GENETIC DIVERSITY, STRUCTURE AND MATING SYSTEM OF THE CRITICALLY ENDANGERED *GREVILLEA CURVILOBA*

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ABSTRACT

Grevillea curviloba is a critically endangered woody shrub, restricted to a narrow range across Muchea limestone and northern ironstone communities in the South West of Western Australia. It occurs in a series of highly disturbed, fragmented populations that are under threat by weed invasion, road and rail maintenance, inappropriate fire regimes, over-grazing and disease. The species has been previously described as consisting of two subspecies, subsp. *curviloba* and subsp. *incurva* based on variations in leaf morphology.

This project investigated the genetic diversity, structure and mating system of 11 populations of *G. curviloba* using three microsatellite loci. The markers revealed that *G. curviloba* was capable of vegetative reproduction and in some cases populations were highly clonal, consisting of a small number of genets. Observations of morphology showed that both subspecies built up 'thickets' of plants from horizontal above and below ground structures. Estimates of allelic richness (A) were low in comparison to other *Grevillea* species, but this was attributed to the high levels of clonality present. The percentage of polymorphic loci (P) was high (91%), although this was expected, given the highly variable nature of microsatellites as molecular markers. Despite the clonality present, levels of genetic diversity (H_e) were similar to those obtained for other *Grevillea* species.

Genetic differentiation was apparent; however this was shown to occur between populations rather than between the two subspecies. Further analysis of genetic structure indicated that the separation of *G. curviloba* into subsp. *curviloba*

and *incurva*, based on morphology, was not reflected by genetic differentiation and it is suggested that this taxonomic division be removed.

Only one population of the 11 studied, which possessed a comparatively high level of genetic diversity, set seed. Seeds that were produced, germinated with high frequency (~95%) over a period of two weeks. Problems were encountered with the extraction and amplification of seedling DNA, most likely due to the high concentration of inhibitors present in the seedlings. Subsequent trials indicated a modified version of a Qiagen DNeasy Plant Mini Kit, provided the best method of extracting seedling DNA. Problems with seedling DNA extraction prevented a study of outcrossing levels in populations within the time frame available

This study highlighted the need to conserve four populations of high genetic diversity that are currently un-protected, and the potential for re-introduction of seed or plant material into sites that are currently protected, in an effort to raise genetic diversity levels. Difficulties associated with the analysis of the clonal data also indicated the need for the development of appropriate statistical models for dealing with plant populations that are extensively clonal.

DECLARATION

I declare that this thesis is an account of my own work and that the work presented has not been previously submitted for a degree or diploma at this, or any other Tertiary Institution.

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Heidi Maria Nistelberger

June 2006

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LIST OF ABBREVIATIONS

AFLP	Amplified fragment length polymorphisms
AMOVA	Analysis of molecular variance
CALM	Department of Conservation and Land Management
GPS	Global positioning system
PCR	Polymerase chain reaction
RAPD	Random amplified polymorphic DNA
RFLP	Restriction Fragment Length Polymorphisms
SSR	Simple sequence repeats

CHAPTER 1
GENERAL INTRODUCTION

1.1 Endangered plant species in Western Australia

The South West of Western Australia is home to an exceptional diversity of endemic, vascular plant species (Hopper, *et al*., 1996; Cochrane *et al*., 2002). This species richness, which consists of relictual and recently evolved taxa (Hopper, 2000) is the result of long periods of isolated evolution that were generally free of large-scale extinction episodes, commonly associated with processes of glaciation, volcanism and mountain uplifting (Hopper, 1979; Hopper *et al*., 1996). Today, this area of extreme species richness and diversity is under threat. Overall, roughly two thirds of the recorded vascular flora extinctions that have occurred on the continent have taken place in Western Australia (Greuter, 1994). Of the 320 Western Australian taxa listed as rare under the Wildlife Conservation Act (1950), 310 are found in the South West Botanical Province shown in Fig. 1.1 (Cochrane *et al*., 2002).

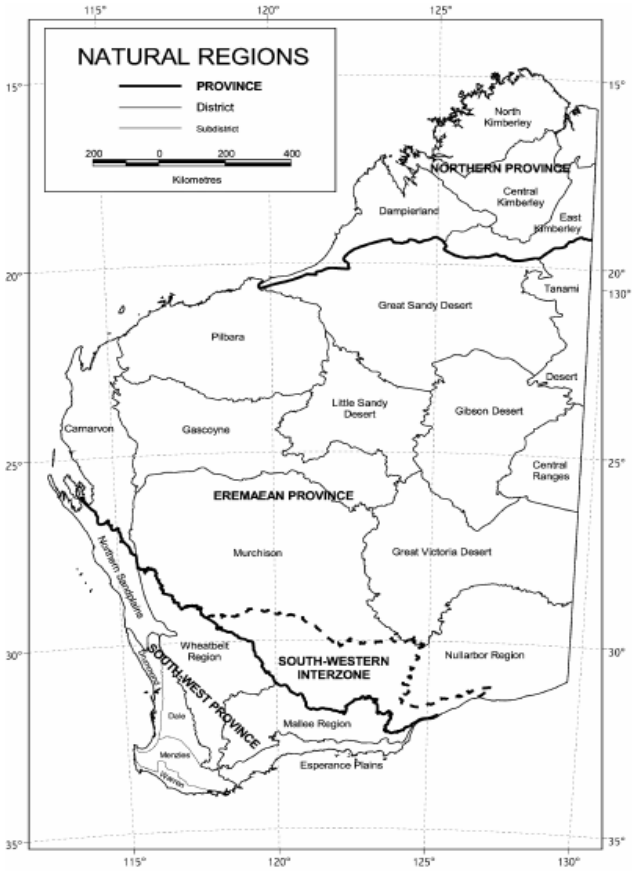

Fig 1.1 Map showing the South-West Botanical Province according to Beard *et al.*, (2000).

The South West Botanical Province has a long history of human use and disturbance, with at least 65% of the area completely cleared for agriculture and other purposes (Cochrane *et al*., 2002). The remaining remnant vegetation is highly fragmented and is threatened by disease, including *Phytophthora cinnamomi* (dieback), weed invasion, salinity and erosion (Cochrane *et al*., 2002). The fragmentation of previously continuous populations is perhaps the greatest threat facing species in this region today. Fragmentation reduces the number of breeding individuals within a population and can also prevent gene flow between neighbouring populations (Dudash and Fenster, 2000). This alteration in the normal mating pattern of a species can severely compromise the persistence of natural populations (Dudash and Fenster, 2000).

1.2 Genetic consequences of habitat loss/fragmentation on plant species

The role of genetic diversity in the survival of a species has become a fundamental aspect of study in the field of conservation biology. In order to form effective conservation strategies, it is essential to understand how a disturbance, such as habitat fragmentation or habitat loss, affects the genetic viability of a population or species.

1.2.1 Loss of genetic diversity

When a population is reduced in size, the overall genetic diversity of the population, in general, decreases (Frankham *et al*., 2002). Rare and endangered species in particular are well known for a characteristically low level of genetic variation (Gitzendanner and Soltis, 2000). Exactly how does a loss in genetic variation affect the survival of a species/population? Genetic diversity is essential for

the maintenance of natural populations, enabling continual adaptation to changing environmental conditions (Lowe *et al*., 2004). Loss of genetic diversity reduces the capacity of a population to respond to new environmental factors, such as disease (Frankham *et al*., 2002), and is often associated with a reduced population size and extinction (Lemes *et al*., 2003). The genetic diversity of a population is more or less dependant on the interaction of four important processes, known as mutation, recombination, selection and genetic drift (Moran and Hopper, 1983; Frankham *et al*., 2002).

Genetic mutations and recombination events give rise to genetic diversity (Savolainen and Kuittinen, 2000; Frankham *et al*., 2002) and the processes of selection and drift maintain/alter this diversity over time (Frankham *et al*., 2002). In smaller populations, genetic drift may play a greater role than selection in the reduction of diversity (Frankham *et al*., 2002).

In small populations, the effects of genetic sampling or random genetic drift are more apparent than in larger populations. In other words, chance events have greater significance in smaller populations (Savolainen and Kuittinen, 2000). As a result, low frequency alleles tend to be lost rapidly in these populations and the frequency of heterozygosity declines as alleles become fixed (Savolainen and Kuittinen, 2000). In conservation genetics, diversity in small populations can be measured simply as $1/2.(N_e)$ where N_e refers to the genetically effective population size (Yeh, 2000). Using this equation as a simple model, the effect of genetic drift on small populations is clearly seen (Fig. 1.2). After approximately 40 generations, nearly all individuals of the N_e=8 population have become homozygous for the allele,

where as the N_e=16 population retains approximately 30% heterozygosity levels (Fig. 1.2).

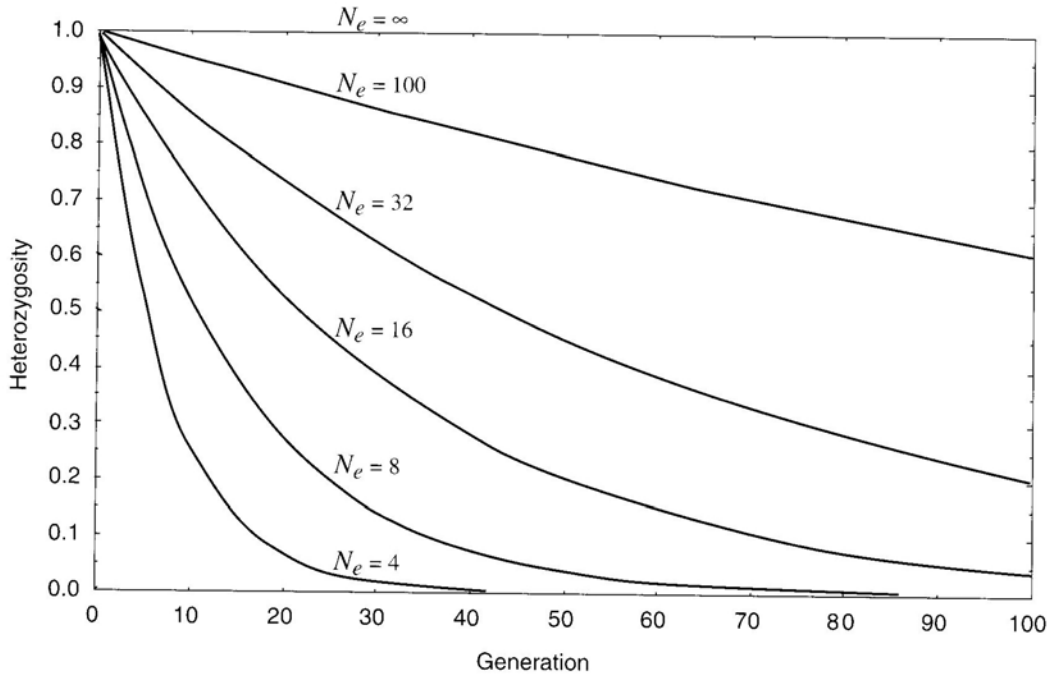

Fig. 1.2 Loss of genetic diversity in a randomly mating population of variable effective size (N_e) over time (Adapted from Yeh, 2000).

What then, are the disadvantages of reduced heterozgosity? Low heterozygosity (or high homozygosity) is associated with a decline in the fitness of populations, often due to inbreeding (Charlesworth and Charlesworth, 1987). Increased homozygosity unmasks the presence of deleterious recessive genes, and the ability to tolerate new environmental pressures is reduced (Charlesworth and Charlesworth, 1987). Increased homozygosity is also associated with a loss of heterosis, which refers to the improved vigour that is often associated with heterozygote genotypes (Burgman and Lindenmayer, 1998).

1.2.2 Inbreeding depression

As the effective population size decreases, the frequency of alleles that are identical by descent increases (Frankham *et al.*, 2002). This leads to a greater probability of breeding between genetically related individuals (Burgman and Lindenmayer, 1998). Inbred individuals exhibit higher levels of homozygosity (Vogl *et al.*, 2002) and, as mentioned above, are therefore associated with a reduction in fitness, known as inbreeding depression.

1.2.3 Restriction of gene flow

Gene flow refers to the proportion of newly immigrant genes that move into a given population (Endler, 1997). This transfer of genes occurs via pollen and seed dispersal. Biologists are primarily concerned with the movement of pollen between populations, as the movement of seed is often highly restricted (Lowe *et al*., 2003). Human interference in an ecosystem often results in the spatial isolation or fragmentation of plant populations, and this can greatly inhibit the movement of pollen between previously mating populations (Juan *et al*., 2004). Restricted gene flow leads to a reduction in effective population size and can result in a loss of genetic diversity and inbreeding depression (Konuma *et al*., 2000; Juan *et al.*, 2004).

1.2.4 The impact of self-incompatibility systems

Self-incompatibility systems are thought to have evolved as a means of protecting populations from the deleterious effects of inbreeding (Boshier, 2000). These genetic systems actively increase molecular diversity by enforcing outcrossing (Wendel and Doyle, 1998) and exist in approximately half of all flowering plants

(Frankham *et al*., 2002). They operate by reducing or preventing self- fertilisation when a pollen grain and ovule come from the same plant (Frankham *et al*., 2002).

Self-incompatibility systems can be characterised as pre- or postzygotic depending on how they operate (Boshier, 2000). Prezygotic (gametophytic) incompatibility systems are regulated by one or more (S) loci, which in large populations often display an abundance of alleles (Frankham *et al*., 2002). When a pollen grain and stigma both possess the same (S) locus allele, fertilisation is prevented via inhibition of pollen tube growth (Boshier, 2000). As genetic diversity is lost from small populations, the number of compatible (S) genotypes is reduced (Young *et al*., 2000) and therefore, the potential number of mating partners in a population is reduced (Frankham *et al*., 2002). In effect, the loss of diversity at these (S) loci lowers the effective population size and can lead to a reduction in population seed set (Boshier, 2000), which in turn, raises the risk of extinction (Frankham *et al.*, 2002).

A loss of diversity at (S) loci can also result in a shift in the fundamental mating system of a population. As the number of compatible genotypes decreases, selection begins to favour self-compatible plants that may exist at low frequencies in a population (Young *et al.*, 2000). In this manner, a predominately outcrossing population can gradually become more reliant on selfing as a means of reproduction (Young *et al*., 2000).

1.3 The role of molecular markers in the conservation of plant species

As understanding of genetic processes and their effects on plant population viability increases, information gained through the use of molecular markers has become integral in aiding in the creation of conservation strategies. Molecular markers have come a long way since the development of protein electrophoresis in the early 1950's (McMillan, 1983). Whilst techniques such allozymes remain useful in genetic analyses, progress in the development of new methods has focussed on DNA based markers that allow for the study of both extant and extinct species (Parker *et al*., 1998). In general, molecular markers can be categorised as either co-dominant or dominant.

1.3.1 Co-dominant markers

Co-dominant markers are those that expose both alleles at a locus, allowing heterozygous individuals to be distinguished from either of the homozygous states (Lowe *et al*., 2004). Also known as single –locus markers (Coates and Byrne, 2005), they include techniques such as protein electrophoresis, restriction fragment length polymorphisms (RFLP) and microsatellites (or simple sequence repeats (SSR)). Known for their high information content and ability to derive information on allele frequencies (Lowe *et al*., 2004), co-dominant markers are a popular choice for conservation biologists, and in general are more cost effective than their dominant counterparts (Sunnucks, 2000).

1.3.2 Dominant markers

Dominant markers are those that show dominant inheritance, where homozygous, dominant individuals are indistinguishable from heterozygotes (Lowe *et al*., 2004). Also referred to as multi-locus markers due to their ability to screen many

arbitrary loci simultaneously, these markers are useful as no knowledge of the target DNA sequence is required prior to their use (Sunnucks, 2000). The most well known dominant markers include random amplified polymorphic DNA (RAPD) and amplified fragment length polymorphisms (AFLP).

Despite the wealth of molecular markers available to date, no single technique has yet been found to function optimally under all conditions (Hillis *et al*., 1996). In order to ensure conservation strategies based on these markers are sound, the discerning biologist must select the most useful marker for the task at hand, while remaining cognisant of potential time, equipment and budget constraints. The discussion below briefly considers the mode of action, advantages/disadvantages and uses of the above molecular markers (Table 1.1).

Conservation biologists can use molecular markers to answer a range of questions regarding the genetic viability of plant species at risk. In general, the four main areas of interest are; the genetic diversity and structure of a population/species, phylogeny, mating systems and gene flow.

1.3.3 Genetic diversity and structure

Analyses of genetic structure and diversity provide biologists with invaluable information regarding the genetic dynamics of species (Degen *et al*., 2001). Conserving genetic diversity is vital in enabling a species/population to maintain its evolutionary potential (Byrne, 2003). Biologists can investigate genetic diversity at many levels including within and between populations, at the species level and even

Table 1.1 Summary of common molecular markers used in the genetic analysis of endangered plant species. RFLP; Restriction fragment length polymorphism, RAPD; Random amplified polymorphic DNAs, AFLP; Amplified fragment length polymorphisms, SSR; Simple sequence repeats (microsatellites).

Assay	**Origin of polymorphism**	**Co-dominant/ dominant**	**Level of polymorphism**	**Marker abundance**	**Seq. info. required**	**Radioactive resources required**	**Costs initial**	**Costs development**	**Development time**	**Application**
Allozymes Isozymes	Charge A.A. substitutions	Co-dominant	Low	Low	No	No	Low	Low	Nil	Popn. Structure, Species Boundaries, phylogenetic relationships
RFLP	Inertion/deletion events, inversions	Co-dominant	Med.	High	No	Yes/no	Mod.-high	Mod.	Mod.	Popn. and sub-popn. structure, diversity
RAPD	Insertion/deletion events, inversions	dominant	Med.	V. high	No	No	Low	Low	Low-mod.	Hybrid zones, species boundaries, gene flow
AFLP	Insertion/deletion events, inversions	dominant	Med.	High	No	Yes/no	Mod.	Mod.-high	Limited	Popn. studies, finger-printing, clonality, gene flow
SSR	Repeat number changes	Co-dominant	High	Med.	Yes	No/yes	High	Mod.	Considerable	Mating systems, popn. structure, diversity, parentage analysis (ex.), genome mapping

across a group of species (Frankham *et al.*, 2002). Genetic structure refers to the partitioning of genetic diversity between populations (Lowe *et al*., 2004). Plant populations often develop a unique genetic structure due to their sedentary nature (Cavers *et al*., 2005). Knowledge of these patterns of diversity is important, providing insight into the driving forces behind genetic dynamics and aiding in the prediction of population responses to disturbance (Degen *et al*., 2001). A wide range of molecular markers both co-dominant and dominant have been used to study genetic diversity and structure. In general, factors of time, cost and the availability of equipment will determine which marker is used.

The ability of co-dominant markers to reveal genotypic and allelic frequencies makes them a powerful choice for studying genetic diversity and structure. These markers, owing to their ability to reveal both alleles at a locus, can provide estimates of heterozygosity and therefore information as to the degree to which a population is in Hardy-Weinberg equilibrium (Bussell, 1999). Allozymes/isozymes are a useful and inexpensive choice of marker for this task, for which an abundance of data is available for comparative analysis (Hamrick and Godt, 1990). Microsatellite markers however, with their high levels of diversity (Parker *et al*., 1998) and ability to examine as many as 30-50 alleles per single locus (Amos *et al*., 1993) provide the most effective method of revealing diversity and structure.

Dominant markers are also useful for analyses of diversity and structure, particularly in studies of species for which no *a priori* knowledge of the DNA sequence exists (Bradeen and Simon, 1998; Parker *et al*., 1998). The disadvantage of these markers is that a large sample size is required in order to provide any statistical

power in a population genetic analysis (Kremer *et al*., 2005). In a recent article, Cavers *et al.*, (2005) used simulated population model data to determine the optimal sampling strategy (minimum sample size and number of loci) needed to produce a meaningful estimate of genetic structure, based on the use of AFLP and microsatellites. They found the lower information content of AFLP resulted in the need for a far greater sample size. Their mean correlation distograms illustrate this clearly (Fig. 1.3). Take for example, a scenario in which 100 individuals are examined at 5 loci. Using microsatellites this would result in a mean correlation (between the real distogram and the one drawn for the sub sample) of approximately 0.85. For AFLP to achieve a correlation of 0.85 would require a sample size of nearly 150 individuals and the examination of at least 100 loci (Fig. 1.3) (Cavers *et al.,* 2005).

1.3.4 Phylogenetic analysis

Phylogenetic studies reveal relationships of ancestry and descent within and between species (Lowe *et al*., 2004). This knowledge provides biologists with a method for identifying independent lineages or evolutionary significant units. Previously, conservation strategies have often focussed on the degree of threat to taxonomically defined species (Hopper, 2000; Byrne, 2003), which were generally defined based on morphology (Byrne, 2003). However, morphological relationships between taxa do not always reflect common ancestry. Occasionally, the similarities in morphology witnessed are indicative of processes such as convergent evolution, where similar phenotypes have developed in distinct species in response to similar environmental conditions (Frankham *et al*., 2002). Thus species definitions based on morphology may not reflect evolutionary units and can lead to the misdirection of

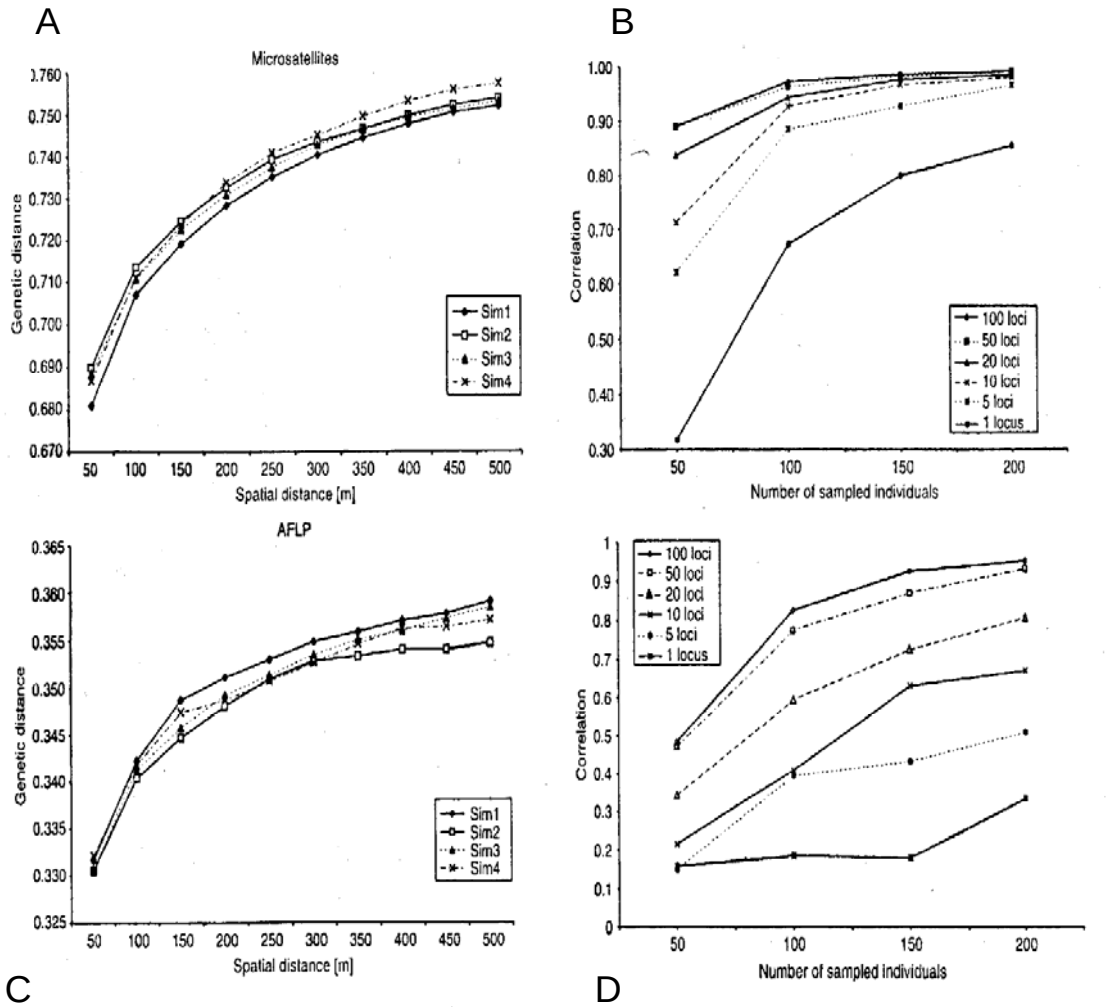

Figure 1.3 A) Distogram of the spatial genetic structure at 100 microsatellite loci for four repetitions after 1000 simulated years. **B)** Mean correlation between the 'real' distogram and the distogram drawn from series of subsamples for microsatellites (number of sampled loci=1,5,10,20,50,100; sampled individuals=50,100,150,200). **C)** Distogram of the spatial genetic structure at 100 AFLP loci for four repetitions after 1000 simulated years. **D)** Mean correlation between the 'real' distogram and the distogram drawn from series of subsamples for AFLP (number of sampled loci=1,5,10,20,50,100; number of sampled individuals=50,100,150,200).(Adapted from Cavers *et al*. 2005).

conservation efforts. Clarifying taxonomy through phylogenetic analysis on the other hand, can provide a useful means of identifying evolutionary lineages and applying conservation strategies at this level. Both co-dominant and dominant markers are useful for phylogenetic analyses; however the use of RAPD is often associated with problems of homoplasy where identical sized bands have arisen separately in different evolutionary lineages (Lowe *et al.,* 2004). Size homoplasy can also occur in some co-dominant markers such as microsatellites, but is uncommon due to the high specificity of the primers used (Estoup and Cornuet, 1999). In the rare case that homoplasy is suspected when using microsatellites, the fragment can be sequenced in order to determine whether or not the band is homologous (Estoup and Cornuet, 1999).

Plant phylogenies can also be determined using chloroplast DNA (cpDNA) markers (Byrne, 2003). These markers assay variation in the slowly evolving chloroplast genome (Byrne, 2003) and are useful in phylogenetic studies (Provan *et al.*, 2001). In most angiosperms, the genome is maternally inherited (Parker *et al*., 1998) and by following matrilines it is possible to elucidate the contributions of gene flow through seed dispersal to the genetic structure of natural populations (Provan *et al*., 2001). Microsatellites have been found in all chloroplast genomes sequenced to date, and in general, show higher levels of diversity than other markers such as cp RFLP (Provan *et al*., 2001) making them an effective marker for this purpose.

1.3.5 Mating systems

Plants utilise a wide range of reproductive strategies (Coates and Byrne, 2005). These modes of reproduction can vary between species but also within populations of the same species. A change in the usual mating system of a population

is often the response to variation in environmental factors. Whether a plant is predominantly outcrossing or selfing (self-fertilising) is dependant upon a number of factors including its mode of pollination (i.e. wind/insect/animal), the size of the population, the structure of the flower itself and the presence of incompatibility systems (Coates and Byrne, 2005). Conservation biologists observe these factors in order to determine the mating system of a species. Studies which involve observing pollinator behaviour, provide information on the 'potential' mating system, whereas crossing experiments, allow inference of the 'preferred' mating system of a species (Coates and Hopper, 2000). Genotypic analyses are used to deduce the 'realised' mating system of a species (Coates and Hopper, 2000).

The genetic diversity of a species is largely dependant on its realised mating system (Coates and Byrne, 2005). Although not always the case, in general, species that are outcrossing contain higher levels of genetic diversity than those that are selfing (Hamrick and Godt, 1989). This is due to the higher homozygosity that occurs through the restricted assortment of alleles in selfing species. In situations where a given plant exists in a small population of low density, that experiences frequent bottlenecks, selfing can be advantageous (Lowe *et al*., 2004). However, reproducing in this manner also places the population at risk of inbreeding depression (Charlesworth, 1998). Understanding the realised mating system is essential for providing information regarding the viability of populations/species and how they are best conserved (Coates and Hopper, 2000).

To estimate the degree of inbreeding or outcrossing in a population, two parameters are needed. The first is a single locus estimate of outcrossing (t_s) and the second, a multi-locus estimate of outcrossing (t_m). Differences between the two are

then used to provide information on the proportion of bi-parental inbreeding in a population (See Lowe *et al*., 2004 for more detail). Dominant markers can be used for some aspects of mating systems studies, such as identifying clonality, but their use is limited (Parker *et al*., 1998). Co-dominant markers such as microsatellites are much more informative and capable of yielding data on paternity along with inbreeding coefficients and both selfing and outcrossing estimates.

1.3.6 Gene flow

Gene flow is an important process that heavily influences primary genetic structure and the adaptive ability of populations (Burczyk and Koralewski, 2005). As mentioned previously, restricted gene flow reduces a population's effective size and can lead to processes of genetic drift, resulting in possible inbreeding depression followed by divergence and speciation (Konuma *et al.*, 2000; Juan *et al.*, 2004). Understanding gene flow not only enhances knowledge of the mating and dispersal systems of target species, it is vital in identifying the ecological constraints that potentially inhibit pollen movement between populations, and this provides the basis for an effective conservation strategy (Konuma *et al.*, 2000). Gene flow estimates can be determined indirectly with Wright's F statistics or directly using paternity analysis (Dunphy and Hamrick, 2005).

Indirect gene flow estimate

This estimate of the effective number of immigrants per generation is based on Wright's (1951) F_{ST} estimate, 'the proportion of genetic diversity due to differences among populations' (Wright 1951) where gene flow (*Nm*), 'the degree of genetic isolation among populations,' is calculated as $Nm=(1-F_{ST}/4F_{ST})$ (Dunphy and

Hamrick, 2005). Indirect estimate calculations operate under the assumption that populations are in migration-drift equilibrium (Rousset, 1997), that is, that the homogenising action of migration is balancing the genetic divergence among populations produced by genetic drift (Dunphy and Hamrick 2005). Often, recently established populations are not in migration-drift equilibrium, and in these instances, direct gene flow estimates are more reliable (Dunphy and Hamrick 2005). Wright's F_{ST} index is based on knowledge of allele frequencies. Therefore, co-dominant markers are the ideal choice for studies of gene flow (due to their capacity to accurately reveal allelic frequency).

Direct gene flow estimate

Paternity analysis is the most common and reliable method of calculating gene flow in a population that is not in migration-drift equilibrium (Burczyk and Koralewski, 2005). Performing a paternity analysis is however, a comparatively laborious process, involving extensive field and laboratory work (Dunphy and Hamrick, 2005). In plants, paternity analysis is effectively a direct measure of pollen flow (Slavov *et al.*, 2005). Initially, the multi-locus genotypes of all reproductive mature plants and a sample of their progeny are determined and from this, the paternal contribution is identified (Slavov *et al*., 2005). The genotypes of the sample progeny are then compared to those of the potential male parents in the local (chosen) population and the most likely male parents are identified (Slavov *et al.*, 2005). Progeny that exhibit multi-locus pollen genotypes that are not found in any of the putative parents, are assumed to have resulted from 'outside' pollen immigration. In this manner, pollen-flow events within and between populations can be observed.

1.4 Assessing and conserving remnant plant genetic diversity

The assessment of genetic diversity in rare/endangered plants has increased in popularity over the last 15 years (Hogbin *et al*., 2000) enhancing our understanding of the genetic processes that occur in natural plant populations. Although proposed conservation outcomes often form an integral component of these studies, few have actually demonstrated the practical outcomes of incorporating knowledge of genetic viability into specific conservation strategies (Hogbin *et al*., 2000). The discerning biologist must ask a number of questions before undertaking the often lengthy and expensive process of genetic analysis. Firstly, given the current management (or lack thereof) of the target species, would new information regarding the genetic viability of populations provide any new possibilities for management, or merely reinforce the present practice? And; if the species is unmanaged, how could the information gained through a genetic study aid in the creation of a feasible conservation strategy?

1.4.1 Molecular markers and clonality

One area in which genetic analysis is undoubtedly helpful is in determining whether or not a population is capable of asexual reproduction (clonality). A study conducted on the endangered New South Wales herbaceous shrub *Haloragodendron lucasii* used allozymes and RAPD markers to determine the genetic variation present in this species that was previously believed to be extinct (Sydes and Peakall, 1998). Results showed that *H. lucasii* was in fact, extensively clonal. Indeed a site that had previously been recorded as having 700 individuals was actually composed of only three unique genotypes (genets) (Sydes and Peakall, 1998). This information provided an important basis for the management of this species, namely for the collection of material for *ex-situ* populations. As there were so few genets and these were largely

localised within single populations it was imperative that all populations were sampled for genetic analysis, however, extensive within population sampling was not necessary (Sydes and Peakall, 1998). The study also called for a more extensive search of the surrounding area, in an attempt to locate more populations. As a result, a group known as the *Haloragodendron lucasii* Rediscovery team was formed, and they did in fact discover a further five populations (Sydes and Peakall, 1998).

1.4.2 Comparison of genetic diversity with a common congener

In order to effectively manage an endangered species based on information of its genetic diversity, we need to know how this diversity compares to that of a common congener (Soltis and Gitzendanner, 1999). Stating that a species is genetically depauperate provides little information as to how it is best conserved. More information is required, such as, whether the lack of diversity is a common feature of related species or unique to the particular taxon. By comparing the genetic diversity of a rare/endangered species to that of common, widespread relatives, biologists can begin to ascertain the cause of the differences between the levels of diversity.

1.5 Grevilleas in Western Australia.

With over 250 species, *Grevillea* is the largest genus in the family Proteaceae (McGillivray, 1993). Most members of this genus are endemic to Australia with a small number of species occurring in southern Indonesia, New Guinea and New Caledonia (McGillivray, 1993). Western Australia is home to more than half of the total *Grevillea* species that exist in Australia, and of the 164 species recorded in Western Australia, 142 are considered endemic (McGillivray, 1993). The majority of

grevilleas occur as woody shrubs with hermaphroditic flowers. They are generally long lived and are found in temperate zones (Hermanutz *et al.,* 1998). *Grevillea* species are predominantly outcrossing (Goldingay and Carthew, 1998), but some are known to possess complex mating systems that can vary both within a species and within populations of a species (Hermanutz *et al*., 1998; Richardson *et al*., 2000). Many species are lignotuberous or root suckering, and regenerate from underground parts following a fire (McGillivray, 1993). Seeds are typically stored in the soil (Auld and Denham, 2001) and are stimulated to germinate following a fire event (Burne *et al*., 2003).

Although little is known about the breeding system of this genus it is thought that self-incompatibility systems are most likely widespread (Pharmawati *et al*., 2004). Of all the studies conducted on members of the Proteaceae thus far, only the gametophytic form of self-incompatibility has been reported (Hoebee and Young, 2001). Several species are characterised by a low to non-existent seed set (Hermanutz *et al*., 1998) and are thought to exist solely as clonal populations (McGillivray, 1993).

Dieback (*Phytophthora cinnamomi*), is a serious threat to members of the Proteaceae family, and members of the *Grevillea* genus are particularly susceptible (Wrigley and Fagg, 1989). Grevilleas are also commonly afflicted with a pest known as the 'grevillea looper caterpillar', a well-camouflaged insect that causes severe defoliation across an extensive range of species (Wrigley and Fagg, 1989). Potentially the greatest threat to grevilleas today however, is that posed by the fragmentation of formerly widespread populations (Lamont *et al*., 1993; Rossetto *et al*., 1995).

1.6 Taxonomy, habitat and distribution of *Grevillea curviloba* subsp. *curviloba* and *G. curviloba* subsp. *incurva*

The study deals with the critically endangered *Grevillea curviloba*, and its two subspecies *curviloba* and *incurva*.

1.6.1 Species description

Grevillea curviloba subsp. *curviloba*, commonly known as 'Curved–leaf grevillea' can occur as either a prostrate, ground-covering shrub or a tall erect shrub with broad, dark green leaves that are oval/wedge shaped and around 1.5-5 cm long (Wrigley and Fagg, 1989). It has inflorescences that are 1 to 3 cm long and 3 cm wide with individual flowers that are creamy-white in colour, 7 to 10 mm long and 0.5mm across (Fig. 1.4). Flowering occurs between September and October (English and Phillimore, 2000).

Fig. 1.4 The creamy, white flowers of *Grevillea curviloba* subspecies *curviloba* (Photo credit, G. Walker).

Grevillea curviloba subsp. *incurva*, also known as 'Narrow curved-leaf grevillea', also occurs as a ground covering or tall erect shrub. Leaves are 1.8 to 5.2 cm long and consist of 3 to 5 incurved, narrowly linear lobes. They have inflorescences that are 1 to 3 cm long and 3 cm wide. Like subsp. *curviloba*, individual flowers are creamy white, 7 to 10 mm long and 0.5 mm across (Phillimore and English, 2000). Flowering occurs between September and October.

1.6.2 Species habitat

Grevillea curviloba subsp. *curviloba* occurs over a small range (less than 20km) in the south west of Western Australia in the rare Muchea limestone habitat (English and Phillimore, 2000). This species typically grows on deep, peaty, winter wet soils overlying limestone and is associated with other species such as *Melaleuca hueglii*, *M. systena* and *Acacia saligna.*

Grevillea curviloba subsp. *incurva* is more widespread than subsp. *curviloba* and occurs between Muchea and Badgingarra over both Muchea limestone and northern ironstone communities (Phillimore and English, 2000). This species typically grows on winter wet sands overlying limestone or, over ironstone at sites with a high water table (Olde and Marriot, 1995).

1.6.3 Cultivation

Grevillea curviloba subsp. *incurva* has been widely used in horticulture as a ground cover, but is often sold, or wrongly identified as *G. biternata* or *G. tridentifera* (McGillivray, 1993). Once a popular ground cover in the 1970's and 1980's, *G. curviloba* subsp. *incurva* now appears on 'Burke's Backyard's, Grevilleas to avoid list' (www.burkesbackyard.com.au) owing to its unpopular habit of sending up tall vertical shoots and its capacity to die quickly and unexpectedly (due to root rot) (McGillivray, 1993).

1.6.4 IUCN status

Both subspecies are currently ranked as critically endangered (CR) according to World Conservation Union IUCN (1994) Red-list criterion. Threats are identified as weed invasion, road, rail and firebreak maintenance, inappropriate fire regimes, grazing by kangaroos and rabbits and dieback disease (*Phytophthora* spp.) (English and Phillimore, 2000; Phillimore and English, 2000).

1.7 Taxonomy, habitat and distribution of the common *Grevillea vestita* subsp. vestita and *G. vestita* subsp. *isopogoides*

Grevillea vestita, the closest suspected relative of *G. curviloba* was used in this study to provide a comparison of genetic diversity, structure and mating system.

1.7.1 Species description

Grevillea vestita subsp. *vestita* is a medium to tall, spreading shrub with grey/green leaves that are wedge-shaped with three broad or narrow lobes and up to 40 mm long (Wrigley and Fagg, 1989). Flowers are white in colour and heavily perfumed. Each is found on a slender stalk approximately 8 mm long (Wrigley and Fagg, 1989). The species is known to be capable of vegetative reproduction via root-suckering (McGillivray, 1993).

Grevillea vestita subsp. *isopogoides* is similar in appearance to subsp. *vestita* except the leaf lobes are deeply cut and spreading. Flowering of both subspecies can occur year round but is predominantly between June and October.

1.7.2 Species habitat

Grevillea vestita subsp. *vestita* occurs over a wide range from Yanchep to Cape Naturaliste along the coast, and from Badgingarra to Pingelly inland. This species is typically found in sandy soils associated with mixed heath or Jarrah/Marri woodland (McGillivray, 1993).

G. vestita subsp. *isopogoides* occurs within a restricted range between Kalbarri, Three Springs, Mullewa and Mingenew in the north of Western Australia (McGillivray, 1993). This species typically grows on sandy or gravely soils associated with heath and mixed sclerophyll scrub (McGillivray, 1993). Neither subspecies are listed on the World Conservation Union IUCN Red list.

1.8 AIMS

Conservation of taxa that occur in highly disturbed, fragmented environments requires an understanding of the genetic processes acting to maintain populations. The critically endangered *G. curviloba* exists today, as a series of small, fragmented populations, occurring in highly disturbed sites. Threatened by fire, weed invasion, trampling, and grazing, the populations were also considered to be at risk from lowered genetic diversity and inbreeding depression. Initial research by M. Byrne (pers. comm.) also suggested that the species may have been reproducing asexually, which would alter the estimated number of individuals present at a site. It was proposed that molecular microsatellite markers developed for two eastern states grevilleas (England *et al*., 1999; Hoebee and Young 2001), be used to determine the genetic diversity occurring in populations of *G. curviloba* and to investigate the potential for clonal growth to occur in this species. G. *curviloba* has typically been

described as consisting of two subspecies, *curviloba* and *incurva*, based on variations in leaf morphology (English and Phillimore, 2000). Often however, distinguishing between the two groups has been difficult and a morphological study has failed to separate them (G. Keighery, pers. comm.). Therefore a genetic study of the species was suggested to clarify whether there is genetic structure that supports the continual recognition of the two subspecies. Information gathered from this study will help determine whether current management practices are sufficient to maintain the viability and persistence of *G. curviloba*. In summary the overall aims of this thesis were:

- To investigate the genetic diversity occurring within and between populations and subspecies of the critically endangered *Grevillea curviloba* in comparison to the common relative *Grevillea vestita*.
- To determine whether the classification into subspecies *curviloba* and *incurva* based on morphology is reflected in the analysis of genetic structure.
- To determine whether current management strategies in place are suitable for the long-term conservation of this species.

CHAPTER 2
ASSESSING CLONALITY IN POPULATIONS OF *Grevillea curviloba*

2.1 INTRODUCTION

2.1.1 What is clonality?

Asexual or vegetative reproduction is the process by which one plant gives rise to another, in the absence of sex (Eckert, 1999). This form of reproduction, also known as clonal growth, results in progeny that are genetically identical to their parent, and is a common occurrence in plants (Raven *et al*., 1999). Various types of vegetative reproduction include above ground stolons, below ground rhizomes, root suckering, bulbs, stem and root tubers (Eckert, 1999), and apomixis (the asexual production of seed) (Raven *et al*., 1999). Clonal plants are defined as those in which genets (genetic individuals originating from a single zygote) are comprised of a number of ramets (distinct sub-units produced via vegetative growth) (Eckert, 1999).

2.1.2 Advantages and disadvantages of clonality in natural populations

Clonal plants can share resources among their ramets through the maintenance of physiological connections (Pennings and Callaway, 2000), and this allows them to colonise a wide range of habitats which vary in nutrient availability (Bushakra *et al*., 1999). The clone as a whole is able to support individual ramets that are experiencing physical stress, competition or herbivory (Hartnett and Bazzaz, 1985) and this confers a specific advantage to populations that are existing in patchy environments (Eckert, 1999). However, there are also disadvantages in terms of survival, for clonal populations. As clones increase in size there is a greater chance of self pollination and

inbreeding depression occurring in species that are self-compatible, or in reduced seed set occurring in those that are self-incompatible (Bushakra *et al*., 1999). This can lead to a reduction in genetic diversity, compromising the ability of a species to respond to new environmental conditions (Lowe *et al*., 2004)

2.1.3 Identifying the extent of clonality in populations

It is often difficult to identify the effective population size in clonal species (Sydes and Peakall, 1998; Rossetto *et al*., 2004) as one clone (genet) usually consists of several plants (ramets) (Suvanto and Latva-Karjanmaa, 2005). When assessing the presence of clonality in populations of rare or endangered species, it is not always ethical to excavate plants in order to obtain morphological evidence of asexual reproduction (Neel and Ellstrand, 2003). Molecular markers can be used to facilitate the non-invasive analysis of suspected clonal populations and provide information needed for accurate genet recognition (Bushakra *et al*., 1999; Waits *et al*., 2001; Neel and Ellstrand, 2003). Microsatellites are perhaps one of the best markers available for the detection of clones in a population, due to their high variability, co-dominant nature and statistical power that allows for the accurate identification of individuals (Suvanto and Latva-Karjanmaa, 2005).

2.1.4 The impact of clonality on the genetic analysis of populations

The methodology used when sampling populations of suspected clonal plant species, can greatly influence the outcome of a genetic analysis (Ellstrand and Roose, 1987). Over-sampling of one clone (that may be larger in distribution) can result in an over-representation of a particular genotype in a population. Similarly, widespread

sampling, in an attempt to avoid sampling the same clone more than once, can result in an underestimation of the total genotypic diversity present in a population. This bias in sampling can greatly alter the diversity statistics derived for clonal populations. For example, a clone which has been over-represented during sampling will cause that genotype to appear to occur at very high frequencies within the population. Alternatively, sampling which is too widespread may result in numerous unique genotypes being missed altogether, altering the genetic differentiation observed between populations. Whilst conducting accurate analyses of clonality in rare plant populations can be difficult (Rossetto *et al*., 2004), the data obtained provides vital information as to the true conservation status of a species, as the total number of genetic individuals present in these populations is often largely overestimated (Sydes and Peakall, 1998).

The aims of this chapter were:

-To determine whether there is genetic evidence of clonal reproduction in populations of *Grevillea curviloba* subsp. *curviloba* and *G. curviloba* subsp. *incurva*.

- If clonality exists, to determine the extent to which it occurs in the populations of both subspecies.

2.2 MATERIALS AND METHODS

2.2.1 Sampling strategy

A preliminary trial conducted by M. Byrne, on the efficacy of microsatellite primers on *G. curviloba* indicated that this species may have been capable of clonal reproduction (M. Byrne, pers. comm.). Therefore the sampling strategy used in this study needed to minimise the chances of sampling numerous ramets of the one genet. Consequently, plants occurring in close proximity (generally < 2 m) were not selected for analysis. During the sampling process it was noted that *G. curviloba* was capable of spreading across the soil surface via a series of horizontal surface stems. These stems were difficult to see amongst the weeds and litter, compounding the difficulty of sampling. A total of 4 populations of subspecies *curviloba* and 7 populations of subspecies *incurva* were sampled. Voucher herbarium specimens had been collected from each of the above populations in the past and deposited in the Perth Herbarium. The 11 populations occurred over an area of approximately 30 km (Fig. 2.1).

2.2.2 Sites: *Grevillea curviloba* subsp. *curviloba* (Fig. 2.1)

Leaf material was collected at four populations of *G. curviloba* subsp. *curviloba* between July and October 2005. Approximately 10 cm of healthy material was collected from a single stem tip. Plants were then tagged with numbered aluminium labels and GPS (Garmin) co-ordinates were recorded. Plant material was placed in labelled plastic zip-lock bags and transported to the laboratory in an ice – chilled, insulated container.

Table 2.1 Number of *Grevillea curviloba* subsp. *curviloba* plants sampled from the four populations.

Population	Number of samples
1- Railway reserve west	30
2- Railway reserve east	20
3- West Rd	15
4-Maralla Rd	17

2.2.3 Sites*: Grevillea curviloba* subsp*. incurva* (Fig. 2.1)

Leaf material was collected at seven populations of *G. curviloba* subsp. *incurva* between July and October 2005. Again, approximately 10 cm of healthy shoot and leaf material was collected from a single stem and numbered aluminium tags were attached and GPS co-ordinates (Garmin) recorded. Material was then placed into labelled zip-lock plastic bags and transported in an ice-chilled, insulated container.

Table 2.2 Number of *Grevillea curviloba* subsp. *incurva* plants sampled from the seven populations.

Population	Number of samples
1- Muchea Nature Reserve (west)	20
2- Muchea Nature Reserve (east)	20
3- Vines resort	5*
4- Great Northern Hwy (4b)	15
5- Great Northern Hwy (7a)	11
6- Great Northern Hwy (11)	22
7- North Muchea Nature Reserve	21

* The Vines population consisted of just five individuals, all of which were sampled.

2.2.4 Sites: *Grevillea vestita* subsp. *vestita*

Leaf material from *Grevillea vestita* subsp. *vestita* was collected from two separate populations in July 2005 in the same manner as above (2.2.3) with the exception that GPS locations were not recorded for each individual.

Table 2.3 Number of *Grevillea vestita* subsp. *vestita* samples taken from the two populations.

Population	Number of samples taken
1- Wanneroo	20
2- Guilderton	20

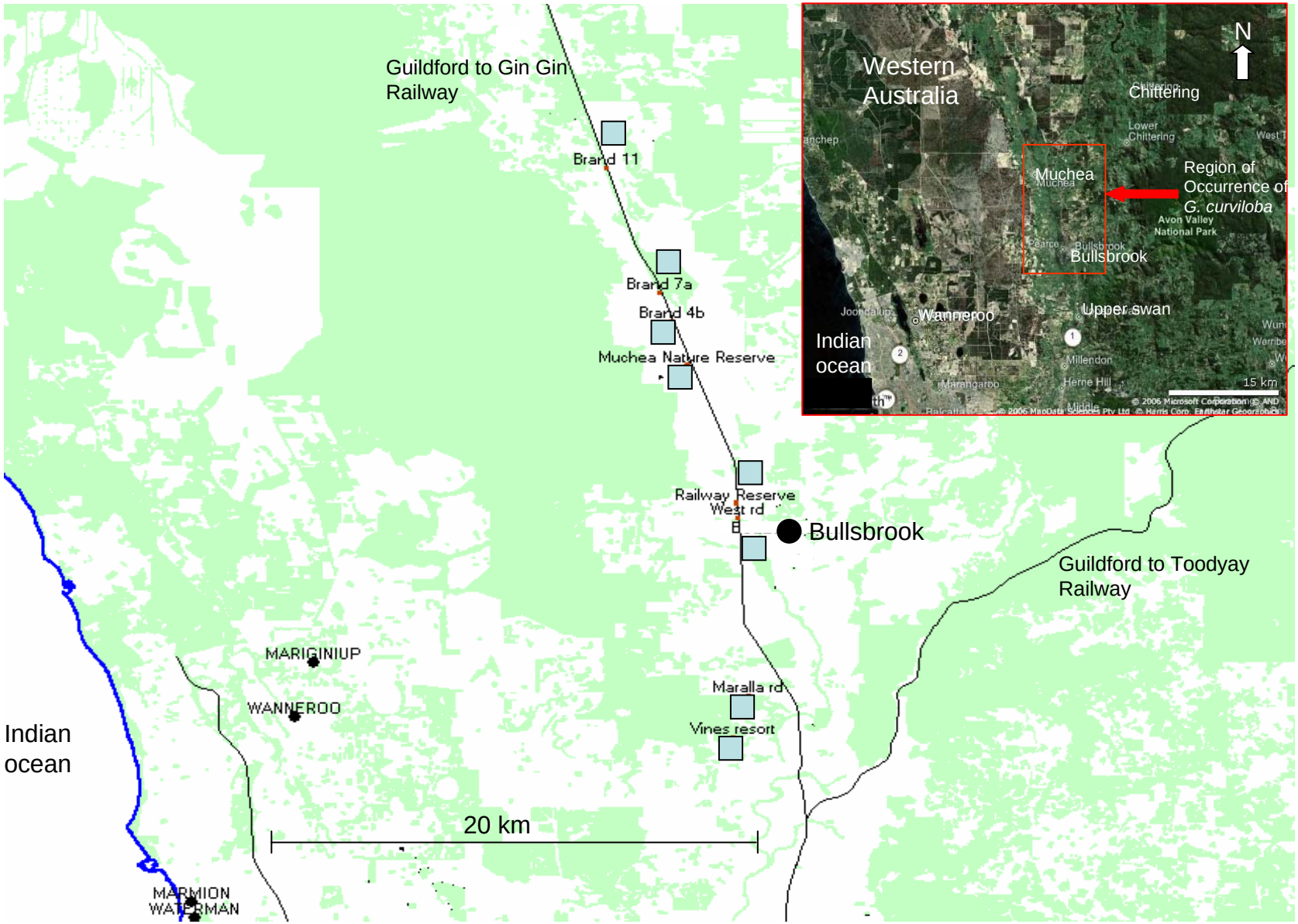

Fig. 2.1 Population sites of *Grevillea curviloba* subspecies *curviloba* and *incurva*

2.2.5 Sites:*Grevillea vestita* subsp. *isopogoides*

Leaf material from *Grevillea vestita* subsp. *isopogoides* had been collected previously from two populations by M. Byrne in September (2004).

Table 2.4 Number of *Grevillea vestita* subsp. *isopogoides* samples taken from the two populations.

Population	Number of samples taken
1- Olgivie Rd	20
2- Binnu Rd	20

All leaf material was stored at 4°C for a maximum of 48 h before extraction.

2.2.6 DNA extraction from adult leaf material

DNA was extracted from fresh material using a Qiagen DNeasy Plant Mini Kit, following the manufacturer's instructions with modifications. Approximately 120 mg of plant material was frozen in liquid nitrogen and ground to a powder using a mortar and pestle. The powder was then homogenised in 400 µl of lysis (AP1) buffer, and mixed thoroughly. A further 3 µl of RNase A stock solution was then added to samples which were vortexed and heated at 65°C for 10 min. Following incubation, 130 µl of precipitation buffer (AP2) was added to samples which were inverted to mix and incubated for 5 min on ice. The lysate was then added to a QIAshredder spin column and centrifuged for 2 min at maximum speed. The flow through was added to a new tube with care taken to avoid disturbing the cell-debris pellet. Volumes (1.5 times) of AP3/E precipitation buffer were added to the cleared lysate and mixed by pipetting. Following mixing, 650 µl of the lysate/AP3/E buffer mixture were added to a DNeasy mini spin column and centrifuged for 1 min at 8000 rpm. The flow through was discarded and the procedure repeated with the remaining mixture. The DNeasy

column was then placed in a new collection tube, and 500 µl of AW wash buffer was added. The tube was centrifuged for 1 min at 8000 rpm and the flow through discarded. Another 500 µl of AW wash buffer was added to the DNeasy tube which was then centrifuged for 2 min at maximum speed to dry the membrane, the flow through was again discarded. The DNeasy column was then added to a clean 1.5 ml microcentrifuge tube and 50 µl of AE elution buffer was added directly onto the DNeasy membrane. The tube was then incubated at room temperature for 5 min before being centrifuged for 1 min at 8000 rpm to elute. The elution step was then repeated once. DNA was stored at -20ºC and spare leaf material was stored at -85ºC.

2.2.7 DNA concentration

The concentration of extracted DNA was determined using a Hoefer DyNA Quant 200 Fluorometer, following manufacturer's instructions using the low range assay solution (A). Prior to testing, DNA samples were incubated in a 35°C water bath for a minimum of 15 min, flick-mixed and pulse spun. After determination of DNA concentration, samples were diluted to a working concentration of 10 ng/µl with sterile dH_20 and stored at -20°C.

2.2.8 DNA quality check

Electrophoresis of sample aliquots of 10 ng/µl DNA in a 0.8% agarose/TAE gel were carried out at 80 V for 1.5 h and stained with ethidium bromide (0.5 mg/ml) to check for DNA degradation. A single high molecular weight band with no smearing indicated high quality DNA.

2.2.9 PCR

Three microsatellite markers, one designed for *Grevillea macleayana* (Gm-37) (England *et al*., 1999) and two for *G. iaspicula* (Gi-4 and Gi-6) (Hoebee and Young, 2001) were used to screen DNA extracted from the 15 populations. Other *Grevillea* primers had previously been trialled (M. Byrne, pers. comm), but only these three were successful. DNA amplification was performed in 15µl volume reactions containing 20-30ng of DNA, 0.2mM of each dATP, dCTP, dGTP and dTTP; 1.5 to 1.75 mM of $MgCl_2$, 2µM of primer, 0.2 µM of betaine (Gm-37 and Gi-4 only) and 0.75 U of *Taq* DNA polymerase (Invitrogen) (Appendix A). DNA samples were heated to 35°C for 15 min, flick-mixed and spun, prior to use. PCR was carried out using an Eppendorf Thermocycler (Eppendorf), using two programs of varying stringency. The program for amplification using primer Gm-37 and Gi-4 consisted of a denaturation cycle at 94°C for 2 min, followed by 30 cycles of 94°C for 3min, 68°C with a touch-down of -0.3°C per cycle for 30 sec and 72 °C for 5 sec, followed by 3 cycles of 94°C for 30 sec, 55°C for 30 sec and 72°C for 5 sec and a final extension cycle of 72°C for 5 min. The program used for primer Gi-6 consisted of an initial denaturation at 96°C for 2 min followed by 30 cycles of 95°C for 30 sec, 56°C for 30 sec and 72°C for 30 sec and a final extension cycle of 72°C for 5 min (appendix A).

2.2.10 Resolution and manual scoring of alleles

Amplified products were resolved on an 8% polyacrylamide gel in TBE (1 x) buffer. All gels were run at 400 V for 3 h and stained in 0.5 mg/ml ethidium bromide for 20 min. Bands were visualised using a Gene Flash Syngene Bio Imaging transillumator and images printed on a Sony Syngene video graphic printer. A pUC 19/Hpa II marker was used as a size standard to estimate the size of fragments and

ensure consistency of scoring. This method was used throughout the study to ensure primer efficacy and for the manual scoring of bands. Bands were scored according to size, for example, the largest fragment would be scored as 1, the second largest 2 etc.

2.2.11 Fragment Analysis

Dye-labelled fluorescent forward primers Gm-37-FAM (Geneworks), Gi-4-PET (Applied Biosystems) and Gi-6-VIC (Applied Biosystems) were used to amplify DNA. DNA fragments were separated by capillary electrophoresis on an ABI377 Sequencer at Murdoch University. Amplification was carried out as per 2.2.9 using the labelled forward primers. In a preliminary assay, aliquots of amplified product were diluted at 1in 20, 1in 50, 1 in 75 and 1 in 100 and multiplexed in a 1:1:1 ratio using 2.5 µl of product per primer in 8 µl of a formamide/Liz size standard master mix, in order to determine the most effective combination for fragment analysis. Alleles were analysed using GENEMAPPER software (ver. 3.7. ABI®). The conditions producing the optimal results for identification of genotypes required the dilution of Gm-37-FAM products at 1/20, and Gi-6-VIC products at 1/20. The weaker of the dyes, PET (Gi-4) did not require the dilution of amplified products. In the final assay for genotype identification, aliquots (2.5 µl) from each primer product (diluted as required) were combined to make a total product mix of 7.5 µl. This was then added to 8 µl of fresh formamide (Applied Biosystems) containing approx. 0.2 µl of Liz size standard (Applied Biosystems), resulting in a final volume of 15.7 µl.

2.2.12 Statistical analyses and the impact of sampling bias

Genotypes were determined from genotyping data using GENEMAPPER software. Despite attempts to avoid sampling putative clonal ramets, many samples

with identical genotypes were revealed. This added a degree of bias to the results which has been dealt with in a number of ways throughout this thesis. As mentioned above, the growth habit of *G. curviloba* was such that one ramet often spread over several meters via a radiating array of above-ground, horizontal stems. Despite attempts to avoid sampling stems from the same plant, it is possible that in some instances the identical genotypes revealed represented not only one clone, but one ramet of a clone that was spread over a wide area where multiple branches were sampled. This presented a problem when it came to analysing the results and determining the extent of clonality in a population. Were two genotypes indicative of two ramets or a single ramet that had been sampled twice? It also placed a heavy bias on the diversity measures of the populations, such that it was thought best to calculate estimates using data in which the identical genotypes of ramets had been removed. Assessment of clonality in populations was made using three different algorithms.

The Ellstrand Roose ratio (G/N) (Ellstrand and Roose, 1987) for describing genotype diversity across populations was calculated from the ratio of the proportion of distinct genotypes (G) to the number of individuals sampled (N). This simple but effective method is useful for comparing genotype diversity present in populations. Values vary between 0 and 1, where 0 indicates that all individuals sampled possess identical genotypes.

Simpson's Diversity Index (d) was first developed by Simpson (1949), to determine the probability that two individuals selected at random, would belong to the same species. If the probability was high, then the species diversity of the area could be said to be low (Pielou, 1969). In a similar manner, the rule can be used to

determine the probability that two individuals selected at random from a population would have different genotypes as a result of sexual reproduction. High values would indicate a high potential for sexual reproduction whereas low values represent a high potential for vegetative reproduction. The index (d) can be calculated as follows:

$$d = 1 - \Sigma \{[n_i (n_i - 1)] / [N (N - 1)]\}$$

-where n_i is the number of individuals with genotype i and N is the number of individuals in a population.

The probability of multiple occurrences of specific multi-locus genotypes that result from sexual reproduction was calculated using GENALEX 6 (Peakall and Smouse, 2006) which follows the method of Parks and Werth (1993). This involves calculating the probability of a single occurrence of each multi-locus genotype (P_{GEN}) and then (for those that occurred more than once) the probability of a second occurrence (P_{SEC}). The probabilities are calculated as follows:

$$P_{GEN} = (\Pi_{i=1}^{L} Pi)\, 2^h$$

-where Pi is the frequency of each allele at locus i in a population, h is the number of heterozygous loci in an individual and L is the number of loci.

$$P_{SEC} = 1 - (1 - P_{GEN})^G$$

-where G is the number of genotypes.

P_{GEN} values represent the probability that two consecutive ramet samples that belong to different genets, would by chance, have the same genotype. Small values (<0.05) would indicate that clusters of two or more closely spaced ramets with

identical genotypes, most likely belong to the same genet (Parks and Werth, 1993). Values of $P_{SEC} < 0.05$ are assumed with greater than 95% confidence to comprise a single genet.

2.3 RESULTS

2.3.1 Ellstrand Roose Ratio

The Ellstrand Roose ratio (G/N) was initially calculated for data across all loci, however this method required the removal of any individuals with data missing at one or more loci. Primer Gi-6 did not always consistently amplify all samples; consequently there were 50 individuals which had data missing at this locus. These 50 samples were re-assayed twice but failed to amplify in both cases. The removal of 50 individuals severely reduced the sample sizes of some populations and so as an alternative, the ratio was also calculated using just two loci, Gm-37 and Gi-4, where only one individual required removal. Throughout the 11 populations of *Grevillea curviloba*, Maralla Rd, possessed the highest proportion of similar genotypes with G/N = 0.12 (Table 2.5). The population with the smallest proportion of similar genotypes varied according to the number of loci used (Table 2.5). Both Brand 4b and Brand 7a had a G/N of 1.00 using three loci, indicating that all individuals have unique genotypes. However when the sample size was increased by using just two loci, these figures were much lower (Brand 4b = 0.33 and Brand 7a = 0.64). When comparing both the two and three locus estimates, the population with the highest G/N ratio was Brand 7a, indicating that a larger proportion of the individuals sampled had unique genotypes (Table 2.5). Overall G/N was higher in subspecies *incurva* than subspecies *curviloba*, indicating that *incurva* populations in general had a greater proportion of unique genotypes.

2.3.2 Simpsons diversity index (d)

Values of 'd' were also calculated using all three loci and loci Gm-37 and Gi-4 only, for reasons stated above (2.3.1). Analysis using three loci resulted in values which ranged from 0.058 (Maralla Rd) which indicated a very high potential for vegetative reproduction to 1.000 (Brand 4b) signifying a high potential for sexual reproduction. When two loci were used,'d' was still lowest in the Maralla Rd population (0.117) however the change in sample size resulted in the highest 'd' value of 0.931 occurring in the North Muchea Nature Reserve population (Table 2.5). Overall, Simpson's diversity index was higher in subspecies *incurva* than *curviloba*, indicating a higher potential for vegetative reproduction in subspecies *curviloba*.

2.3.3 Parks and Werth method

The methods of Parks and Werth (1993) were carried out to assess the probability of multiple occurrences of specific multi-locus genotypes in a population resulting from sexual reproduction. The probability of any particular genotype occurring (P_{GEN}) was always less than 0.0038 (genotype N) (Table 2.6). As these values were small for all genotypes, clusters of two or more closely spaced ramets with identical genotypes were assumed to belong to the same genet. The probability of a second occurrence of each multi-locus genotype (occurring more than once) (P_{SEC}) ranged from 0.0034 (genotype U) to 0.4179 (genotype N) (Table 2.6). These results indicated that genotypes H, R, S, T and U (with a $P_{SEC} < 0.05$) could be assumed with greater than 95% confidence, to comprise a single genet.

Table 2.5 Ellstrand Roose ratio and Simpson's diversity index for populations of *Grevillea curviloba* and *Grevillea vestita*. calculated over all loci (requiring the removal of 50 individuals with data missing) and over two loci (requiring the removal of 1 individual with data missing), where G is the proportion of distinct genotypes and N is the number of individuals sampled.

	Ellstrand Roose index (G/N)						Simpson's diversity index (d)	
	N	G	G/N	N	G	G/N		
Number of individuals removed	50			1			50	1
population	**3 loci**			**2 loci**			**3 loci**	**2 loci**
Grevillea curviloba* subsp. *curviloba								
Railway Reserve west	29	6	0.21	29	6	0.21	0.61	0.625
Railway Reserve east	9	3	0.33	14	4	0.29	0.416	0.582
West rd	8	3	0.37	14	4	0.29	0.428	0.648
Maralla rd	17	2	0.12	17	2	0.12	0.058	0.117
mean	**15.75**	**3.5**	**0.26**	**18.5**	**4**	**0.23**	**0.378**	**0.493**
subsp. *incurva*								
Muchea Nature Reserve (west)	15	4	0.27	19	6	0.32	0.257	0.543
Muchea Nature Reserve (east)	14	5	0.36	20	8	0.4	0.472	0.768
Vines resort	5	2	0.4	5	2	0.4	0.4	0.4
Brand Hwy 4b	3	3	1	15	5	0.33	1	0.73
Brand Hwy 7a	5	5	1	11	7	0.64	0.7	0.927
Brand Hwy 11	21	14	0.66	22	5	0.23	0.938	0.783
North Muchea Nature Reserve	17	13	0.76	20	10	0.5	0.882	0.931
mean	**11.4**	**6.57**	**0.64**	**16**	**6.14**	**0.4**	**0.664**	**0.726**
Grevillea vestita* subsp. *vestita								
Wanneroo	20	2	0.1	20	2	0.1	0.478	0.478
Guilderton	17	15	0.88	20	13	0.65	0.985	0.926
mean	**18.5**	**8.5**	**0.49**	**20**	**7.5**	**0.37**	**0.7315**	**0.702**
subsp. *isopogoides*								
Olgivie Rd	19	19	1	20	19	0.95	1	0.994
Binnu Rd	16	16	1	19	19	1	1	1
mean	**17.5**	**17.5**	**1**	**19.5**	**19**	**0.97**	**1**	**0.997**

Table 2.6 Probability of occurrence (P_{GEN}) of each multilocus genotype that occurred more than once in populations of *Grevillea curviloba* and *Grevillea vestita* and the probability of encountering that same genotype a second time (P_{SEC}). N is the number of times the genotype occurred. *Genotypes with a $P_{SEC} < 0.05$ are assumed with greater than 95% confidence to comprise a single genet.

Grevillea curviloba				***Grevillea vestita***			
Genotype	**N**	**P_{GEN}**	**P_{SEC}**	**Genotype**	**N**	**P_{GEN}**	**P_{SEC}**
A	2	0.000379	0.052736	**A**	7	0.000628	0.044253*
B	17	0.003266	0.373641	**B**	13	0.001950	0.131130
C	2	0.003663	0.408302	**C**	2	0.000004	0.000317*
D	2	0.002662	0.316920	**D**	2	0.000025	0.001786*
E	7	0.002698	0.320476				
F	7	0.000560	0.077018				
G	2	0.000519	0.071612				
H	3	0.000030	0.004324*				
I	2	0.000530	0.073049				
J	2	0.002488	0.299725				
K	3	0.002919	0.341661				
L	2	0.002121	0.261888				
M	4	0.001786	0.225577				
N	16	0.003777	0.417951				
O	3	0.002002	0.249129				
P	12	0.001455	0.187918				
Q	3	0.000375	0.052264				
R	2	0.000258	0.036255*				
S	3	0.000207	0.029177*				
T	2	0.000091	0.012920*				
U	2	0.000024	0.003394*				
V	10	0.000603	0.082615				

2.3.4 Observations on morphology

Both subspecies were found to exhibit a peculiar growth habit, whereby a plant spreads across the soil surface via a series of horizontal, above-ground stems (Fig. 2.2a and b). This series of ground spreading stems can then send up vertical shoots. This habit was clearly seen in the Muchea Nature Reserve population after a controlled burn occurred in September 2005. The fire removed the dense groundcover surrounding the plants and revealed a radiating array of thick horizontal stems, capable of spreading up to 3 m across. No roots were formed on these horizontal, above-ground structures.

Fig. 2.2a. The horizontal, above–ground stems of *Grevillea curviloba* subsp. *incurva* were easily distinguished following a fire (tape measure scale : 1 m).

Fig. 2.2b. The complicated arrangement of above ground stems in *Grevillea curviloba* subsp. *incurva* revealed following a fire.

In the Muchae Nature Reserve (west) population, new growth was noted soon after the September fire. When the soil surface was removed from between two adjacent individuals at a depth of 10 cm, an underground structure was encountered, from which roots arose (Fig. 2.3a and b).

Fig 2.3a Under-ground structure encountered between two plants at a depth of 10 cm.

Fig. 2.3b Close up of Fig. 2.3a showing the growth of roots off the horizontal, underground structure.

2.3.5 Clonal spread

The growth habit of *G. curviloba* subsp. *curviloba* plants in the highly clonal Maralla Rd population was noticeably different to other populations. Analysis of the multi-locus genotypes present in this population indicated that the 17 individuals sampled consisted of two genetic individuals, one comprising of 16 widespread ramets (Fig. 2.4). The majority of plants here occurred as large shrubs (up to 4 m in diameter) and were in general more prolific, possessing an abundance of healthy foliage (Fig. 2.5). The second individual of the Maralla Rd population occurs approximately 16 m outside the general area of the large clone, and was noticeably smaller in size in comparison to the other clonal ramets (Fig. 2.6).

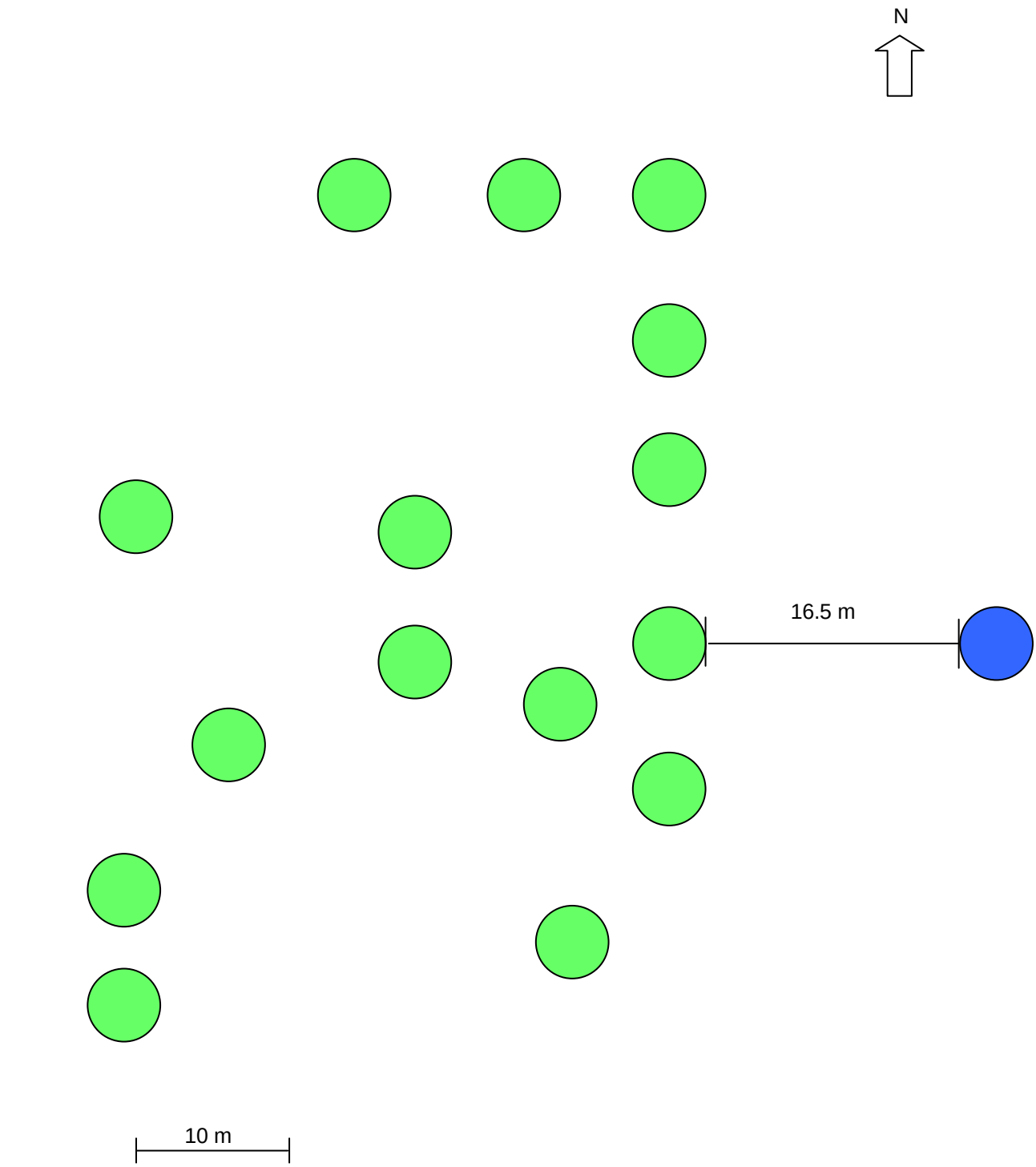

Fig. 2.4 Schematic representation of ramets of the two clones of *Grevillea curviloba* subsp. *curviloba* present at the Maralla Rd population.

Fig. 2.5 Large ramet of clone 1 from the Maralla Rd population

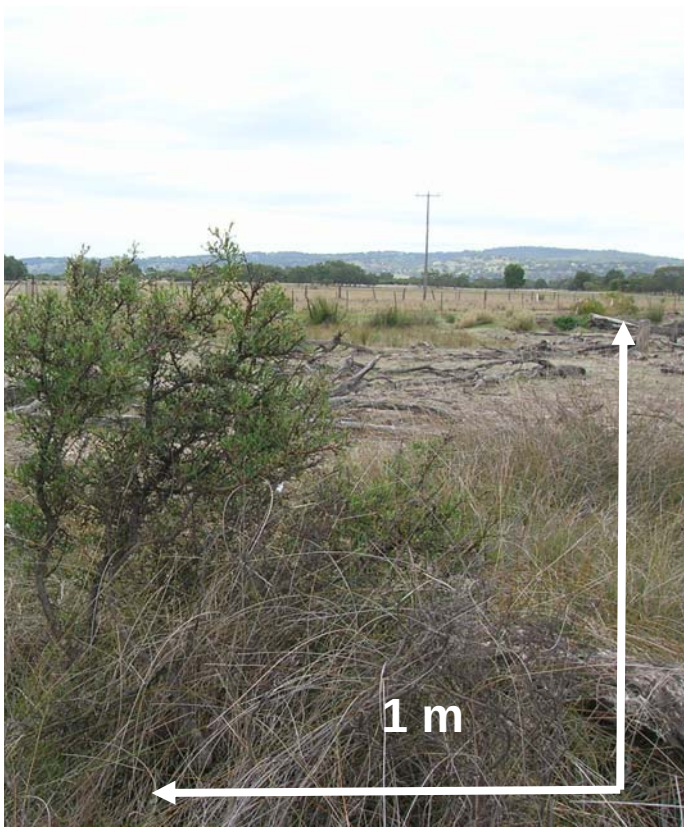

Fig. 2.6 Small individual (genet 2) from the Maralla Rd population.

2.4 DISCUSSION

The genetic data presented in this chapter have indicated that clonality forms a major component of the reproductive strategy in populations of *G. curviloba* (Table 2.5), with both the Ellstrand Roose ratio and Simpson's diversity index revealing a high potential for clonality to occur. Vegetative reproduction as the major reproductive strategy has been reported previously for a number of *Grevillea* species including the critically endangered *G. althoferorum* (Burne *et al*., 2003), the common *G. synapheae* (Burne *et al*., 2003), *G. infecunda* (Kimpton *et al*., 2002) and *G. vestita* which is capable of clonal growth via root suckering (McGillivray, 1993).

The degree of clonality within *G. curviloba* varied considerably from population to population, from Maralla Rd which was found to be highly clonal, consisting of just two genets, both of which were homozygous at all loci, to North Muchea Nature Reserve, a small, road side population that exhibited a surprisingly high proportion of unique genotypes (Table. 2.5). The maintenance of clonality in populations is thought to be controlled by a number of factors including sporadic sexual recruitment and frequency dependant selection involving pathogens, environmental heterogeneity, somatic mutation and gene flow (Eckert, 1999). Maralla Rd provides an interesting example of a population that has, (with the exception of one individual) become fixed for one genotype. A hypothetical model on clonal development in populations developed by Eriksson (1993), described the way in which genotypes can become fixed in clonal populations. The process begins with the selective elimination of certain genets from the initial cohort, together with an increase in the number of ramets. This occurs until an equilibrium level (determined by resource availability) is reached

(Eriksson, 1993). The model assumes that there is no further seedling recruitment following the establishment of the initial cohort. In the Maralla Rd population (subsp. *curviloba*), it is also possible that selection for homozygosity has occurred during the formation of these clones, as these exhibited no heterozygosity.

It is not uncommon for populations within a species to vary considerably in their modes of reproduction. Both sexual and asexual reproduction has been previously reported in *Acacia anomala*, a naturally rare and localised species that occurs in two disjunct groups of populations 30 km apart, in the South West of Western Australia (Coates, 1988). The northern group reproduces sexually whilst the southern populations reproduce entirely by root-suckering (Coates, 1988). These differences in breeding system are thought to result from prolonged geographic isolation, in conjunction with other factors including population size and the amount and distribution of genetic diversity (Coates, 1988). Although the Maralla Rd population (Fig. 2.1) is not significantly geographically isolated from other populations of *G. curviloba*, it may have been historically isolated in terms of gene flow. The small population size of Maralla Rd would also enhance the effects of genetic sampling and random genetic drift over generations, which in turn would result in the loss of low frequency alleles from the population and a general decline in heterozygosity as certain alleles become fixed (Savolainen and Kuittinen, 2000). Interestingly, the growth habit of plants in the Maralla Rd population differed from the usual appearance of *G. curviloba*. In general, plants at this site were widely separated (by as much as 7 m) and occurred as large bushy shrubs up to 4 m in diameter, rather than the predominantly ground covering growth habit with upright stems noted in the other populations (Appendix A).

Whilst it was known that *G. vestita* was capable of vegetative growth via root suckering (McGillivray, 1993), the large variation in the clonality occurring between the Wanneroo and Guilderton populations (subsp. *vestita*) was surprising (Table 2.5). The Wanneroo population presents another example of genotypes becoming fixed in a population. What was initially thought to be a population consisting of many individuals, turned out to be comprised of just two clones, one on either side of a dirt track.

When analysing the results on the presence of clonality in *G. curviloba* consideration must be given for the potential for bias to occur (due to the sampling method used), a problem long associated with the study of vegetative reproduction (Ellstrand and Roose, 1987). Several populations were sampled before the unusual growth habit of *G. curviloba* was revealed following a fire. These populations included the Muchea Nature Reserve (east and west) and the Railway Reserve (east and west). The larger sample size of the Railway Reserve population is more likely to contain a representative sample of genotypes, as the larger area allowed for samples to be taken from individuals up to 10 m apart. However the Muchea Nature Reserve population was smaller in area, and did not allow for such widespread sampling. Therefore, many of the identical genotypes encountered in the Muchea Nature Reserve population may have represented the same ramet of a genet, rather than different ramets of the same genet. Although studies of clonality will always produce approximate estimates (due to this sampling bias), a more detailed knowledge of the growth habit of *G. curviloba* and its mode of vegetative reproduction prior to genetic analysis, would result in a more accurate estimation of the extent of clonality in populations.

Microsatellite markers have proven to be useful for the study of clonality in *G. curviloba* populations, providing enough statistical power to enable the identification of individual genets with high probability (evident through the small probabilities of obtaining specific genotypes (P_{GEN})) (Table 2.6). Ideally, all microsatellites used in a genetic analysis should be working effectively. However, one of the downfalls associated with the use of these markers is the difficulties often experienced in cross amplification between species (Zane *et al*., 2002). Previous trials conducted by M, Byrne, had found only three primers that work on *G. curviloba*. These were isolated from the eastern states species *G. macleayana* and *G. iaspicula*, which are unrelated and likely to be genetically distant from *G. curviloba*.

Complications were associated with the calculation of the Ellstrand Roose ratio and Simpson's diversity index due to the difficulties encountered when removing individuals with data missing at one or more loci. Removing individuals severely reduced the sample size in some instances (for example population Brand Hwy 4b, Table 2.5), yet removing 1 locus altogether (to eliminate the problems associated with data absent at the Gi-6 locus), reduced the number of unique genotypes occurring per population. Ideally, the ratios would be calculated across populations of large sample size and complete data.

There were also difficulties in calculating P_{SEC} values, due to the high clonality present in some populations. When a clone has been sampled frequently, for example genotype N which occurred 16 times (Table 2.6), the frequency of the alleles in that clone are so high that the probability of obtaining the genotype a second time is also high (0.417) (Table 2.6) However the method states that only values less than 0.05 can

be considered with high confidence to indicate ramets comprising a single genet. The high frequency of genotype N is likely to represent a single genet, but one that has been over-represented during sampling. Moreover, the Parks and Werth method (1993) is based on a number of assumptions, one of them, that mating is random in populations. When such extensive clonality exists in a population, mating cannot be assumed to be random, violating one of the main assumptions required for this method. Therefore in situations where clonality is extensive in populations and sampling difficult, the Parks and Werth method may not provide a useful means of analysis, particularly for the determination of P_{SEC} values.

A large proportion of plants possess the capacity for clonal reproduction (Eckert, 1999), yet when dealing with plant populations that are highly clonal, there does not appear to be any reliable guidelines indicating how to conduct, assess and interpret analyses of genetic diversity. As interest in this area grows, future research should focus on developing appropriate models for the statistical analysis of clonal populations and on reducing the degree of bias associated with sampling.

THE GENETIC DIVERSITY AND STRUCTURE OF *Grevillea curviloba*

3.1 INTRODUCTION

3.1.1 Analysis of genetic diversity and structure in endangered plant species

Knowledge of the genetic diversity present in an endangered plant species can provide important information on the viability of its populations, including levels of inbreeding, total diversity and the genetic structure (partitioning of diversity within and between populations). The genetic structure of a plant species is governed by a number of factors including mode of reproduction, geographic distribution, mating system and population size (Coates, 1988). Analyses of structure are useful for identifying groups of populations that are genetically distinct and may warrant classification as an independent conservation unit. They can also clarify whether proposed subspecies, based on morphology, are reflected in terms of genetic differentiation (Moran and Hopper, 1983). *Grevillea curviloba* has typically been regarded as consisting of two subspecies. Individuals of subspecies *curviloba* are identified by their wider, slightly cupped shaped primary leaf lobes, and subspecies *incurva* by their more incurved, narrowly linear leaf lobes (Phillimore and English, 2000). However a continuum has often been noted in the leaf morphology between the two subspecies, prompting a request for the review of the current taxonomy (Phillimore and English, 2000). The incorrect designation of subspecies can have enormous repercussions for the management of a species, by potentially over or under-estimating the total number of populations and individuals present.

The analysis of genetic structure also indicates whether the bulk of genetic diversity is occurring within populations or between them, information which is vital in

the creation of a suitable conservation strategy (Haig, 1998). For example, if most of the diversity is occurring between populations, management should aim to preserve all populations in order to maximise the total genetic diversity conserved. If the majority of variation is occurring within populations, priority can be given to those of highest genetic diversity.

3.1.2 The importance of molecular markers in studies of genetic diversity and structure

Molecular markers are useful in interpreting the evolutionary patterns and lineages that often occur in flora due to geographical separation (Coates, 1988) and more recently, due to the wide-spread habitat fragmentation through land clearance that has occurred in south-western Western Australia (Coates and Hamley, 1999). This recent disturbance has often resulted in a reduction in population size (Barrett and Kohn, 1991) and a loss of genetic diversity in populations due to drift and a reduction in gene flow (Lowe *et al*., 2004). As populations become more fragmented and isolated, the genetic divergence between them increases over time. A study on the genetic structure of *Lambertia orbifolia* (Coates and Hamley, 1999) (an endangered woody shrub from the South West) used isozymes to determine the degree of genetic divergence occurring between populations that had been affected by recent habitat fragmentation. The study identified two groups of genetically distinct populations that appeared to have been historically isolated. As a result it was suggested that one of the groups be recognised as a separate conservation unit and given priority for conservation management (Coates and Hamley, 1999).

3.1.3 Determining genetic diversity and structure using microsatellite markers

The high resolution and statistical power provided by microsatellites make them a useful molecular marker for the analysis of diversity and structure (Cavers *et al*., 2005). They are particularly useful when dealing with populations that exhibit clonality due their ability to accurately identify individuals (Suvanto and Latva-Karjanmaa, 2005).

The aims of this chapter were:

-To determine how genetic diversity is partitioned between populations of *Grevillea. curviloba.*

-To determine the genetic diversity occurring between the subspecies of *Grevillea curviloba*.

-To determine whether the genetic data support classification of *Grevillea curviloba* into the two subspecies *incurva* and *curviloba* which has been based on morphological criteria.

3.2 MATERIALS AND METHODS

Population sampling, DNA extraction, electrophoresis and genotype analysis were conducted as in section 2.2. Allele frequencies and distributions were recorded across 11 populations of *Grevillea curviloba* and 4 populations of *Grevillea vestita* and analysed in order to establish any patterns that may have occurred between subspecies and between the two species. Diversity estimates were calculated for all populations to determine the polymorphism of loci and the genetic diversity present within populations, subspecies and species. The distribution of genetic diversity was also analysed to determine the genetic structure of both *G. curviloba* and *G. vestita*.

3.2.1 Allele frequencies and diversity estimates

The frequency of alleles at the species and subspecies level that occurred at each of the three loci was calculated using the F_{STAT} statistical package (Goudet, 2001). The following single-locus diversity estimates were calculated using the GDA statistical package (Lewis and Zaykin, 2002):

N- Population sample size.

A- The mean number of alleles per locus, known as allelic diversity or allelic richness (Nei, 1987).

P- The percentage of polymorphic loci per population (Nei, 1987).

H_o- The observed heterozygosity, calculated as the number of heterozygotes at a locus divided by the total number of individuals sampled (Frankham *et al*., 2002)

H_e- Gene diversity which measures the expected heterozygosity at a locus, and is also known as the expected panmictic heterozygosity, and is calculated as:

$$H_e = 1 - \sum_{i=1}^{k} P_i^2$$

-where P is the frequency of the ith allele summed over k alleles.

F_{IS}- The inbreeding coefficient, measures the probability that two alleles in an individual are identical by descent. It describes the reduction of heterozygosity within individuals relative to the total population as a result of non-random mating within subpopulations. F_{IS} values can be positive or negative, the former indicating a deficit of heterozygotes and the latter an excess. It is calculated as:

$$F_{IS} = (H_S - H_I)/H_S$$

-where H_S is the mean expected heterozygosity within populations and H_I is the mean observed heterozygosity per individual.

3.2.2 Partitioning of genetic diversity

The following estimates of Nei's distribution of genetic diversity were calculated using FSTAT (Goudet, 2001):

H_T- Total gene diversity, measures total expected heterozygosity and is broken down to determine the proportion of total diversity that is found within populations (H_S) and among populations (D_{ST}) (Nei, 1973).

H_S- The amount of gene diversity within populations.

D_{ST}- The proportion of the total gene diversity that is expected among populations.

G_{ST}- The proportion of variation between populations.

3.2.3 F statistics

Weir and Cockerham's (1984) estimation of F-statistics, F_{IT} and theta (θ) were calculated using F_{STAT} (Goudet, 2001).

F_{IT}- The overall inbreeding coefficient, measures the reduction in heterozygosity in individuals relative to the total population, due to non-random mating. Values can be positive or negative, the former indicating a deficit of heterozygotes and the latter an excess.

Theta θ- Genetic differentiation, provides a measure of the distribution of variation within and among populations.

3.2.4 Genetic Structure

The analysis of molecular variance (AMOVA) within both species *G. curviloba* and *G. vestita* was performed in GENALEX (Peakall and Smouse, 2006) using data with ramets of each clone removed. Calculations of population differentiation were based on genotypic variance and provided an estimate of PhiPT (φPT) an analogue of G_{ST}. Output was in the form of a pie graph.

STRUCTURE (Pritchard *et al*., 2000) is a model-based, clustering program that tests for population structure by using multi-locus genotype data. The model assumes there are K populations, each of which is defined by a set of allele frequencies at each locus. Individuals are assigned to populations (based on probability), or to one or more populations when their genotypes are admixed. The program is useful for studying the classification of species into subspecies based on genetic data, and is known to be accurate when using small numbers of microsatellite loci (Pritchard *et al*., 2000). Initially no assumptions were made as to the number of populations present and K was

set from 1 to 4. This analysis did not identify any structure within the data, therefore the data was run again, this time with the prior assumption of the two subspecies based on morphology. In both analyses a burn-in period of 10 000 iterations was used, with 6 iterations at each K value performed.

3.2.5 Maximum likelihood trees

Maximum likelihood trees based on the analysis of gene frequencies were produced using CONTML (Continuous characters maximum likelihood method) in PHYLIP (Felsenstein, 1993) with *G. vestita* subspecies *isopogoides* used as an outgroup. Multiple data sets were generated using SEQBOOT to calculate bootstrap values and a consensus tree was produced using CONSENSE. When all populations of *G. curviloba* and *G. vestita* were used, SEQBOOT was unable to calculate bootstrap values due to the low amount of genetic variation present. Therefore three populations with low genetic variation, Wanneroo, Maralla Rd and the Vines resort, were removed to allow for the creation of multiple data sets and the determination of bootstrap values. Trees were produced in Treefile.

3.3 RESULTS

3.3.1 Allele Frequencies

Within *Grevillea curviloba* there were a total of 20 alleles over all three loci, 12 of which occurred in subspecies *curviloba* and 19 in subspecies *incurva* (Table 3.1). Common alleles included allele A (Gi-4) which occurred in 10 of the 11 populations and allele F (Gi-4) which occurred in all populations of *Grevillea curviloba.* Some alleles were rare, and found in only 1 population, including alleles K and L (Gm-37), E

and I (Gi-4) and allele K (Gi-6). Despite being rare in the *G. curviloba* populations, allele I (Gi-4) occurred at a high frequency (0.500) in the Maralla Rd population. In general the allele frequencies varied greatly between populations, and alleles that were common in some populations often occurred at much lower frequencies in others (Table 3.1). For example allele A (Gi-4) ranges in frequency from 0.100 (Railway Reserve east) to 0.967 (Brand Hwy 11). Allele J (Gi-6) ranges from a frequency of 0.107 in Brand Hwy 11 to 1.000 in Maralla Rd. Six alleles occurred solely in populations of subsp. *incurva* but Maralla Rd (subsp. *curviloba*) was the only population to contain an allele (I, Gi-4) that was not also found in a subsp. *incurva* population.

3.3.2 Population genetic parameters

The mean number of alleles per locus (A) averaged 2.696 over the 11 populations of *G. curviloba*. Values ranged from 1.33 in the Maralla Rd population to 4.00 in Brand Hwy 11. Overall the mean number of alleles per locus was lower in subspecies *curviloba* (2.249) than subspecies *incurva* (2.952) (Table 3.2). The percentage of polymorphic loci (P) was high with a mean of 90.9%. The lowest value of (P) occurred in the Maralla Rd population (33.3%) where only one of the three loci was polymorphic. In the Vines resort population, two out of the three loci were polymorphic resulting in a value of 66.6%. The remaining nine populations were polymorphic at all three loci (Table 3.2).

Table 3.1: Allele frequencies for 3 loci across 11 populations of *Grevillea curviloba* and 4 populations of *Grevillea vestita*. Data with ramets of clones removed.

		Grevillea vestita				*Grevillea curviloba*										
		subspecies *vestita*		subspecies *isopogoides*		subspecies *incurva*							subspecies *curviloba*			
		Wanneroo	Guild-erton	Olgivie	Binnu	MNR (west)	MNR (east)	vines	brand 4b	brand 7a	brand 11	north MNR	RR west	RR east	West rd	Maralla Rd
	N	2	18	20	18	5	10	2	8	10	15	16	6	5	4	2
locus-Gm-37	allele															
	A	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.350	0.233	0.000	0.583	0.100	0.125	0.000
	B	0.000	0.000	0.075	0.111	0.400	0.250	1.000	0.000	0.250	0.567	0.250	0.250	0.700	0.625	1.000
	C	0.000	0.000	0.000	0.167	0.200	0.100	0.000	0.563	0.350	0.000	0.625	0.000	0.000	0.000	0.000
	D	1.000	0.111	0.225	0.194	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	E	0.000	0.167	0.050	0.056	0.400	0.550	0.000	0.438	0.050	0.100	0.063	0.000	0.000	0.125	0.000
	F	0.000	0.194	0.125	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.063	0.167	0.000	0.125	0.000
	G	0.000	0.528	0.100	0.139	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	H	0.000	0.000	0.025	0.028	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	I	0.000	0.000	0.125	0.083	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	J	0.000	0.000	0.225	0.139	0.000	0.000	0.000	0.000	0.000	0.100	0.000	0.000	0.000	0.000	0.000
	K	0.000	0.000	0.000	0.056	0.000	0.100	0.000	0.000	0.000	0.000	0.000	0.000	0.200	0.000	0.000
	L	0.000	0.000	0.050	0.028	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000

		Wann	Guild	Olgiv	Binnu	MNR west	MNR east	Vines	Brand 4b	Brand 7a	Brand 11	NMNR	RR west	RR east	West Rd	Maralla Rd
locus-Gi-4	A	0.000	0.000	0.000	0.000	0.400	0.550	0.750	0.750	0.950	0.967	0.313	0.417	0.100	0.750	0.000
	B	0.000	0.528	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	C	0.000	0.000	0.050	0.056	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	D	0.000	0.000	0.000	0.056	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	E	0.750	0.000	0.000	0.056	0.000	0.000	0.000	0.125	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	F	0.250	0.028	0.500	0.417	0.600	0.350	0.250	0.125	0.050	0.033	0.563	0.583	0.900	0.250	0.500
	G	0.000	0.000	0.275	0.083	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	H	0.000	0.000	0.050	0.111	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	I	0.000	0.250	0.050	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.500
	J	0.000	0.083	0.050	0.083	0.000	0.100	0.000	0.000	0.000	0.000	0.125	0.000	0.000	0.000	0.000
	K	0.000	0.111	0.025	0.139	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
locus-Gi-6	A	0.000	0.000	0.132	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	B	0.000	0.000	0.000	0.031	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	C	0.000	0.563	0.132	0.125	0.000	0.500	0.500	0.000	0.000	0.000	0.192	0.000	0.667	0.000	0.000
	D	0.000	0.000	0.079	0.094	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	E	0.000	0.000	0.132	0.281	0.000	0.000	0.000	0.333	0.400	0.286	0.115	0.000	0.000	0.000	0.000
	F	0.000	0.000	0.026	0.063	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	G	0.000	0.219	0.000	0.000	0.333	0.000	0.000	0.000	0.000	0.000	0.000	0.167	0.000	0.000	0.000
	H	0.000	0.000	0.158	0.188	0.000	0.500	0.500	0.500	0.600	0.321	0.038	0.167	0.000	0.667	0.000
	I	0.500	0.000	0.211	0.156	0.667	0.000	0.000	0.000	0.000	0.179	0.192	0.000	0.333	0.333	0.000
	J	0.500	0.219	0.000	0.063	0.000	0.000	0.000	0.167	0.000	0.107	0.154	0.667	0.000	0.000	1.000
	K	0.000	0.000	0.132	0.000	0.000	0.000	0.000	0.000	0.000	0.071	0.000	0.000	0.000	0.000	0.000
	L	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.036	0.308	0.000	0.000	0.000	0.000

Table 3.2: Genetic diversity parameters for eleven populations of *Grevillea curviloba* and four populations of *Grevillea vestita*. C.R.; clone ramets removed from data, C.I.; clone ramets included in data, N; number of samples, A; allelic diversity, P; percentage polymorphic loci, H_o; observed heteozygosity, H_e; gene diversity and F_{IS}; fixation index. Standard errors in parentheses.

population	**N**		**A**	**P (%)**	**H_o**		**H_e**		**F_{IS}**	
Grevillea curviloba subspecies *incurva*										
	C.R.	C.I.			C.R.	C.I.	C.R.	C.I	C.R.	C.I
MNR (west)	4.333	17.666	2.333 (0.33)	100	0.266 (0.133)	0.298 (0.224)	0.592 (0.059)	0.332 (0.124)	0.593 (0.217)	0.102 (0.389)
MNR (east)	8.333	18.000	3.000 (0.57)	100	0.300 (0.057)	0.307 (0.175)	0.599 (0.026)	0.416 (0.074)	0.519 (0.079)	0.265 (0.281)
Vines	2.000	5.000	1.666 (0.33)	66.6	0.166 (0.166)	0.267 (0.266)	0.388 (0.200)	0.296 (0.157)	0.667 (0.333)	0.111 (0.467)
Brand 4b	6.333	11.000	2.666 (0.33)	100	0.319 (0.181)	0.355 (0.212)	0.563 (0.088)	0.582 (0.076)	0.472 (0.355)	0.400 (0.429)
Brand 7a	8.333	9.000	2.666 (0.66)	100	0.500 (0.208)	0.479 (0.207)	0.453 (0.185)	0.451 (0.188)	-0.125 (0.236)	-0.058 (0.254)
Brand 11	14.666	21.666	4.000 (1.15)	100	0.339 (0.175)	0.307 (0.174)	0.495 (0.220)	0.461 (0.211)	0.322 (0.228)	0.340 (0.238)
NMNR	15.000	19.000	4.333 (0.88)	100	0.306 (0.066)	0.293 (0.065)	0.656 (0.084)	0.620 (0.090)	0.543 (0.151)	0.535 (0.164)
mean	**8.428**	**14.476**	**2.952 (0.61)**	**95.2**	**0.313 (0.141)**	**0.329 (0.189)**	**0.535 (0.123)**	**0.451 (0.131)**	**0.427 (0.228)**	**0.242 (0.317)**
subspecies *curviloba*										
RR west	6.000	29.000	2.666 (0.33)	100	0.388 (0.147)	0.724 (0.105)	0.565 (0.028)	0.506 (0.026)	0.333 (0.229)	-0.441 (0.136)
RR east	4.333	13.333	2.333 (0.33)	100	0.133 (0.066)	0.244 (0.212)	0.414 (0.107)	0.288 (0.163)	0.718 (0.292)	0.152 (0.352)
West rd	3.666	12.000	2.666 (0.66)	100	0.416 (0.220)	0.381 (0.281)	0.534 (0.062)	0.412 (0.127)	0.268 (0.400)	0.069 (0.443)
Maralla rd	2.000	17.000	1.333 (0.33)	33.3	0.000 (0)	0.000 (0)	0.222 (0.222)	0.038 (0.038)	1.000 (0.333)	1.000 (0.333)
mean	**3.999**	**17.833**	**2.249 (0.41)**	**83.3**	**0.234 (0.108)**	**0.337 (0.150)**	**0.433 (0.105)**	**0.311 (0.060)**	**0.579 (0.314)**	**0.193 (0.316)**
species mean	**6.817**	**15.697**	**2.696**	**90.9**	**0.284**		**0.498**		**0.482**	
Grevillea vestita subspecies *vestita*										
Wanneroo	2.000	20.000	1.666 (0.33)	66.6	0.500 (0.288)	0.550 (0.293)	0.388 (0.200)	0.321 (0.161)	-0.500 (0.333)	-0.747 (0.289)
Guilderton	17.333	19.000	4.000 (0.57)	100	0.196 (0.060)	0.181 (0.058)	0.641 (0.017)	0.631 (0.022)	0.700 (0.103)	0.719 (0.104)
mean	**9.666**	**19.500**	**2.833 (0.45)**	**83.3**	**0.348 (0.174)**	**0.366 (0.176)**	**0.514 (0.109)**	**0.476 (0.092)**	**0.100 (0.218)**	**-0.014 (0.197)**
subspecies *isopogoides*										
Olgivie	19.666	19.666	8.000 (0.57)	100	0.511(0.133)	0.511 (0.112)	0.808 (0.064)	0.808 (0.064)	0.373 (0.099)	0.373 (0.099)
Binnu	17.333	17.333	8.666 (0.66)	100	0.442(0.032)	0.442 (0.032)	0.847 (0.029)	0.847 (0.029)	0.486 (0.057)	0.486 (0.057)
mean	**18.498**	**18.498**	**8.333 (0.62)**	**100**	**0.476 (0.083)**	**0.476 (0.072)**	**0.827 (0.047)**	**0.827 (0.047)**	**0.429 (0.078)**	**0.429 (0.078)**
species mean	**14.082**	**18.999**	**5.583**	**91.6**	**0.412**		**0.671**		**0.264**	

Observed heterozygosity (H_o) values ranged from 0 in the Maralla Rd population to 0.500 in the Brand 7a population. Overall, when clones were removed from the data, (H_o) was higher in subspecies *incurva* (0.313) in comparison to subspecies *curviloba* (0.234). However when ramets of clones were left in the data, subspecies *curviloba* exhibited higher heterozygosity (0.337) in comparison to *incurva* (0.329). This is due primarily to the high value of heterozygosiy (0.724) in the Railway Reserve west population, which consisted of a large number of heterozygous clones. This high figure was reduced when clone ramets were removed from the data set (0.388) (Table 3.2).

Gene diversity (H_e) ranged from 0.222 in the Maralla Rd population to 0.656 in the North Muchea Nature Reserve population. Overall gene diversity was higher in subspecies *incurva* (0.535) than *curviloba* (0.433). H_e was consistently higher than the observed heterozygosity (H_o) with the exception of the Brand 7a population. When clone ramets were left in the data, gene diversity, in general, was reduced as a result of the increased sample size. With the clones included, population Brand 7a still exhibited a higher H_o than H_e and population Railway Reserve west also had a higher H_o than H_e owing to the presence of extensive, heterozygous clones (Table 3.2). It is interesting to note that when clones were left in the data, genetic diversity was similar in *G. curviloba* subsp. *incurva* (0.535) and *G. vestita* subsp. *vestita* (0.514).

F_{IS}, the inbreeding coefficient, provides a measure of the divergence of observed heterozygosity from the expected heterozygosity within populations assuming panmixia (random mating between individuals in a population) (Lowe *et al*., 2004). The lowest F_{IS} value occurred in Brand 7a (-0.125) indicating that this population contained an excess of

heterozygotes. The highest F_{IS} value of 1.000 occurred in the Maralla Rd population in which all 17 individuals sampled were homozygous. Overall F_{IS} was higher in subspecies *curviloba* (0.482) than subspecies *incurva* (0.427). In general however, both subspecies, with the exception of population Brand 7a, had more homozygotes than would be expected under panmictic conditions (Table 3.2).

3.3.3 Nei's (1987) estimates of the distribution of genetic diversity

Nei's estimate of total gene diversity, measured in terms of the total expected heterozygosity (H_T), was higher in subspecies *incurva* (0.673) than in *curviloba* (0.639). Values decreased when clone ramets were included in the data and sample size increased (Table 3.3). The mean gene diversity within populations, measured in terms of the mean expected heterozygosity (H_S) was again greater in subspecies *incurva* (0.573) than in subspecies *curviloba* (0.489). Values (H_S) decreased when clone ramets were added to the data as sample sizes were increased (Table 3.3). The mean amount of genetic diversity occurring within populations of subspecies (D_{ST}) was greater in subspecies *curviloba* (0.151) than in subspecies *incurva* (0.102) (Table 3.3). The proportion of gene diversity occurring between populations of subspecies (G_{Sp}) was also greater in subspecies *curviloba* (0.235) than in *incurva* (0.151) (Table 3.3). The gene diversity occurring between subspecies of *G. curviloba* (0.222) was high in comparison to *G. vestita* (0.191).

3.3.4 Weir and Cockerham's (1984) estimation of F statistics

Weir and Cockerham's estimation of F statistics, F_{IT} and theta (θ) were generated using both data with clonal ramets removed and with clones included. The overall inbreeding coefficient (F_{IT}) provides a measure of the reduction of heterozygosity within individuals

relative to the total population, due to non-random mating. Values greater than 0 indicate a deficit of heterozygotes in a population. F_{IT} was higher in subspecies *curviloba* 0.594 than in subspecies *incurva* 0.539 (Table 3.4).

Genetic differentiation or theta (θ) was higher in subspecies *curviloba* (0.280) than subspecies *incurva* (0.178). Including the clonal data increased theta values, as diversity within the populations decreased and diversity between the populations increased (Table 3.4). Genetic differentiation between subspecies of *G. curviloba* (0.217) was high in comparison to *G. vestita* (0.140). The low theta value for G. *vestita* subsp. *isopogoides* (0.001) indicated that genetic diversity was maintained within populations rather than between. Overall *Grevillea vestita* showed less genetic differentiation than *G. curviloba*, however differences in the level of clonality present in each species may be a major cause of this.

3.3.5 Genetic structure of *Grevillea curviloba*

The partitioning of genetic diversity within and between populations and subspecies of *G. curviloba*, determined using an AMOVA analysis, indicated that 27% of genetic variation was occurring between populations within subspecies, with only 6% of variation occurring among the subspecies *curviloba* and *incurva* (Fig. 3.1a). Overall 67% of the variation was contained within populations. The *Grevillea vestita* AMOVA analysis (Fig 3.1b) indicated that a higher proportion of genetic variation was maintained within populations (79%) compared to *G. curviloba*.

Table 3.3: Nei's (1987) Distribution of genetic diversity within and between populations of *Grevillea curviloba* and *Grevillea vestita* at the species level and subspecies level.

	H_T H_T	H_S H_{PS}	D_{ST} D_{PS}	G_{ST} G_{SP}
Grevillea curviloba	0.697 (0.074) 0.679 (0.081)	0.543 (0.061) 0.404 (0.046)	0.154 (0.015) 0.275 (0.049)	0.222 (0.009) 0.405 (0.036)
subsp. *curviloba*	0.639 (0.064) 0.579 (0.081)	0.489 (0.008) 0.311 (0.069)	0.151 (0.076) 0.268 (0.103)	0.235 (0.062) 0.463 (0.133)
Subsp. *incurva*	0.673 (0.010) 0.653 (0.098)	0.573 (0.092) 0.460 (0.047)	0.102 (0.035) 0.193 (0.052)	0.151 (0.036) 0.296 (0.044)
Grevillea vestita	0.843 (0.016) 0.827 (0.016)	0.717 (0.028) 0.658 (0.028)	0.127 (0.022) 0.169 (0.014)	0.191 (0.021) 0.204 (0.020)
subsp. *vestita*	0.746 (0.046) 0.704 (0.046)	0.567 (0.069) 0.479 (0.071)	0.179 (0.029) 0.225 (0.031)	0.386 (0.052) 0.320 (0.066)
subsp. *isopogoides*	0.838 (0.044) 0.838 (0.044)	0.838 (0.046) 0.838 (0.046)	0.000 (0.001) 0.000 (0.001)	0.000 (0.002) 0.000 (0.002)

H_T, total gene diversity; H_S, mean gene diversity within populations; H_{PS} mean gene diversity of populations within a subspecies; D_{ST}, mean gene diversity between populations; D_{PS}, mean gene diversity between populations within subspecies; G_{ST} gene diversity between populations; G_{PS}, gene diversity between populations within a subspecies. Standard errors in parentheses.

Table 3.4 : Weir and Cockerham's (1984) estimation of F-statistics; F_{IT} and theta.

	F_{IT}	Theta
	C.R C.I.	C.R. C.I.
G. curviloba	**0.563** (0.110) 0.511 (0.146)	**0.217** (0.032) 0.429 (0.045)
subsp. *curviloba.*	0.594 (0.159) 0.348 (0.229)	0.280 (0.057) 0.469 (0.111)
subsp. *incurva*.	0.539 (0.103) 0.556 (0.133)	0.178 (0.044) 0.326 (0.032)
G. vestita	**0.561** (0.064) 0.517 (0.113)	**0.140** (0.029) 0.260 (0.024)
subsp. *vestita*	0.762 (0.103) 0.596 (0.195)	0.331 (0.037) 0.486 (0.071)
subsp. *isopogoides*	0.427 (0.040) 0.427 (0.040)	0.001 (0.004) 0.001 (0.004)

Standard errors in parentheses, C.R. = data with clone ramets removed and C.I. = data with clone ramets included.

There was also a greater proportion of diversity occurring amongst subspecies (15%). Only 6% of the variation occurred among populations.

The degree to which the genetic data correlated with the morphological classification into subspecies was revealed using STRUCTURE. Analysis of *G. curviloba* indicated there was no distinct genetic differentiation into two groups. This was clearly seen in the bar plot (Fig. 3.2), where there was no relationship between individuals labelled by subspecies (denoted as (1) for *incurva* and (2) for *curviloba*) and the clusters generated (red and green). The triangle plot (Fig. 3.3) also showed there was no genetic distinction into two groups (individuals did not cluster into either corner).

The STRUCTURE analysis performed on *G. vestita* (Fig. 3.4 and 3.5) highlights a clear genetic distinction into two groups, with the exception of the two individuals from the Wanneroo (subsp. *vestita*) population which appeared to share a greater genetic similarity to subsp. *isopogoides*. This is particularly obvious in the bar plot in which the individuals numbered one and two, thought to be subspecies *vestita*, have been placed in the cluster (red) representing subsp. *isopogoides* (Fig. 3.4).

The maximum likelihood consensus tree (with populations of low diversity removed), further indicated a lack of genetic relationship between the two subspecies, with populations of *curviloba* and *incurva* failing to group together (Fig. 3.7).

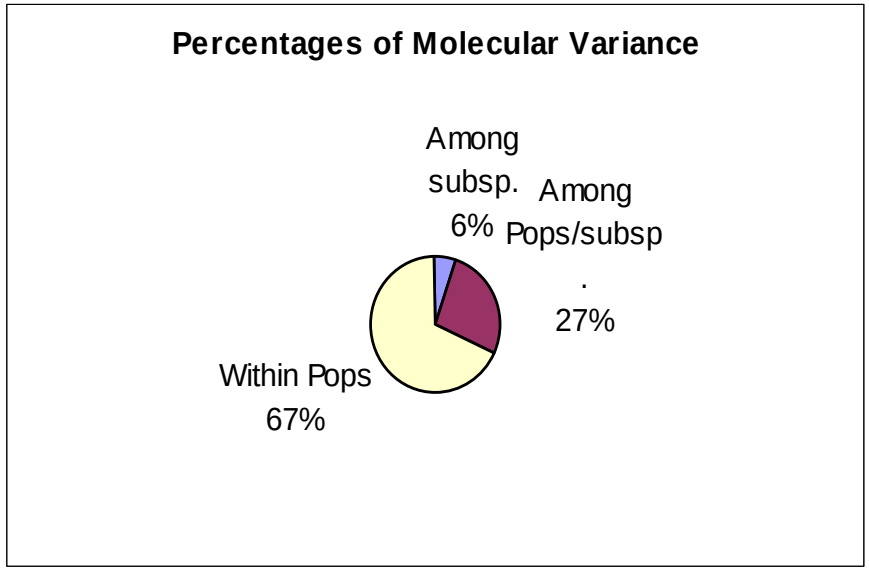

Fig. 3.1a *Grevillea curviloba*

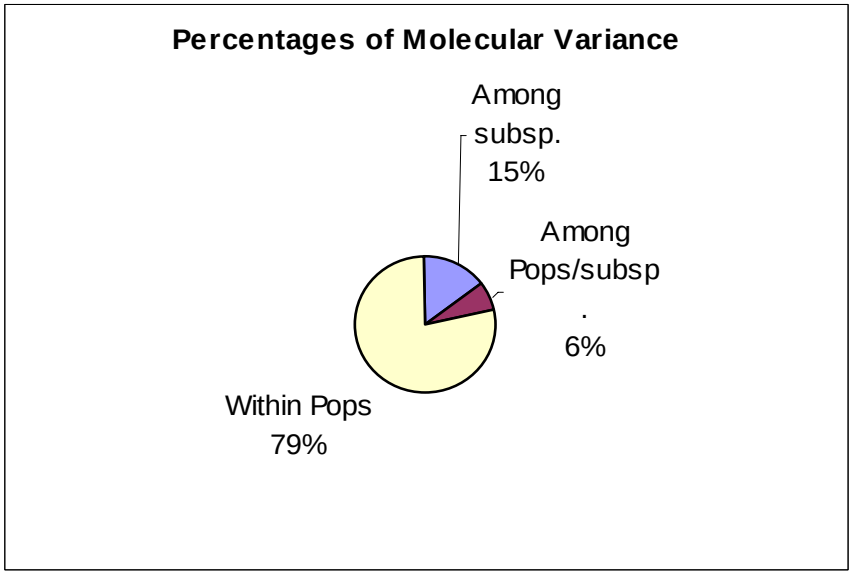

Fig. 3.1b *Grevillea vestita*

Fig. 3.1a and **b**: Pie charts illustrating the distribution of molecular genotypic variance (PhiPT) within and between populations and subspecies of *Grevillea curviloba* and *Grevillea vestita.*

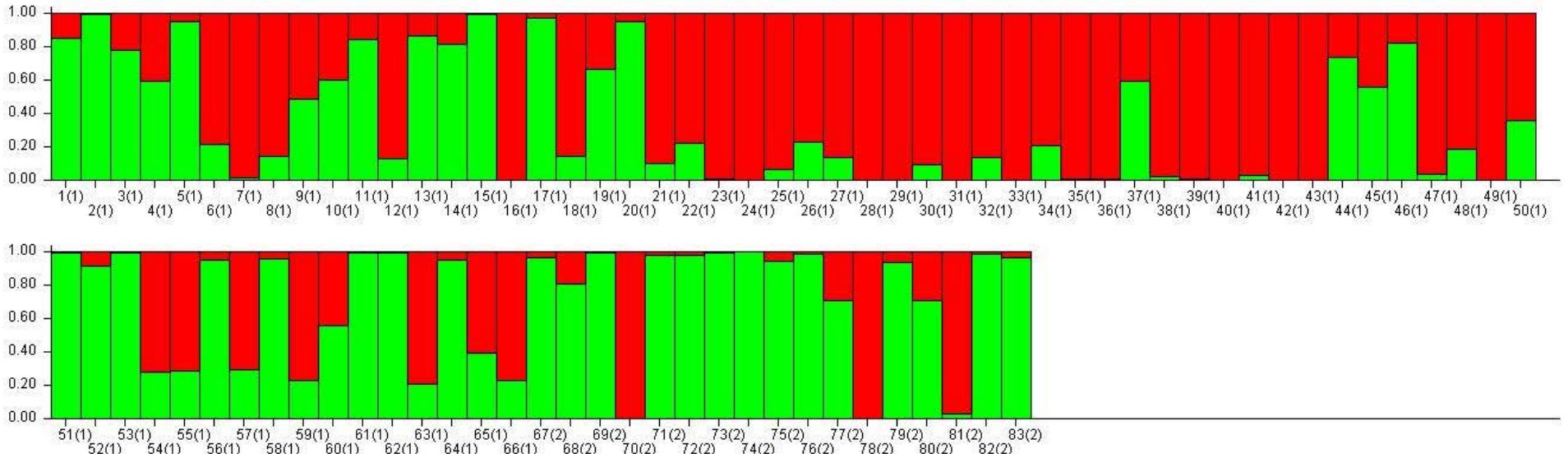

Fig. 3.2 Bar plot indicating the relationship between the prior assumed subspecies of *Grevillea curviloba*, subsp. *incurva* (denoted (1)) and subsp. *curviloba* (2) and the clusters generated using STRUCTURE 2.1 (red and green).

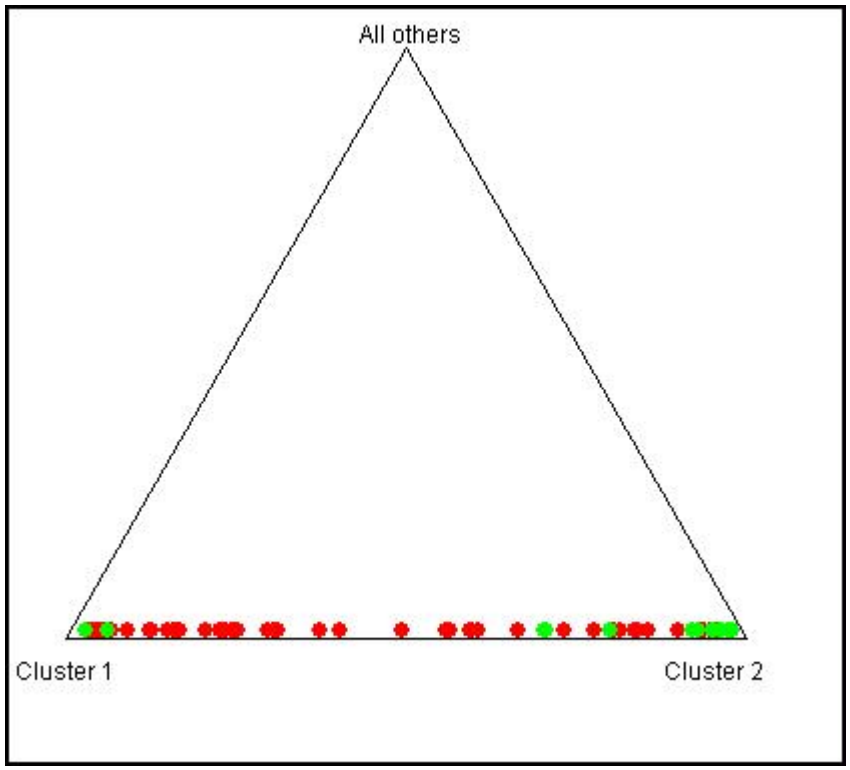

Fig. 3.3 Triangle plot indicating the relationship between the prior assumed subspecies of *G. curviloba*, subsp. *incurva* (red) and subsp. *curviloba* (green) and the clusters generated using STRUCTURE 2.1.

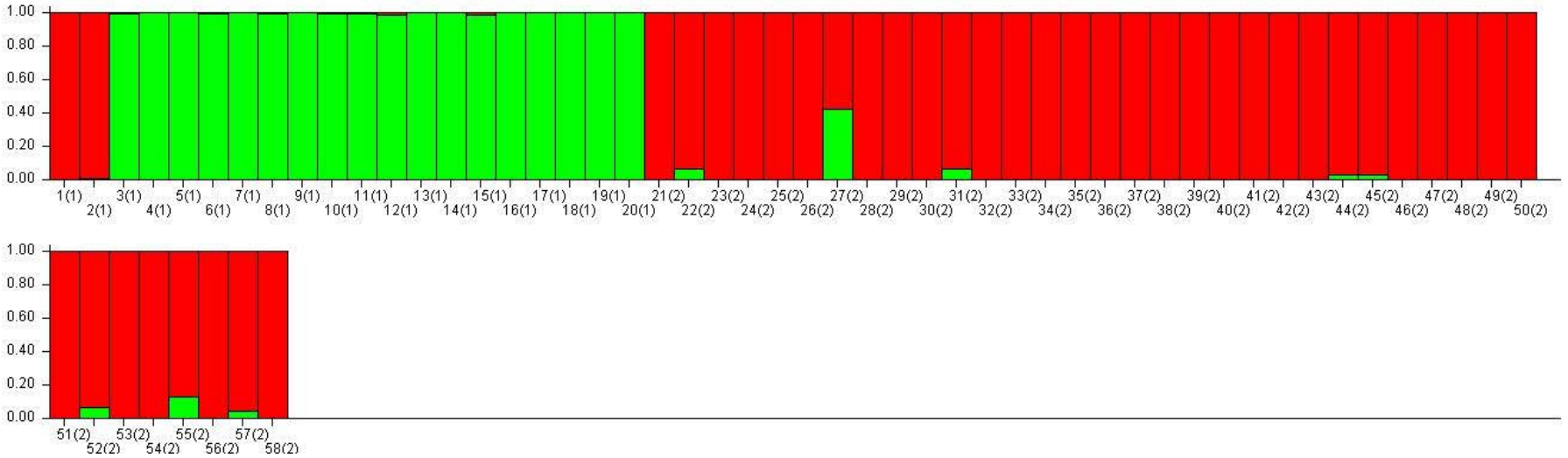

Fig. 3.4 Bar plot indicating the relationship between the prior assumed subspecies of *Grevillea vestita*, subsp *vestita* (denoted (1)) and subsp. *isopogoides* (2) and the clusters generated using STRUCTURE 2.1.

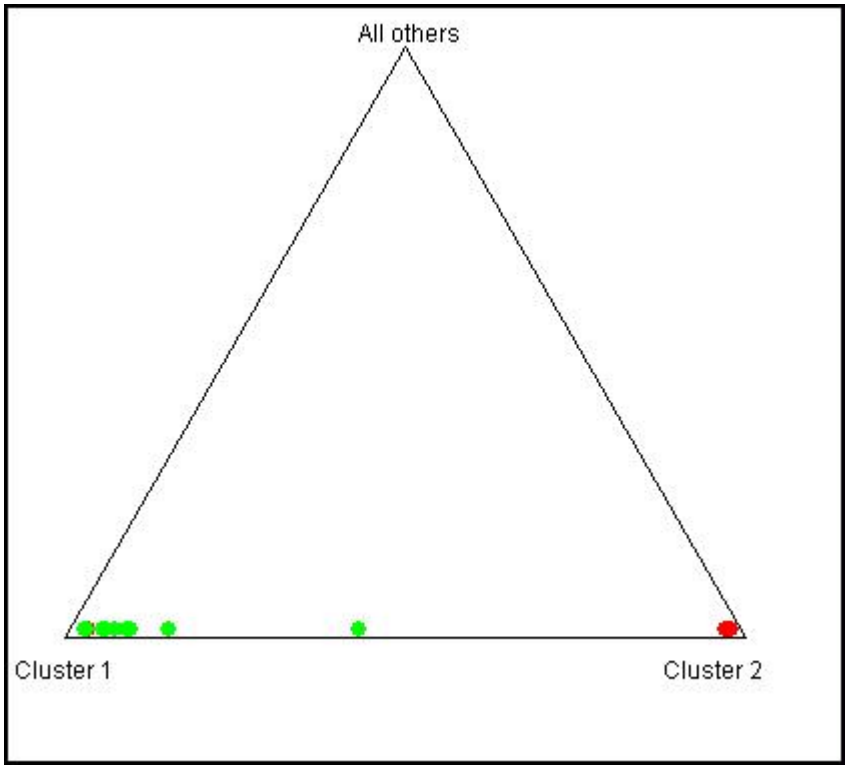

Fig. 3.5 Triangle plot indicating the relationship between the prior assumed subspecies of G. *vestita*, subsp. *vestita* (red) and subsp. *isopogoide*s (green) and the clusters generated using STRUCTURE 2.1. Note the red dot behind the green in cluster 1, representing the two individuals from population Wanneroo.

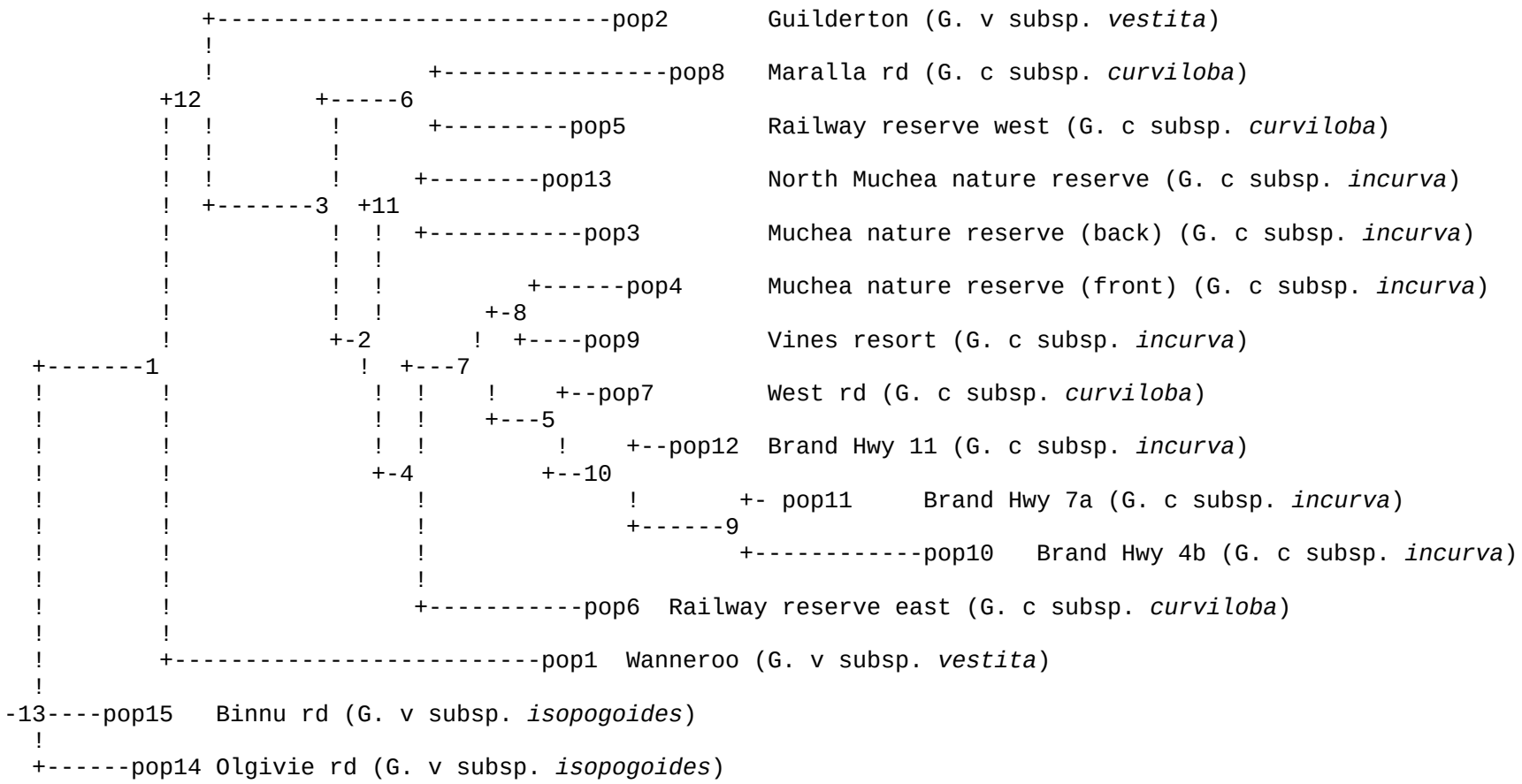

Fig. 3.6 Maximum likelihood tree of populations of *Grevillea curviloba* (G. c) and *Grevillea vestita* (G. v), with populations of low diversity included.

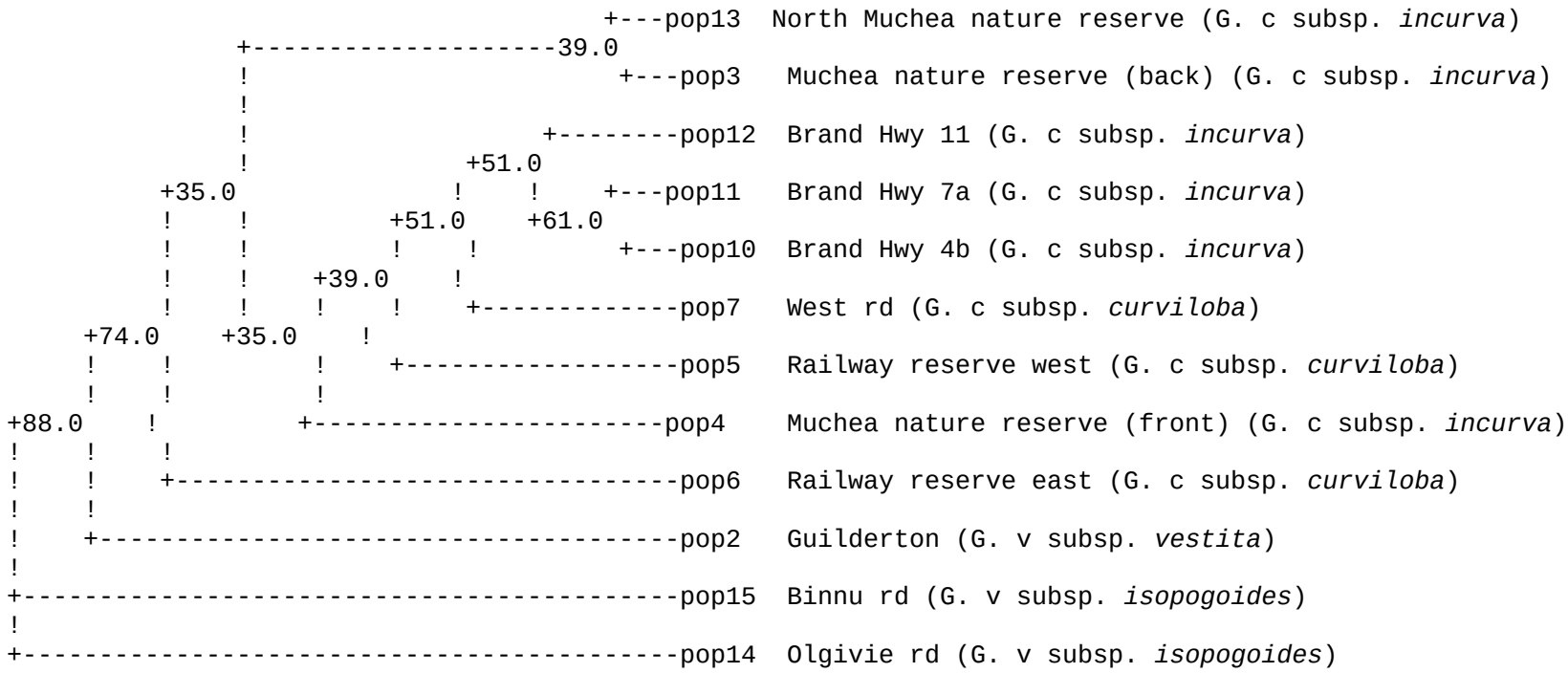

Fig. 3.7 Maximum likelihood consensus tree of 11 populations of *Grevillea curviloba* (G. c) and 4 populations of *Grevillea vestita* (G. v). * Three populations with low genetic variation (Wanneroo (*vestita*), Maralla rd (*curviloba*) and Vines (*incurva*)) were removed to enable calculation of bootstrap values, shown at nodes of the tree.

3.4 DISCUSSION

The genetic data presented in this chapter reflects the high level of clonality present in some populations of *G. curviloba*. However, whilst clonality is often associated with paucity in genetic diversity, the levels present overall are generally consistent with other *Grevillea* species.

3.4.1 Genetic diversity parameters

Estimates of allelic richness in populations of *G. curviloba* were low (2.696) in comparison to other *Grevillea* species that have been assayed with microsatellites such as *G. caleyi* (5.29) and *G. longifolia* (5.30) (Llorens, 2004). *Grevillea* species in general, appear to show low levels of allelic diversity in comparison to other woody shrubs such as *Melaleuca alternifolia* which can average as high as (19.6) alleles per locus (Rossetto *et al*., 1999b). In comparison to *G. vestita* (5.583), the allelic diversity present in *G. curviloba* populations was also low (2.696) (Table 3.2), and this was due to the presence of high clonality. Populations consisting of only two or three genetically unique individuals would be expected to maintain a limited number of alleles, and this was clearly seen in the differences between the highly clonal populations such as Maralla Rd population (1.333) and the more diverse North Muchea Nature Reserve population (4.333) (Table 3.2). The study of the asexual and sexual population groups of *Acacia anomala*, provides a useful comparison of the impact of clonality on population allele richness. In that study, the average allelic diversity estimated for the clonal Kalamunda populations (1.2) was almost half of that in the outcrossing Chittering populations (2.0) (Coates, 1988).

As a consequence of the extensive clonality present in some populations, genetic diversity (H_e) varied according to whether data with ramets of clones were included or excluded. When clonal data was included, the overall population sample size was increased, however the

actual genetic diversity remained the same, therefore values of H_e appear reduced (Table 3.2). The calculation of genetic diversity was further compounded by the presence of bias in the clonal data. For example, if one ramet in a population has been sampled more than once, or if one clone has been over-represented in sampling (likely to occur in small populations), the population size appears increased, lowering the overall genetic diversity. Thus, in order to better understand genetic diversity in clonal plant species, it may be necessary to remove clonal data, thereby removing the bias associated in sampling these populations.

Despite levels of genetic diversity within *G. curviloba* varying widely across populations, overall, H_e (0.498) was similar to and in some cases higher than the values obtained for the other *Grevillea* species such as *G. caleyi*, and *G. longifolia* where the maximum expected heterozygosity for any population was (0.456) and (0.443) respectively (Llorens, 2004). *G. macleayana*, an endangered shrub from south-eastern NSW was also recorded as having a similar genetic diversity to G. *curviloba* with an average across populations of (0.482) (England *et al*., 2003). The high variation in genetic diversity across populations of G. *curviloba* was the result of variations in the degree of clonality present. The impact of clonality on reducing diversity in populations can be seen in the similar ratios of difference between the clonal (0.079) and sexual (0.209) populations of *A. anomala* (outcrossing:clonal = 2.6) (Coates, 1988) in comparison to the clonal (i.e. Maralla Rd) (0.222) and more diverse (eg. North Muchea Nature Reserve) (0.656) populations of G. *curviloba* (outcrossing: clonal = 2.9).

3.4.2 Genetic diversity occurring between populations and subspecies of *G. curviloba*

Estimates of genetic differentiation (G_{ST}) (with clonal ramets removed) in *G. curviloba* (0.222) (Table 3.3) were average to low in comparison to other fragmented taxa

of the South West, including *A. anomala* (0.422), *Banksia cuneata* (0.227) and various *Eucalyptus* species (0.256) (Coates, 2000). The differences in results produced by the two data sets (clones included/removed) (Table 3.3) provide an interesting example of how clonal data can effect estimates of genetic differentiation. When ramets of clones were included in the data, the degree of differentiation between populations increased, as clonal genotypes occurred at high frequencies in their populations. The level of genetic differentiation maintained between subspecies (0.222) was similar to the level of differentiation between populations within subspecies for *curviloba* (0.235) and *incurva* (0.151). This indicated that differentiation was occurring between populations, rather than subspecies (Table 3.3). The analysis of the distribution of genotypic variance further supports these findings, with the majority of differentiation occurring between populations of subspecies (27%) rather than between the two subspecies (6%) (Fig. 3.1). It is not uncommon for taxa from the South West to exhibit high levels of genetic differentiation between populations (Coates, 2000). Past studies have shown this pattern occurs primarily in species that are historically fragmented and rare, and consequently are usually a target for conservation management (Moran and Hopper, 1983). Although the values for subspecies were similar, differentiation between populations was highest in subspecies *curviloba*, which in general consisted of populations with higher clonality than those of subspecies *incurva* (see chapter 2). Selfing populations are usually more genetically differentiated than outcrossing ones (Levin, 1978) and clonal populations, such as those of subsp. *curviloba*, would be expected to exhibit similar characteristics to selfing populations (Coates, 1988) resulting in a higher level of genetic differentiation.

3.4.3 Evidence of structure within *G. curviloba*

The analysis of genetic differentiation occurring within G. *curviloba* indicated an absence of structure between the two subspecies. This lack of differentiation was further confirmed in the maximum likelihood tree generated, where there was no clear relationship between the two subspecies (Fig. 3.7). The division of *G. curviloba* into the two subspecies *curviloba* and *incurva* was initially based on morphological variations noted on leaf structure (i.e. broader leaf lobes for subsp. *curviloba*, and incurved, narrow leaf lobes for subsp. *incurva*) (English and Phillimore, 2000), however grevilleas are notorious for the considerable variation in leaf morphology that can occur within a species (Kimpton *et al*., 2002). For example, *G. infecunda*, a narrow endemic of Victoria, has leaf morphology that is consistent within each population but that differs between populations (Kimpton *et al*, 2002).

G. vestita provided an interesting comparison for the genetic structure present in *G. curviloba*. The results indicated a clear distinction into two genetically differentiated groups; however the Wanneroo population (subsp. *vestita*) (which consisted of two widespread clones) appeared to be more similar genetically, to subspecies *isopogoides* than to the other sampled population (Guilderton) of subspecies *vestita* (Fig 3.4 and 3.5). This may be an artefact of the presence of only two clones in this population which happen to have alleles similar to those in subspecies *isopogoides*. In addition, only two populations of subspecies *vestita* were sampled and analysis of more populations may reveal that these also share similar allele frequencies to the Wanneroo population. It has been noted in the past that some specimens of *G. vestita* are difficult to assign to either subspecies (McGillivray, 1993). Due to the similarity of the Wanneroo clones to

subspecies *isopogoides*, it is suggested that a review of the identification of the Wanneroo population be conducted.

3.4.4 Microsatellites and the study of diversity and structure

Three microsatellite markers should provide enough statistical power to enable the accurate estimation of genetic structure and diversity in a species (Cavers *et al*., 2005). A case study using simulated population model data on *Symphonia globulifera* showed that three microsatellite markers were sufficient to provide a good estimate of the 'real' genetic structure present in a population of 1900 trees, resulting in a mean correlation greater than 0.8 (Cavers *et al*., 2005). Although the number of individuals sampled for this study was far less than that used in the Cavers *et al*., (2005) simulation, three microsatellite markers would still be expected to produce a more accurate estimation of genetic structure across the small populations than other molecular markers due to their high resolution and level of polymorphism. Indeed, it was not a lack of marker power that made interpretation of some of the results reported here on *G. curviloba* difficult, but rather the extensive clonality that was often present. In some populations (e.g. Maralla Rd and Wanneroo) the high level of clonality resulted in such a lack of genetic diversity that this prevented the calculation of bootstrap values for the maximum likelihood trees generated (Fig. 3.6). In the case of G. *curviloba* (where the presence of sampling bias was likely) the removal of ramets of clones from the data was believed to produce a more accurate estimation of diversity and structure within this species.

The results presented in this chapter have highlighted the difficulties associated in dealing with the genetic data of extensively clonal plant populations. Traditional methods of calculating diversity statistics may not always be suitable for clonal plant species and

should be approached with caution. An important outcome of the chapter was the finding that the genetic structure *G. curviloba* does not reflect the current classification into the subspecies *curviloba* and *incurva*.

CHAPTER 4

THE MATING SYSTEM OF *Grevillea curviloba*

4.1 INTRODUCTION

4.1.1 Mating systems and the conservation of plant populations

Understanding the mating system of endangered plant populations provides vital information on the minimum population size and genetic diversity required for the maintenance and conservation of the species (Ayre and Whelan, 1994). Population fragmentation, whether caused by recent or historical events can affect the mating system of a species, particularly in those that consist of small populations (Sampson *et al*., 1996). A significant proportion of genetic analyses on rare and endangered plants now involve an assessment of mating systems in order to determine factors such as levels of outcrossing, selfing, the presence of incompatibility systems and paternity analysis.

The ability of a species to self-fertilise (selfing) can have a large impact on its genetic viability. Two forms of self-fertilisation exist, autogamy, known as within flower fertilisation and geitonogamy which is fertilisation occurring between flowers of the same plant or in the case of clonal species, between ramets of the same clone (Eckert, 2000). Autogamy is thought to have reproductive advantages, such as the assurance of seed set, with a minimum reproductive effort. Geitonogamy is more common than autogamy, particularly in clonal plants (Eckert, 2000), however this mode of reproduction is believed to be disadvantageous, resulting in inbreeding depression (Eckert, 2000). Species which are outcrossing, generally produce superior seed to those which are selfing, however this can be an expensive mode of reproduction (Eckert, 2000). Many plants engage in both outcrossing and geitonogamy (Schemske and Lande, 1985) the degree of which can alter, depending on external pressures and environmental changes. Obtaining information on the various breeding strategies present

in populations can be used to interpret population viability and provide the best possible management strategy for the species at hand (Coates and Hopper, 2000).

4.1.2 Low fruit set in the Proteaceae

Members of the Proteaceae are known for their low fruit to flower ratios (FR:FL) when compared to other woody perennials (Ayre and Whelan, 1989; Hermanutz *et al*., 1998) and compatibility is believed to play an important role in this family's low level of fruit set (Hermantuz *et al*., 1998). Comparatively little is known about the breeding systems of grevilleas, however previous studies indicate FR:FL ratios are low, in keeping with the general trend seen in the Proteaceae (Hermanutz *et al*., 1998). *Grevillea* species have the potential to possess complex mating systems that vary among species and among populations of species (Hermanutz *et al*., 1998; Richardson *et al*., 2000). These variations can be dependant on the mode of pollination, the size of the populations and the presence of incompatibility systems (Coates and Byrne, 2005). In general, *Grevillea* species are outcrossing and bird pollinated (Hermanutz *et al*., 1998), however *G. curviloba* is thought to be insect pollinated, possibly by native bees or wasps (Olde and Marriott, 1995). Interestingly, previous studies have suggested that self-incompatibility systems are more likely to occur in insect-pollinated species (Richardson *et al*., 2000).

The aims of this chapter were to:

- Assess whether *G. curviloba* was capable of setting seed in the absence of pollinators (autogamous).
- Assess the levels of outcrossing and the degree of pollen dispersal occurring in populations of *G. curviloba* that set seed.

4.2 MATERIALS AND METHODS

4.2.1 Checking for autogamy

During flowering (Sep, 2005), white insect-proof gauze was used to bag unopened flowers on 10 individuals at the Railway Reserve population, and five individuals from each of the West Rd and Maralla Rd populations, in order to test for the ability of plants to produce fruit in the absence of pollinators (autogamy).

4.2.2 Seed collection and storage

Seed was collected from North Muchea Nature Reserve (the only population to set seed) between November and December 2005 using light-weight, cotton drawstring bags that were attached to the tops of branches bearing maximum fruit. As pods ripened and opened, seeds fell into the collection bags which were checked and emptied on a weekly basis in order to prevent predation by insects. Collected seeds were stored in paper envelopes at room temperature. Approximately 50 seeds were collected from each seeding individual.

4.2.3 Seedling germination

Seeds (150) were soaked for 24 h in 10% Smokemaster 2000 solution (Regen), rinsed with deionised water and the seed coat nicked with a scalpel blade. Seeds were then soaked in a 50% solution of PPM (Plant Preservative Material supplier, (Plant Cell Technology)) for 15 min before being placed onto 0.75% water agar (20 ml in 9 cm glass petri dishes) containing 25 mg/L Gibberellic Acid (GA_3). Gibberellic Acid (filter sterilised) was added to autoclaved water agar that had cooled to a temperature of 60°C. Plates were incubated at 15°C with light/dark cycles of 12 h over a period of 2 weeks.

4.2.4 DNA extraction from seedlings using the Doyle and Doyle method

DNA was initially extracted from seedlings using the Doyle and Doyle seedling DNA extraction procedure (Doyle and Doyle, 1990) with modifications. Seedlings (approximately 100 mg weight) were placed in 1.5 ml microcentrifuge tubes and ground in 50 µl of Doyle and Doyle extraction buffer using a hand drill. After mixing well, a further 150 µl of buffer was added to make a final volume of 200 µl. Samples were then placed in a 65°C water bath for 30 min. Following incubation, 20 µl of 10% sarkosyl was added and the samples left to incubate at room temperature for 15 min. Chloroform/IAA (isoamyl alcohol) (220 µl) was then added to samples and placed on a shaker for 15 min, to ensure mixing. After mixing, samples were centrifuged at maximum speed for 5 min. The supernatant was then pipetted to a new tube and 2/3 volumes of isopropanol added and mixed gently by inverting the tube a number of times. Samples were kept overnight at -20°C. The following day these were thawed at room temperature briefly and centrifuged at 13 000 rpm for 5 min. The supernatant was discarded and 500 µl of isopropanol/NH_4Ac added. Samples were then incubated at room temperature for 20 min and centrifuged at maximum speed for 5 min. The supernatant was discarded and the samples pulse spun to pull down any remaining liquid which was then pipetted off. The resulting DNA pellet was air-dried at room temperature for approximately 3 h. The pellets were then resuspended in 10 µl of sterile distilled water.

4.2.5 DNA extraction from seedlings using Qiagen DNeasy Plant Mini Kit

Five seedlings were also extracted using the Qiagen DNeasy Plant Mini Kit modified protocol as outlined in section 2.2.6.

4.2.6 Seedling DNA phenol extraction and ethanol precipitation

Three seedling DNA samples that had been extracted using the Doyle and Doyle method were purified using a phenol extraction and ethanol precipitation method with modifications. DNA (~2 µl) was initially diluted to 100µl using TE buffer in a 1.5 ml microcentrifuge tube. An equal volume of phenol/chloroform/isoamyl alcohol was then added to the DNA and the tube vortexed for 10 sec followed by centrifugation at maximum speed for 2 min. The top (aqueous) phase containing the DNA was then carefully removed to a new microcentrifuge tube using a pipette. Volume (1/10) of 3 M sodium acetate (pH 5.2) were then added to the DNA solution and mixed by inverting the tube. Volume (2.5 x) of ice cold ethanol was then added to tubes, which were mixed by vortexing, and incubated at -80°C for 2 h. Following incubation, samples were centrifuged for 5 min at maximum speed and the supernatant discarded. Room temperature 70% ethanol (1 ml) was then added to each sample, which was inverted to mix and then centrifuged at maximum speed for 5 min. The supernatant was then discarded and the resulting pellet air dried. Once the pellet was completely dry it was resuspended in 50 µl of sterile, de-ionised water.

4.2.7 DNA quality check

Agarose gel (0.8%) electrophoresis with sample aliquots (10 µl) of extracted DNA was carried out at 80 V for 1.5 h to check the DNA was not degraded.

4.2.8 DNA concentration of seedlings

The concentration of extracted DNA was determined as per section 2.2.8. DNA was diluted to a working concentration of 10ng/µl using sterile de-ionised water.

4.2.9 DNA amplification

DNA was amplified as described in section 2.2.9 and the product run on a polyacrylamide gel as per section 3.3.0 to check for successful amplification.

4.3 RESULTS

4.3.1 Seed production and autogamy

All 11 populations of *G. curviloba* flowered prolifically between September and November, 2005, however only one population, the North Muchea Nature Reserve, contained individuals that set seed. The 10 individuals of this population that did produce seed bore prolific amounts of it, and roughly 95 % of these germinated in the lab over a 10 day period. The populations in which inflorescences were bagged to exclude insects did not set seed, therefore the capacity of *G. curviloba* to produce fruit in the absence of pollinators remains unclear.

4.3.2 Extraction and amplification of seedling DNA

Following seedling DNA extraction using the Doyle and Doyle method, electrophoresis of sample aliquots showed that the DNA was degraded. In a second attempt at Doyle and Doyle extraction, seedlings were ground using a mortar and pestle in liquid nitrogen (rather than using the hand drill) in order to homogenise plant material more effectively. Once again, agarose gel electrophoresis indicated that DNA was partially degraded (Fig. 4.1). In an attempt to clean up the extracted DNA, an ethanol precipitation step was performed on three seedling DNA samples. Following the cleanup, DNA was amplified and run on a polyacrylamide gel. Results showed that with the exception of one sample (using primer Gi-4), DNA failed to amplify (Fig. 4.2). A new set of seeds were

germinated and DNA extraction of 5 seedlings, was attempted using the Qiagen DNeasy Plant Mini Kit modified protocol. Extracted DNA was amplified and polyacrylamide gel electrophoresis indicated that both the Qiagen extraction modified procedure and amplification had been partially successful, with clear bands produced (Fig. 4.3) with the exception of 2 samples using primer Gi-4 and 4 samples using primer Gi-6.

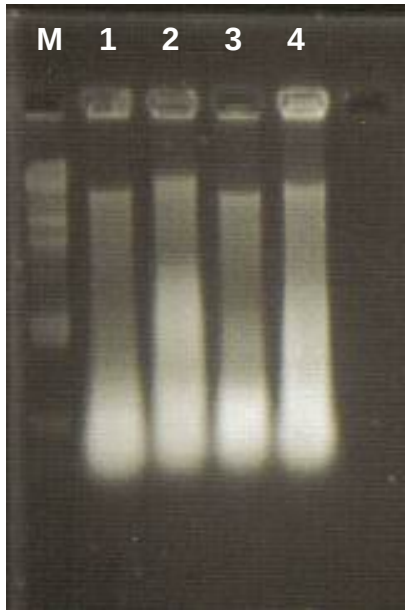

Fig. 4.1 Agarose gel showing degraded *G. curviloba* seedling DNA in lanes 1-4 following a Doyle and Doyle seedling extraction procedure. M = Promega lambda/*Hind*III molecular weight marker.

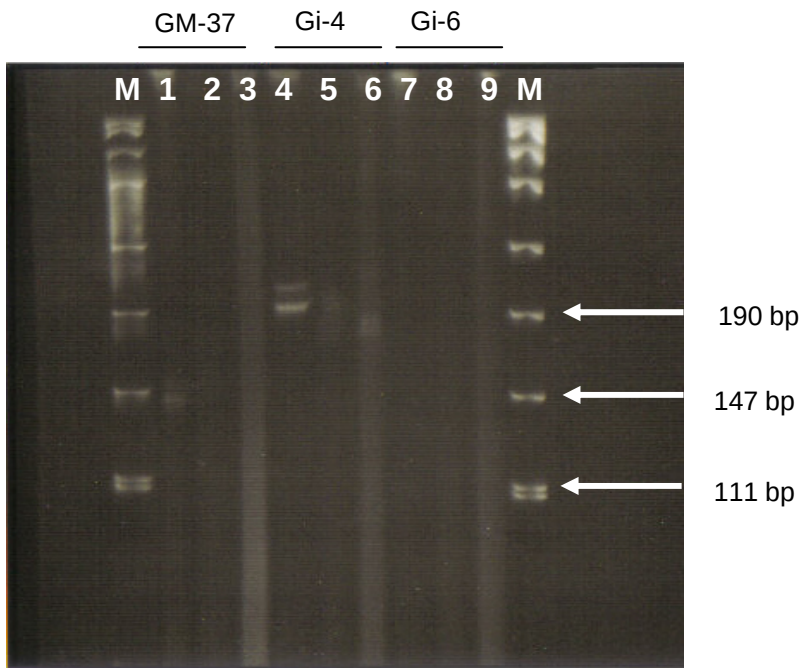

Fig. 4.2 Polyacrylamide gel showing amplified DNA product following a phenol-ethanol clean up of extracted template DNA from 3 seedlings. Lanes 1-3 (primer GM-37), no bands visible. Lanes 4-6 (primer Gi-4) band approx. 190 bp in size in lane 4, Lanes 7-9 (primer Gi-6),no bands visible. M = pUC19/*hpa*II Molecular weight markers.

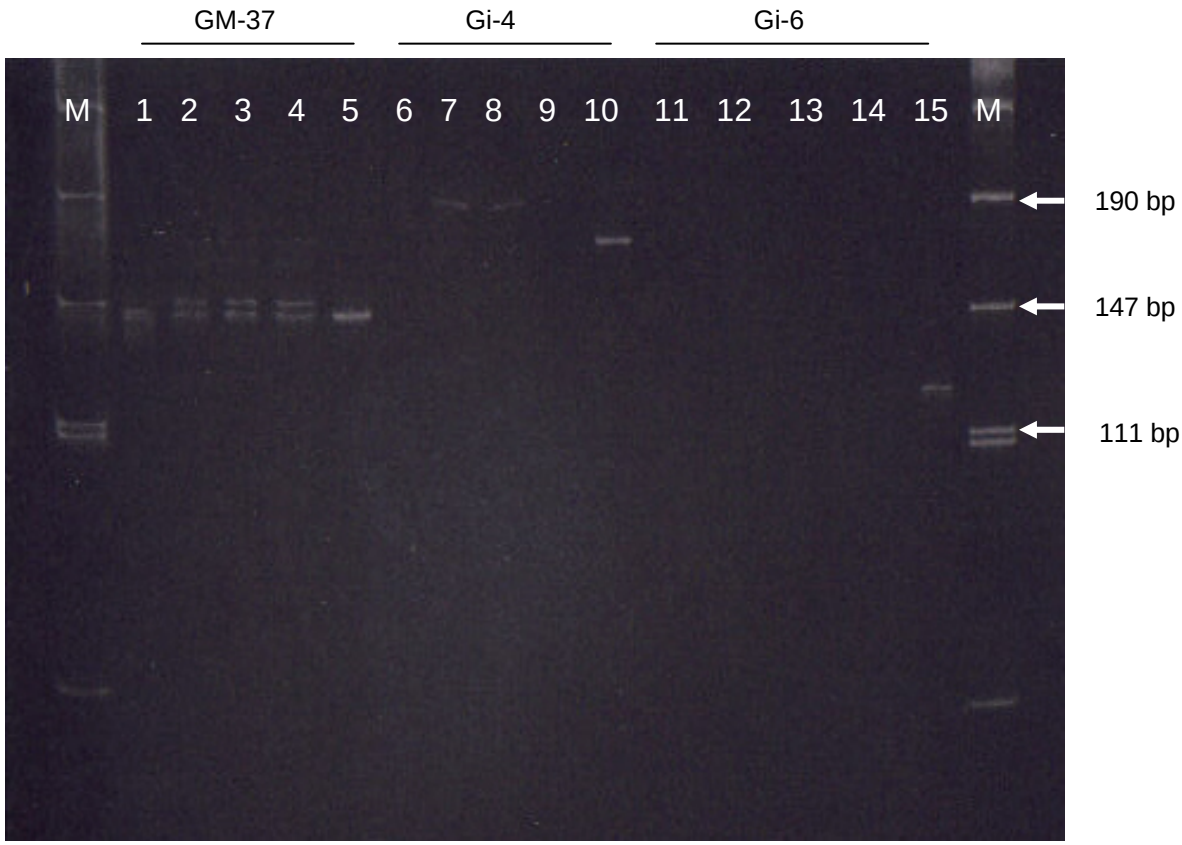

Fig. 4.3 Polyacrylamide gel showing amplified DNA of 5 seedlings extracted using the modified Qiagen Mini Plant Kit protocol. Lanes 1-5; using primer Gm-37, bands visible in each lane approx. 150 bp in size. Lanes 6-10; using primer Gi-4, bands visible in lanes 7,8 and 10 approx. 180 bp in size. Lanes 11-15; using primer Gi-6, band visible in lane 15 approx. 120 bp in size. M = pUC19/*HpaII* molecular weight markers.

4.4 DISCUSSION

4.4.1 Difficulties in seedling DNA extraction

The degradation of DNA after extraction using the Doyle and Doyle procedure is likely to be due to inhibitors present in the *G. curviloba* seedlings. The use of the Qiagen DNeasy Plant Mini Kit, appeared to remove these inhibitors and allowed for successful amplification of DNA with the exception of 2 samples amplified using Gi-4 and 4 samples using Gi-6 (Fig. 4.3). For future analyses requiring *G. curviloba* seedling DNA, it may be necessary to perform a DNA cleanup method in conjunction with a Qiagen DNeasy Plant Mini Kit extraction, to allow for the successful amplification of all samples. DNA isolation from grevilleas has proved difficult in the past (Pharmawati *et al*., 2004) and could be due to a number of factors including variations in the composition of the cell wall and/or the presence of polysaccharides and other compounds (particularly phenolics) (Mason and Schmidt, 2002; Pharmawati *et al*., 2004). In the case of *G. curviloba* it appears that seedlings contained higher levels of inhibitors than the adult leaf tissue.

4.4.2 Factors affecting seed set in plant populations

Several factors are thought to contribute to a reduction in seed set in plant populations including; space limitation, climatic variation, small population size, genetic load (Fuss and Sedgley, 1991), resource limitation (Lamont *et al*., 1985) and the disruption of native pollinator activities (usually as a result of fragmentation) (Whelan *et al*., 2000). Resource limitation is thought to be particularly important in the Proteaceae, as taxa in this family tended to evolve in low resource habitats (Lamont *et al*., 1985). However populations of *G. curviloba* exist in close proximity to one another and are unlikely to experience variations in climate, resource availability or pollinator activity. The presence of foreign pollinators such

as the European honeybee (*Apis mellifera*) have also been known to result in a reduction in seed set by reducing the amount of pollen available and/or by shifting the transfer of pollen so that it occurs predominantly within a plant rather than between plants. This was witnessed in populations of *G. macleayana* and is thought to have contributed to a reduced seed set in this species (Whelan *et al*., 2000). Once again, given the close proximity of *G. curviloba* populations, foreign pollinators would be expected to influence all populations, and so can be reasonably assumed not to have resulted in the lack of seed set in this species.

4.4.3 The impact of clonality on self incompatibility systems

The evolution of clonality is often associated with a loss of sexual reproduction and extensively clonal plant populations that are self-incompatible often exhibit extremely low seed set (Sydes and Peakall, 1998; Vekemans *et al*., 1998). As plant populations develop extensive clonality and genetic diversity is lost, the number of compatible genotypes present are reduced. This in effect, lowers the effective population size and reduces the potential number of mating partners available in a population.

A lack of seed production in *G. curviloba* has been reported in the past (McGillivray, 1993). In this study, it was significant that the only population to set seed was the population of highest genetic diversity (see chapter 3). Given the knowledge we have of the impact of genetic diversity on mating systems, it is reasonable to assume that the high degree of clonality present in some populations of this species has resulted in a general reduction in outcrossing. This loss of capacity for sexual reproduction has been noted in other clonal *Grevillea* species such as *G. althoferorum* (Burne *et al*., 2003) and *G. infecunda*, a root suckering species which also flowers prolifically, but that has never been recorded to produce seed (Kimpton *et al*., 2002).

G. curviloba may represent a species in which extensive clonality has resulted in a reduction of sexual reproduction as a response to decreasing genetic diversity. The high diversity present in the North Muchea Nature Reserve has most likely allowed for the production of seed in this small population. It is possible that *G. curviloba* was once a predominantly outcrossing species that has become more clonal over time and lost the capacity or requirement for sexual reproduction. Given this information, a loss in genetic diversity in the North Muchea Nature Reserve due to genetic drift and selection may be expected to result in a reduction in sexual reproduction in this population.

4.4.4 Future study

Due to problems with the initial extraction of seedling DNA and subsequent time constraints, the mating system study could not be completed. Ideally, future research on *G. curviloba* will provide information on the levels of outcrossing and pollen dispersal occurring in seeding populations. It is also recommended that trials of autogamy are conducted on individuals known to set seed in the North Muchea Nature Reserve in September 2006. The knowledge provided by a mating system study will aid in determining whether self-incompatibility mechanisms are present in *G. curviloba* and if these are the likely cause of a general lack of sexual reproduction in this species. Assessing the ability of populations of *G. vestita* to set seed, particularly in the extensively clonal Wanneroo population, would also provide an interesting comparison to the trends witnessed in *G. curviloba*.

CHAPTER 5
GENERAL DISCUSSION

5.1 The genetic diversity and structure of *G. curviloba*

The genetic analysis of *G. curviloba* using three microsatellite primers has revealed a number of important findings. The fact that this species was capable of clonal reproduction was not surprising, given that preliminary genetic studies had suggested the potential for vegetative reproduction, however the extent of clonality which occurred in some populations was unexpected. Indeed in the Maralla Rd population the 17 'individuals' sampled, were found to consist of just two genetic individuals (genets), one of which consisted of 16 widely spaced, robust ramets, suggesting that clonal fixation could be occurring. Despite the high level of clonality, levels of genetic diversity within *G. curviloba* were comparable to other *Grevillea* species. Genetic differentiation was present in *G. curviloba*, but was occurring between populations rather than between subspecies. High population differentiation is not uncommon in taxa of the South West (Coates and Hamley, 1999) and in the case of *G. curviloba* this was likely a reflection of the high frequency of clonal genotypes occurring within populations. There was no apparent genetic structure occurring within *G. curviloba* that would validate the recognition of the two subspecies *curviloba* and *incurva*. Therefore it appears that variations in leaf morphology are a normal feature of this species, rather than an indicator of genetic differentiation. The population that was recorded as having the highest genetic diversity of all those sampled was the North Muchea Nature Reserve, a small, road side population. It was also the only population (of the 11 sampled) to set seed, despite prolific flowering in all populations. It is possible that self-incompatibility mechanisms are present in *G. curviloba* and that the low levels of genetic diversity present in some populations, as a result of clonality, are preventing the production of seed.

Grevillea vestita, a common relative of *G. curviloba*, provided an interesting comparison. Although previously known to be capable of clonal reproduction via root-suckering (McGillivray, 1993), it was unknown that populations, such as Wanneroo, could be almost entirely clonal. Like *G. curviloba* at Maralla Rd, this population, initially considered to consist of more than 20 individuals actually consisted of just two unique genets separated by a dirt track. Interestingly, these individuals displayed a growth habit similar to that seen in the *G. curviloba* Maralla Rd clonal ramets. Reports in the literature on the growth of dominant clones (Eriksson, 1993; Douhovnikoff *et al*., 2005) have suggested that these are usually the primary colonisers of a site, and experience rapid expansion and growth, particularly in the absence of disturbance. Ellstrand and Roose (1987), suggested that the growth form of these widely distributed clones may result in greater dispersal and a higher competitive ability. The growth morphology and extensive spread of the dominant genets observed at the Maralla Rd and Wanneroo populations may suggest that these particular clones were the primary colonisers at these sites.

5.2 Conducting genetic analyses on clonal plant species

This study of *G. curviloba* has emphasized the need for a greater understanding on how to conduct genetic analyses of extensively clonal plant species. Bias appears to be unavoidable in such a study, whether it occurs as a result of sampling technique or data manipulation. Selecting an appropriate molecular marker for these types of analysis is critical in producing an accurate data set. Co-dominant markers with high resolution such as microsatellites, are ideal, given that they can accurately identify individuals (seen in the low probabilities of genotype occurrence determined (PGEN)) and typically detect high levels of variation (Lowe *et al*., 2004). As interest in the function and viability of clonal plant

populations increases, it is hoped that better statistical models will be developed, resulting in a reduction in bias and maximising the information gained through a genetic analysis.

5.3 Determining the conservation status of clonal plant species

One of the difficulties associated with assessing the status of clonal species is accurately determining the number of individuals present in a population (Rossetto *et al*., 1999a). For example, from the 29 estimated individuals sampled at the Railway Reserve (west) population, only 6 unique genets were identified. Another, more striking example of over–estimation of population size occurred in *Haloragodendron lucasii* where genetic analysis delineated just three genets from a total of 700 'individuals' (Sydes and Peakall, 1998). According to the latest criteria provided by The World Conservation Union (IUCN version 3.1, 2001) a population is defined as "the total number of mature individuals of the taxon", with mature individuals defined as "the number of individuals known, estimated or inferred to be capable of reproduction" (IUCN, 2001). It is then specified that in clonal taxa mature individuals include the number of reproducing units (ramets) within a clone, except where such units are unable to survive alone (such as coral) (IUCN, 2001). Therefore, given that a sufficient number of ramets in a population are capable of reproduction (sexual or asexual) the species is not considered to be at risk. Yet clonal plant species are often associated with a lack of genetic diversity (Maynard-Smith, 1978), and past studies have shown that genetically depauperate populations have an impaired ability to respond to environmental change (Kunin, 1997). This would place clonal plant species, with their often deceptively small effective population sizes, at a higher risk of extinction than those that are capable of renewing diversity through sexual reproduction.

As the number of genetic analyses performed on rare and endangered taxa increases, and our understanding of the complexity of clonal plant populations increases, conservation unions need to accommodate knowledge of genetic diversity into their criterion to enable a more accurate identification of threatened taxa. Therefore, when dealing with small, fragmented and disturbed populations of clonal plants species, it is suggested that the definition of mature individuals, in solely asexual species, include the number of genetically unique clones capable of reproduction, and in plant populations exhibiting both sexual and asexual reproduction; as the number of individuals setting seed and/or the number of genetically unique clones capable of reproduction. Unfortunately, it is not always feasible or affordable to determine the genets occurring in a population through a genetic analysis, making evaluation of the number of individuals difficult.

Both subspecies *incurva* and *curviloba* are currently ranked as critically endangered (CR) according to the IUCN criterion B1; where the extent of occurrence is less than 100 km^2, and criteria 2 B and C; where there is an observed decline in the quality of the habitat and populations. The results presented in this thesis (chapter 3) have shown that the morphological classification into subspecies is not supported in terms of genetic differentiation. By removing the two subspecies, the total number of populations of *G. curviloba* is increased. However, as the CR ranking for this taxon has been based on habitat range rather than population number, the status would be expected to remain the same.

It is essential that species are classified accurately if they are to be conserved appropriately. With species which are ranked according to low population number, the incorrect delineation of subspecies could result in an over-estimation of the threat to that taxon; alternatively, the failure to recognise distinct taxonomic units within a species could result in an under-estimation of the degree of threat. In cases where the taxonomy is unclear,

molecular markers can provide a useful tool for the confirmation of genetic structure, ensuring that the correct status is assigned to a taxon.

5.4 Conservation implications

Of the eleven *G. curviloba* populations sampled, only four are currently protected, three of which occur in CALM nature reserves (Muchea Nature Reserve east and west, and Maralla Rd) and one of which is found in a Bush Forever reserve (Vines resort). Unfortunately, genetic analysis has indicated that the populations of highest genetic diversity (eg. North Muchea Nature Reserve, Brand Hwy 4b, 7a and 11), and therefore greatest conservation value, are all found outside these reserves in highly disturbed roadside or rail-side locations that are overrun with weeds such as Watsonia (*Watsonia* spp.) and Veldt grass (*Ehrharta* spp.).

The primary goal of conservation biology is to maintain viable populations of endangered species (Coates and Hopper, 2000) through protection of existing populations or the re-establishment of populations in protected areas. This can be achieved through the creation of new populations by translocation of seed or plant material or by re-establishing current populations with seed/cuttings from neighbouring populations. In plant species that are highly clonal, or have low seed set, growing new plants from cuttings or tissue culture are often the only options available for population restoration (Burne *et al*., 2003). As the populations of *G. curviloba* are in relatively close proximity to one another, and do not appear to be composed of genetically distinct lineages, it may be possible to use either seed or leaf material from the diverse, unprotected populations for planting in the protected areas. Ideally this would boost the levels of genetic diversity present in these populations, with the ultimate aim of re-initiating sexual reproduction and the production of viable seed. As a result of this

study several recommendations can be made to aid in the long term conservation of *G. curviloba* and at a broader level, for the conservation of clonal plant species.

Recommendations to the Department of Conservation And Land Management (CALM) for the future conservation of *G. curviloba*:

- That populations of high genetic diversity are given priority for management, in particular the North Muchea Nature Reserve, which is currently the only population observed to set seed and is not protected.
- That all populations are repeatedly checked for production of seed during Sept-Jan in following years, and in the event of seed production, that a proportion of the output is conserved for potential use in later translocations and/or reintroductions.
- That weed control in areas of high infestation is carried out (although this may prove difficult due to the highly disturbed nature of the sites and the surrounding regions).

Recommendations to CALM for further research include:

- The development of microsatellite primers specific for *G. curviloba*.
- That a genetic study on the mating system of *G. curviloba* is conducted, in order to determine levels of paternity and outcrossing occurring in populations.
- That the potential presence of self incompatibility systems are determined via detailed cross and self pollination experiments and by the examination of pollen tube growth between natural, self- and cross- pollinated treatments.
- That protocols are developed for dealing with the genetic analysis and conservation of extensively clonal, endangered plant populations.

G. curviloba is typical of many taxa of the South West of Western Australia, persisting in fragmented, highly disturbed sites, displaying a range of reproductive strategies across populations, regenerating vigorously from fire and setting low levels of seed. Despite the extensive clonality present in some populations, the species as a whole appears to maintain normal levels of genetic diversity in comparison to other *Grevillea* species. However the general lack of seed set may indicate that these levels are still inadequate to allow for sexual reproduction to occur in the majority of populations. To ensure the successful, long-term conservation of *G. curviloba*, future research should focus on determining the mating system and levels of gene flow occurring both within and between current populations.

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Appendix A

Primers and PCR reactions.

Table 1 Sequence data for the three microsatellite primers used. Both forward and reverse sequences are shown.

Microsatellite locus	Primer sequence
GM-37	F 5'-TTT gCT gAA Agt CCC CAT TC-3' R 5'-gTT gTC AAA CCC TgC CAC TT-3'
GI-4	F 5'-AAC CAT AAg ggC gAC AAg-3' R 5'- gCC TAC AgA TAT ggT ggA AC-3'
GI-6	F 5'-AgC CAC TTg TCT ATC ACT ATC-3' R 5'-TCT ATC TAT CCC CAC TCT TC-3'

Table 2a Microsatellite primer **GM-37** reaction mix, using 1.75mM $MgCl_2$, for amplification of *Grevillea curviloba* and *Grevillea vestita*.

	Reagents	1x (µl)
	Sterile dH_20	1.65
2 mM	5xPCR buffer	3
50 mM	$MgCl_2$	0.525
10 pmol/µl	Labelled forward primer	1.875
10 pmol/µl	reverse primer	1.875
5M	Betaine	3
10 units/µl	TAQ polymerase	0.075
	sample DNA(10ng/µl)	3
	Total volume	**15**

Table 2b Microsatellite primer **GI-4** (Hoebee *et al.* 2002) reaction mix, using 1.75mM $MgCl_2$, for amplification of *Grevillea curviloba* and *Grevillea vestita*.

	Reagents	1x (µl)
	Sterile dH_20	4.15
2 mM	5xPCR buffer	3
50 mM	$MgCl_2$	0.525
10 pmol/µl	Labelled forward primer	1.125
10 pmol/µl	reverse primer	1.125
5M	Betaine	3
10 units/ul	TAQ polymerase	0.075
	sample DNA(10ng/µl)	2
	Total volume	**15**

Table 2c Microsatellite primer **GI-6** (Hoebee *et al*. 2002) reaction mix, using 1.5mM $MgCl_2$ for amplification of *Grevillea curviloba* and *G. vestita.*

	Reagents	1x (µl)
	Sterile dH_20	0.975
2 mM	5xPCR buffer	3
50 mM	$MgCl_2$	0.45
10 pmol/µl	Labelled forward primer	3.75
10 pmol/µl	reverse primer	3.75
	TAQ polymerase	0.075
	sample DNA(10ng/µl)	3
	Total volume	**15**

Table 3 Eppendorf Thermocycler programs used to amplify microsatellite loci in *Grevillea curviloba* and *G. vestita.*

Program **8** Primers **GM-37** and **GI-4**	Program **9** Primer **GI-6**
94°C for 2min	96°C for 2min
94°C for 30sec 68°C for 30sec - 30 cycles [-0.3°C/cycle to 56.3°C] 72°C for 5sec	95°C for 30sec 56°C for 30sec - 30 cycles 72°C for 30sec
94°C for 30 sec 55°C for 30sec - 3 cycles 72°C for 5sec	-
72°C for 5min	72°C for 5min

Gel Solutions

8% Polyacrylamide gel

Reagent	Gel (x1)
dH_20	22.0 ml
10xTBE buffer	3.5 ml
30% acrylamide (19:1)*	9.3 ml
10% ammonium persulphate (AMPS)*^	175 µl
TEMED*^	35 µl

* Biorad

^ Added immediately prior to pouring

0.8% Agarose/TAE gel

	Small gel (x1)
DNA grade agarose	0.8g
TAE	100ml

Typical growth habit of *G. curviloba*

Fig.1 Typical growth habit of *G. curviloba*.(photo taken at Muchea Nature Reserve East).