.*, 2000; England *et al.*, 2002; Pither *et al.*, 2003; Lowe *et al.*, 2005; Prentice *et al.*, 2006). Theoretically, fragmentation is expected to have a marked impact on the genetic structure of populations (Hobbs & Yates, 2003; Lowe *et al.*, 2005). The removal of large expanses of vegetation from an area has the potential to significantly reduce the size of natural populations and leave them exposed to stochastic processes (Hobbs & Yates, 2003; Lowe *et al.*, 2005). This is particularly true for species which are represented in large continuous populations (Lowe *et al.*, 2005). For such species, fragmentation often leads to the formation of a series of smaller populations which, depending upon the fine scale genetic

structure within the population prior to clearing, may display markedly different genetic structures (Lowe *et al.*, 2005). Such appears to be the case in the temperate tree *Tetrao urogallus* (Segelbacher *et al.*, 2003). This species, which once occupied a relatively continuous distribution across northern Europe, has now been fragmented across the majority of its range. In recently fragmented areas, Segelbacher *et al.* (2003) observed significant levels of differentiation between populations which was attributed to the fragmentation of a previously larger continuous population. So appears to be the case for *Calothamnus* sp. Whicher. There is some evidence suggesting that many of the natural study populations of *C.* sp. Whicher were connected prior to fragmentation. The majority of this evidence comes from other relatively undisturbed populations of this species in the vicinity of the study area (Western Australian Herbarium, 2006). Those found in large, relatively undisturbed fragments of natural vegetation are comprised of thousands of individuals and encompass large areas of several ha (Western Australian Herbarium, 2006). Similarly large, low density populations of the closely related species *Calothamnus quadrifidus* were observed by Yates *et al.* (in press A) in large vegetation remnants, while smaller discreet populations were found in heavily disturbed roadsides, presumably as a result of fragmentation. Considering the close geographical proximities of the *C.* sp Whicher populations examined in this study, and the high level of clearing on road verges, it is possible that many of these populations were once physically connected. Thus, much of the differentiation observed between the study populations may be a reflection of fine scale genetic structure within a once larger population which was subsequently divided. Although it is reasonable to assume that large populations would exhibit some internal structure (as seed dispersal distances generally decay rapidly with increasing distance from the mother plant; see Howe & Smallwood, 1982), there is no data regarding the fine scale genetic structure of large populations of *Calothamnus* sp. Whicher. However, there are large extant populations which would allow this to be explored.

5.1.2 Impacts on gene flow

Fragmentation is expected to have deleterious impacts on patterns of gene flow, as fragmentation generally results in increased spatial isolation between populations (Hobbs & Yates, 2003; Lowe *et al.*, 2005). As the vast majority of pollinator visits occur over short distances, with the frequency of visits decaying in a leptokurtotic fashion as distance from the pollen source increases (Richards, 1986), it has been hypothesised that rates of gene flow in fragmented landscapes would be

considerably lower than in those which are intact (Slatkin, 1985; Kearns *et al.*, 1998; Lowe *et al.*, 2005). Recently, a number of authors have used direct genetic techniques to describe the effects of landscape fragmentation on patterns of pollen mediated gene flow (White *et al.*, 2002; Sork *et al.*, 2002; Dick *et al.*, 2003; Trapnell & Hamrick, 2005; Byrne *et al.*, in press; Byrne *et al.*, in review). In contrast to widely accepted expectations (Slatkin, 1985), the vast majority of these studies have demonstrated that rates of gene flow in fragmented landscapes are high (Burczyk *et al.*, 2004; Lowe *et al.*, 2005). In fact, some studies actually suggest that isolated populations receive immigrant pollen at a much higher rate than those in intact landscapes, with the rate of pollen immigration increasing with decreasing population size (Trapnell & Hamrick, 2005). For example, White *et al.*, (2002) examined the effects of fragmentation in the tropical tree, *Swietenia humilis* in fragmented and intact tropical forest. In addition to observing considerably higher rates of pollen immigration into isolated populations (61%) than into populations associated with intact forest (36%), the highest rates were associated with smaller populations. Similarly, Trapnell & Hamrick (2005) observed high levels of pollen immigration (up to 43%) into fragmented populations of the neontropical epiphytic orchid, *Laelia rubescens*. They also observed higher rates of gene flow associated with populations which contained fewer individuals.

These observations suggest that the current patterns of gene flow between the populations of *Calothamnus* sp. Whicher examined in this study are likely to reflect those which occurred prior to fragmentation. This is supported by the similarity in historical estimates of gene flow and the direct estimates from paternity analysis. However, given the high levels of inbreeding detected within these populations, and the hypothesis that the initial differentiation resulted from the subdivision of larger populations which exhibited fine scale genetic heterogeneity, one must question how 'historical' these indirect estimates are. The high density of plants within the road verge populations (relative to the densities exhibited within remnant populations) suggests that there has been significant post fragmentation regeneration. The excess of homozygotes and significant allele frequency differences between populations suggest that this regeneration has occurred from intrinsic genetic stocks (Ellstrand & Elam, 1993). As a result, these historical estimates are more likely to be indicative of limited post fragmentation gene flow rather than an indicator of gene flow rates prior to fragmentation.

Rates of gene flow determined for species with similar life history strategies and pollination syndromes may provide a more accurate indication of whether fragmentation has affected rates of gene flow in this species (Lowe *et al.*, 2004). Rates of gene flow observed among the *C.* sp. Whicher populations are significantly lower than the rates observed in other bird pollinated species (Trapnell & Hamrick, 2005; Byrne *et al.*, in press). In fact, the rates observed in this study are amongst the lowest reported in the literature. This suggests that fragmentation may have influenced gene flow in this species, yet the detection of some gene flow events across the breadth of the study site suggests that the level of isolation alone does not prevent gene flow. A possible explanation for the within population patterns of dispersal and high rates of self fertilisation is that the extensive loss of vegetation in the study area has significantly reduced the abundance of bird pollinators (Richards, 1986; England *et al.*, 2001). Current pollinator observations would be required to confirm this hypothesis. Fragmentation is likely to have affected avian pollinator abundance if the abundance of pollinators is lower in heavily fragmented areas than in larger, relatively undisturbed vegetation fragments containing *C.* sp. Whicher. In addition, a study of pollen dispersal could be conducted in these larger intact populations. The use of paternity analysis in such a study would be impractical due to the need to sample all of the potential fathers in the surrounding area. In contrast, the TwoGener analysis of Smouse *et al.* (2001) would be ideal, as it enables dispersal characteristics to be inferred from the pollen pools sampled by maternal plants.

5.1.3 Summary of likely fragmentation effects

It does appear that fragmentation has significantly influenced the genetic characteristics of the study sample (Figure 5.1). The six natural road verge populations observed in this study were likely to be part of one or more larger, continuous populations similar to those which are located in relatively undisturbed fragments of natural vegetation. Initially, differentiation within these populations was probably derived as a result their small sizes and the heterogeneous fine scale genetic structure within the larger population(s) from which they originated. Further differentiation appears to have resulted from extensive inbreeding within populations and the increased vulnerability to drift associated with decreasing population size. Results from other studies, including that conducted on the closely related species, *Calothamnus quadrifidus* (Byrne *et al.*, in press), suggest that fragmentation has reduced rates of gene flow from higher historical levels. The detection of some gene flow events across the breath of the study site suggests

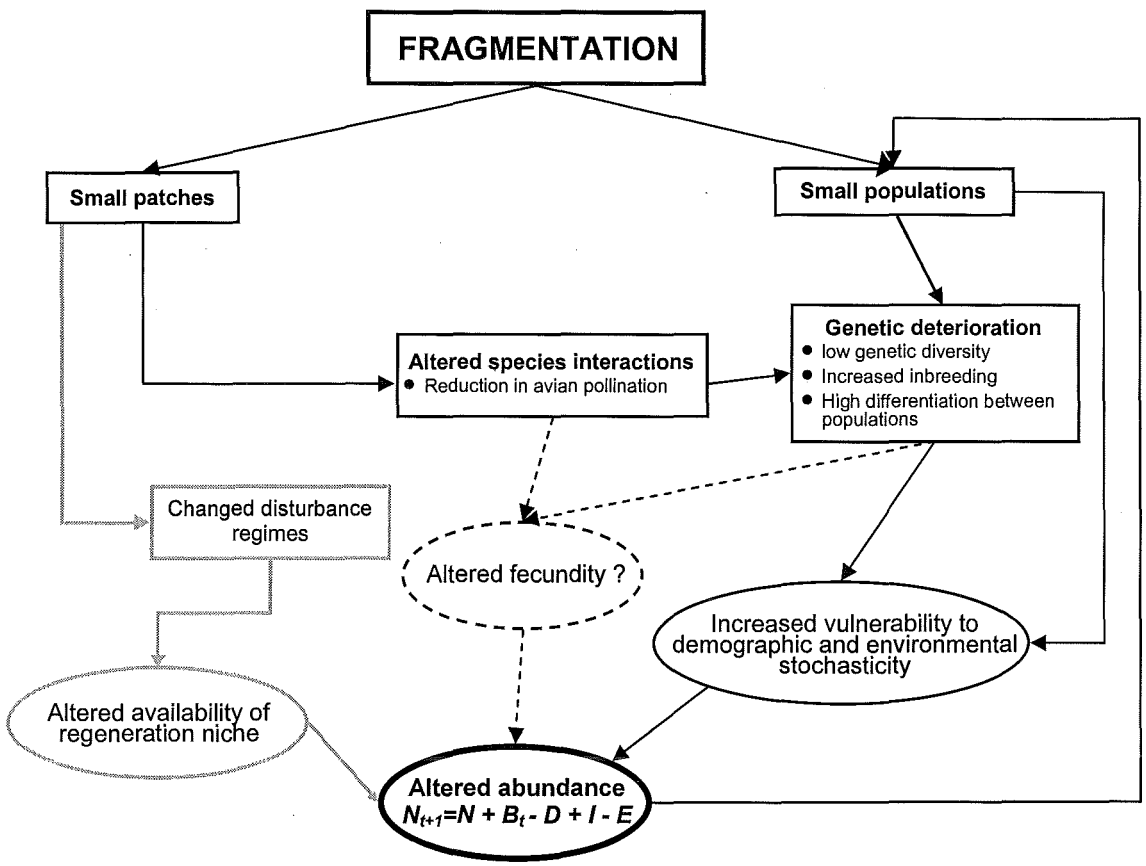


Figure 5.1 Hypothesis regarding the effects of fragmentation on the genetic structure of the six natural study populations. Fragmentation appears to have reduced the size of populations resulting in reduced genetic diversity, increased inbreeding, high interpopulation differentiation and increased vulnerability to stochastic events. In addition, fragmentation has markedly reduced the amount of habitat in the study area (smaller patches), resulting in fewer avian pollinators. The consequences of this has been reduced gene flow. Dashed lines represent possible pathways. Grey boxes and arrows represent demographic consequences which have not directly influenced genetic structure, though these appear to have reduced post fragmentation regeneration. Adapted from Hobbs & Yates (2003).

that spatial isolation itself was not preventing gene flow. Rather, the loss of natural vegetation appears to have reduced the abundance of bird pollinators.

5.2 Evolutionary implications

Given the patterns of diversity, differentiation and gene flow revealed in this study, what is in store for these populations in the future? Although high allelic diversity exists among the study populations, diversity within them is considerably lower. According to estimates of *Nm* derived from paternity analysis, a slow to moderate homogenisation of genetic structure between populations is expected (Wright, 1931). This, however, is dependent upon levels of seed set and recruitment within populations (Slatkin, 1985). Recruitment was only observed in one of the populations (Hairpin Large). There may be several explanations for this. High levels of disturbance on roadsides combined with extensive weed invasion would be likely to limit recruitment success. High population densities provide little opportunity for recruitment in more central areas of the populations where weeds are less abundant due to competition with adult plants. The populations are also associated with little or no remnant vegetation which would provide a buffer against disturbance and weed invasion (Williams & West, 2000). As a consequence of limited recruitment, homogenisation of genetic structure between the study populations is unlikely. In addition, the study populations are highly susceptible to the effects of drift as they are quite small. Given their current circumstances, a further decline in the size of these populations is expected as a consequence of genetic erosion (through drift and inbreeding), limited recruitment and ecological processes which may threaten adult plants. For the smaller populations, extinction in the near future is foreseeable.

5.3 Implications for conservation

Recently, there has been a shift away from the classical predicted theory regarding the effects of fragmentation on population genetic structure (Lowe *et al.*, 2005; Byrne *et al.*, in review). This shift in thinking has been inspired by the observation that the decrease in gene flow associated with increasing isolation is not as strong as the increase in gene flow associated with small population size. (White *et al.*, 2002; Trapnell & Hamrick, 2005). Many authors have suggested that these high levels of post fragmentation gene flow provide small isolated populations with large effective population sizes, potentially negating the effects of inbreeding – a phenomenon which has been referred to as 'genetic rescue' (White *et al.*, 2002;

Trapnell & Hamrick, 2005; Byrne *et al.*, in review). The work presented in this thesis largely contradicts these observations, and therefore has direct implications for the conservation of this species. In addition, it may provide information which will contribute to the conservation of other species in similar ecological scenarios.

5.3.1 Direct conservation outcomes for the study populations

The greatest threat to these populations is their vulnerability to stochastic processes (i.e. road grading and fire) and ecological threats (i.e. weed invasion) (Ellstrand & Elam, 1993). For the very small populations, the most effective management action would be to increase their size. Due to the extensive disturbance in these areas, seedlings originating from other populations should be introduced (Lesica & Allendorf, 1999). This would increase levels of diversity and should result in reduced levels of inbreeding (Ellstrand & Elam, 1993). Although the introduction of seedlings into a population from an exogenous source can lead to outbreeding depression (as well suited gene combinations are diluted resulting in less fit progeny), local adaptation in this species is unlikely due to its restricted geographical distribution and association with a specific substrate (Lesica & Allendorf, 1999). In addition to increasing their size, threatening processes in these heavily disturbed roadside environments must be controlled. For example, the extensive weed invasion which may be a factor limiting recruitment (Williams & West, 2000). In addition, native vegetation, including additional populations of *C.* sp Whicher, should be restored as this will buffer the populations against disturbance and will provide food and shelter for bird pollinating species (Paton, 2000).

5.3.2 Implications for the conservation of other species in fragmented landscapes

One of the most important outcomes of this thesis is the observation that the effects of fragmentation on gene flow are idiosyncratic – even when species with similar life history strategies and pollination syndrome are examined. To date, most authors have concentrated on capturing variation in gene flow patterns associated with different plant groups, which has lead to an extrapolation of these findings to other species in fragmented landscapes with similar life history strategies (Ellstrand, 1992; Lowe *et al.*, 2005; Burczyk *et al.*, 2004). The comparison of the findings of this study with the gene flow study performed by Byrne *et al.* (in press) indicate that variability in ecological setting may have more influence on patterns of gene flow than the inherent variability associated with different plant types. Thus, researchers and managers should exercise caution when making generalisations regarding the effects of fragmentation on gene flow, even between related species

or between populations of the same species. Ideally, where an understanding of gene flow patterns is seen as a vital component in a conservation plan, a gene flow study should be conducted specifically for that situation/species.

The findings reported in this study also have implications for the way that populations themselves are defined. The population concept is central to the studies of ecology, evolutionary biology and conservation biology, as it describes the biological unit at which conspecific organisms interact and, collectively, evolve (Waples & Gaggiotti, 2006). There have been numerous attempts to identify characteristics which distinguish populations (Waples & Gaggiotti, 2006). Despite some variation, all biological definitions are based on cohesion between organisms of the same species – physical and/or reproductive (Waples & Gaggiotti, 2006). In this study, a population was defined as a discrete group of individuals isolated by conspecifics by more than 50 m; reproductive connectivity was not assumed. The lack of reproductive connectivity between these populations may have implications for those attempting to define populations in fragmented landscapes. Due to the close proximities of these populations they may not have been classified as separate under a definition which placed more emphasis on reproductive connectivity, as high levels of gene exchange between them may have been assumed.

5.4 Conclusion and future research priorities

The aims of this thesis were to describe patterns of genetic diversity and gene flow among seven populations of the rare, bird pollinated shrub, *Calothamnus* sp. Whicher, in a fragmented landscape. Considering studies performed on the more common, but closely related species, *Calothamnus quadrifidus* (Byrne *et al.*, in press), high levels of gene flow were expected between populations. As a consequence, levels of genetic differentiation between the populations were expected to be low. In contrast to these expectations, gene flow among the study populations was limited and differentiation between the study populations was high. The low levels of gene flow and genetic diversity appear to be a consequence of fragmentation. The differentiation between populations is possibly a consequence of fine scale variability following the subdivision of a much larger continuous population (or populations), followed by high levels of post fragmentation inbreeding and the effects of drift. Low levels of gene flow may be attributed to a decrease in the abundance of avian pollinators in the study area, presumably as a

result of extensive habitat loss; observations of pollinator behaviour will be required to support or refute this hypothesis.

While this thesis has revealed insight into the genetic scenario associated with these study populations, and the idiosyncratic nature of fragmentation with respect to its effects on gene flow, it has also highlighted the need for further research. In regard to *C.* sp. Whicher, this would include:

1. Investigating the effects of population size on patterns of gene flow. Due to the costs and time required to produce microsatellite profiles, and the need to exhaustively sample all potential fathers in the study area, small seed samples could only be collected from a limited number of mothers within two of the study populations. Thus, the patterns of pollen dispersal observed here may not illustrate those associated with the other populations in the study area, since other studies have suggested that smaller populations may be associated with higher rates of pollen immigration (i.e. White *et al.*, 2002; Trapnell & Hamrick, 2005)
2. Investigating temporal variability in patterns of gene flow. In this study, paternity analysis was only conducted with seed crops from the previous years flowering. Therefore, the patterns of gene flow illustrated in this study may not represent those observed in other years. Although other studies that used paternity analysis to illustrate temporal variation in pollen dispersal reported consistent results between years (i.e., Trapnell & Hamrick 2005), this may not be the case for *Calothamnus* sp. Whicher.
3. Conducting a pollinator observation study. This would support or refute the hypothesis that low levels of gene flow are a consequence of a decline in the abundance of bird pollinators in the study area. This study would also reveal if high levels of insect pollination are responsible for the within population patterns of mating which were observed.
4. Investigating the effects of high self fertilisation rates on seedling abortion and fitness. This will further inform managers as to the implications of the mating patterns observed in this study.
5. Undertaking a large scale study of mating system in *Calothamnus* sp. Whicher. This should include populations of varying sizes and densities, and should also include road verge populations and some located in intact remnant vegetation. This will identify factors which promote a mating system with high levels of outcrossing.

In regard to gene flow more generally, research should concentrate on examining the way that fragmentation influences gene flow. This may be achieved by:

1. Conducting studies of gene flow on populations both prior and subsequent to fragmentation. Although this would require careful planning and a suitable study area, such studies would remove many of the confounding factors which are apparent in studies of gene flow conducted in already fragmented landscapes.
2. Studying the same species within a range of different post fragmentation scenarios. This will control variation in mating system, life form and pollination syndrome which is apparent between species, as these inherent differences often confound ecological effects. This may enable researchers to 'tease apart' the consequences of fragmentation which affect patterns gene flow and identify those which that have the greatest influence upon them.

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